Univerzita Karlova v Praze

2. lékařská fakulta

Validace metody High Resolution Melting (HRM) pro účely DNA diagnostiky; mutační analýza genu cystické fibrózy a vybraných kandidátních genů u mužské infertility

Mgr. Petra Křenková

Disertační práce

Praha, 2013

Doktorský studijní program v biomedicíně

Obor: **Molekulární a buněčná biologie, genetika a virologie**

Školitel: **Doc. MUDr. Milan Macek, CSc.**

Školitel konzultant: **Prof. MUDr. Milan Macek jr., DrSc.**

Školící pracoviště: **Ústav biologie a lékařské genetiky 2. lékařské fakulty Univerzity Karlovy v Praze a Fakultní nemocnice v Motole**

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem řádně uvedla a citovala všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu.

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, dne 25. 2. 2013

Petra Křenková

Identifikační záznam:

KŘENKOVÁ, Petra. *Validace metody High Resolution Melting (HRM) pro účely DNA diagnostiky; mutační analýza genu cystické fibrózy a vybraných kandidátních genů u mužské infertility [Validation of High Resolution Melting (HRM) for the purpose of DNA diagnostics; mutation analysis of the cystic fibrosis gene and selected candidates genes of male infertility]*. Praha, 2013, s. 149, př. 0. Dizertační práce. Univerzita Karlova v Praze, 2. lékařská fakulta, Ústav biologie a lékařské genetiky 2. LF UK 2012. Vedoucí závěrečné práce Prof. MUDr. Milan Macek jr., DrSc.

PODĚKOVÁNÍ

Ráda bych poděkovala svým školitelům Prof. MUDr. Milanu Mackovi jr., DrSc. a Doc. MUDr. Milanu Mackovi, CSc. za příležitost pracovat na zajímavých projektech, za jejich ochotu a odbornou pomoc v průběhu celého mého postgraduálního studia.

Za cenné odborné rady a přátelství děkuji RNDr. Peteru Vasovčákovi, PhD. a Mgr. Patricii Norambueně, PhD.

Děkuji i svým kolegům z pracoviště za vytvoření příjemné pracovní atmosféry a zvláště pak svým rodičům a manželovi za jejich podporu.

ABSTRAKT

V posledních letech došlo k výraznému rozvoji molekulárně genetické diagnostiky (DNA diagnostiky). Jsou zaváděny nové metody a technologie a rozšiřováno spektrum genetických služeb. Jelikož výsledky genetických testů zárodečného genomu mohou celoživotně ovlivnit zdraví a kvalitu života pacienta i jeho rodiny, je kladen důraz na neustálé zvyšování kvality poskytovaných diagnostických služeb. Stále více laboratoří přechází z „domácích metod“ na komerční diagnostické soupravy, ale často pak svévolně modifikují protokoly výrobce. Proto je pro zajištění kvality genetických testů nezbytné všechny metody a technologie před zavedením do rutinní diagnostické praxe řádně validovat a verifikovat.

V této dizertační práci jsem se věnovala otázce vhodnosti a využitelnosti metody High Resolution Melting (HRM) v diagnostice na základě její kompletní validace dle mezinárodních požadavků normy na řízení systému jakosti (ISO 15189). Potvrdili jsme užitečnost této metody a její výhody pro mutační skenování neznámých variant i genotypizaci častých polymorfismů, a to na příkladu různých genů (*BRCA1*, *MTHFR* či *CFTR*). Zároveň jsme poskytli metodické pokyny a postupy pro usnadnění diagnostické implementace této technologie a umožnění jejího úspěšného využití i v dalších genetických laboratořích, a na příkladu validace HRM poskytli návody a model pro validaci dalších DNA diagnostických metod.

O zvýšení kvality poskytovaných genetických služeb jsme se zasadili i v oblasti diagnostiky cystické fibrózy (CF). Toto frekventované monogenní onemocnění je charakterizováno velkým množstvím identifikovaných mutací v genu *CFTR* a molekulární heterogenitou v závislosti na etnicitě probanda, proto je znalost distribuce a četnosti mutací tohoto genu v každé populaci klíčová. Molekulárně genetickými studiemi jsme zmapovali spektrum mutací u českých a ukrajinských pacientů s CF, neboť identifikace obou kauzálních mutací podpoří klinickou diagnózu, umožní předpovědět průběh onemocnění, individuálně stanovit léčbu, poskytnout spolehlivou prenatální diagnostiku a stanovit přenos těchto alel a rizik u dalších rodinných příslušníků. Vysoká populační záchytnost umožní zavedení novorozeneckého skríningu, který vyhledá postižené pacienty

v preklinickém stádiu. Včasné stanovení diagnózy a brzká aplikace léčby příznivě ovlivní průběh choroby a zároveň sníží náklady na léčbu.

V současné době je věnována velká pozornost problematice neplodnosti, neboť postihuje až 15% párů a dochází k jejímu nárůstu. Muž se na neplodnosti páru podílí v přibližně 50% případů. Po vyloučení rutinně vyšetřovaných příčin mužské neplodnosti (např. mutace v genu cystické fibrózy, Klinefelterův syndrom, strukturální abnormality Y chromozomu, prodělané záněty varlat virem příušnic, tumor atd.) nadále zůstává mnoho případů neobjasněno. Proto jsem se v rámci tohoto studia zabývala analýzou protaminových genů (*PRM1, PRM2*), neboť plní stěžejní funkci při diferenciaci spermií a bylo prokázáno, že myši haploinsuficientní pro jeden z protaminových genů vykazují poruchu uspořádání chromatinu, jaderné integrity a produkují spermie abnormální morfologie a snížené pohyblivosti, které nejsou schopné oplodnit oocyt. Na reprezentativním souboru německých teratozoospermických mužů s normálním počtem spermií (simulující fenotyp PRM-haploinsuficientní myši) a mužů se sníženým počtem spermií, včetně souboru normozoospermických mužů jako kontrol, byla provedena mutační analýza protaminových genů zkoumající vliv případných mutací na poruchu spermiogeneze. Podařilo se nalézt statisticky signifikantní asociaci mezi haplotypem ACC, tvořeným třemi častými polymorfismy genů *PRM1/2*, a koncentrací spermií a jejich celkovým počtem. Homozygotní nosiči haplotypu ACC měli dvojnásobně vyšší počet spermií než muži bez tohoto haplotypu ($45x10^6$/ml x $24.2x10^6$/ml). Je možné, že spermie nosičů jiného než ACC haplotypu nejsou životaschopné či podléhají negativní selekci. Pro určení klinického významu tohoto zjištění a jeho případného diagnostického využití je však potřeba nález ověřit studiemi na dalších souborech a populacích.

Klíčová slova: High Resolution Melting, HRM, validace, cystická fibróza, *CFTR* gen, mutace, mužská neplodnost, protaminy

ABSTRACT

During the last years we have observed a rapid development of molecular genetic diagnostics (DNA diagnostics). New methods and technologies are rapidly being introduced and the spectrum of genetic services is gradually extended. Since germline genetic tests might have lifelong influence health and quality of patient's life, all efforts should aim at improvement of the overall quality of provided diagnostic services. An increasing number of laboratories replace their “in-house” developed techniques by the commercial diagnostic assays, but they often modify manufacturer's instructions. Therefore, it is necessary to validate and verify all methods and techniques before their implementation into routine DNA diagnostics.

In this thesis I have focused on evaluation and application of High Resolution Melting (HRM) in clinical diagnostic practice based on its comprehensive validation, according to the major international quality assurance standard ISO 15189. On the model of selected genes (*BRCA1, MTHFR, CFTR*) we have confirmed the high utility of HRM for mutation scanning of unknown variants, as well as genotyping of common variants. Concurrently, we have provided a list of methodical guidelines which could be applied for setting up HRM in other genetic laboratories and provided a diagnostic validation strategy for other DNA diagnostic techniques.

Furthermore, we have contributed to the higher quality of genetic services in the area of diagnostics of cystic fibrosis. This common life-threatening autosomal recessive disease is known for a substantial number of mutations in the *CFTR* gene and for its molecular heterogeneity based on the patient's ethnicity. Therefore, it is important to analyse mutation distribution and frequency of *CFTR* gene mutations among different populations. In this thesis, I have presented a comprehensive overview of CFTR mutations at Czech and Ukrainian CF patients, since identification of both causal mutations will support a clinical diagnosis, allow clinical prognosis, individually assess appropriate medical treatment, provide a reliable prenatal diagnostics and determine the risk for other family members. The high population detection rate will enable implementation of CF newborn screening, which helps to find CF patients before symptom occurrence. Such an

early establishment of CF diagnosis and an immediate application of medical treatment favourably influence the overall clinical outcome and reduce the costs for treatment in this disease.

Human infertility is a serious medical and socio-economic issue since it currently affects approximately 15% of couples and this number is still increasing. The "male factor" in reproductive failure accounts for 50% of all cases, while many causes still remain unknown. Therefore, we performed a mutation analysis of protamine genes (*PRM1* and *PRM2*), as they play a crucial role in differentiation of spermatids. It was demonstrated that knockout (KO) of either protamine gene in mice results in male infertility due to an alteration in sperm chromatin assembly and nuclear integrity. These *Prm1* or *Prm2* haploinsufficient mice produce sperm exhibiting abnormal morphology, combined with reduced motility and are thus unable to fertilize an oocyte. We sequenced both genes in representative groups of German idiopathic infertile patients with distinct teratozoospermia and normal (resembling the phenotype of the KO mice) or reduced sperm concentration and in normozoospermic men as a control, in order to investigate the impact of protamine gene sequence variations on spermatogenesis. We have revealed a statistical significant association of ACC haplotype, formed by the three common SNPs of *PRM1/2* genes, and sperm concentration/count. Homozygous carriers of ACC haplotype had a twofold higher sperm concentration and count than men lacking this haplotype (45x106/mL x 24.2x106/mL). Spermatozoa without the ACC haplotype might not be viable or might be subjected to negative selection. For the clinical impact of this finding and its implementation to the diagnostics it is necessary to confirm results by other studies on different cohorts and/or populations.

Key words: High Resolution Melting, HRM, validation, cystic fibrosis, *CFTR* gene, mutations, male infertility, protamines

OBSAH

1. ÚVOD **11**

1.1 Implementace nových metod do genetické laboratorní diagnostiky na příkladu HRM 11

1.1.1 Validace a verifikace *11*

1.1.2 High Resolution Melting *13*

1.2 Poruchy plodnosti 18

1.2.1 Přehled příčin poruch plodnosti *18*

1.2.2 Spermatogeneze a hodnocení spermiogramu *24*

1.2.3 Protaminy a mužská neplodnost *28*

1.2.4 Cystická fibróza a gen CFTR *33*

2. CÍLE DIZERTAČNÍ PRÁCE **43**

3. VÝSLEDKY, DISKUZE A PŘILOŽENÉ PUBLIKACE **45**

3.1. van der Stoep, et al. (2009) Diagnostic guidelines for high-resolution melting curve (HRM) analysis: an interlaboratory validation of BRCA1 mutation scanning using the 96-well LightScanner. Hum Mutat. (IF: 5.686) 45

3.2. Norambuena, et al. (2009) Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene. Clin Biochem. (IF: 2.076) 80

3.3. Křenková, et al. (2009) Evaluation of high-resolution melting (HRM) for mutation scanning of selected exons of the CFTR gene. Folia Biol (Praha). (IF: 1.151) 91

3.4. Makukh et al. (2010) A high frequency of the Cystic Fibrosis 2184insA mutation in Western Ukraine: genotype-phenotype correlations, relevance for newborn screening and genetic testing. J Cyst Fibros. (IF: 3.190) 98

3.5. Křenková et al. (2012) Distribution of CFTR mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection of novel/de novo alleles and relevance for related/derived populations. J Cyst Fibros. (IF: 3.190) 105

3.6. Tüttelmann et al. (2010) A common haplotype of protamine 1 and 2 genes is associated with higher sperm counts. Int J Androl. (IF: 3.591) 113

4. ZÁVĚR **125**

5. PŘEHLED PUBLIKACÍ, POSTERŮ A PŘEDNÁŠEK **127**

5.1. Publikace 127

5.2. Postery 129

5.3. Přednášky 132

5.4. Ostatní 133

6. SEZNAM ZKRATEK **134**

7. SEZNAM CITOVANÉ LITERATURY **136**

1. ÚVOD

1.1 Implementace nových metod do genetické laboratorní diagnostiky na příkladu HRM

Dosud se v klinické diagnostické praxi rutinně vyšetřovala pouze malá část genů u omezené skupiny známých monogenních onemocnění. Rovněž spektrum vyšetřovaných mutací bylo zpravidla menší, než počet všech známých mutací v konkrétním genu. To bylo dáno především omezenou technickou kapacitou dostupných metod či jejich finanční nákladností. Z tohoto důvodu bylo nutné rozvinout nové metody. S tím souvisí potřeba řešit problematiku organizace práce a kontrol jakosti (akreditace podle norem ISO), potřeba stanovit obecná doporučení a pokyny pro zavádění nových molekulárně genetických metod a jejich validaci.

Za tímto účelem vznikl projekt Eurogentest1 (www.eurogentest.org), který v průběhu let 2005-2010 řešil harmonizaci a standardizaci genetického testování v Evropské unii (EU) pomocí integrace roztříštěných národních aktivit v této oblasti. Projekt vytvořil potřebnou infrastrukturu a prostředky, pokyny a procedury, které měly zajistit zlepšování kvality poskytovaných genetických služeb v EU (Macek et al. 2007, Mattocks et al. 2010). V následném projektu Eurogentest2 došlo k zachycení nových trendů, tj. především zavádění sekvenování nové generace do klinicko-diagnostické praxe.

1.1.1 Validace a verifikace

Výsledky genetických testů zárodečného genomu mohou ovlivnit zdraví a kvalitu života pacienta i jeho rodiny, proto provádět DNA diagnostiku o dostatečné kvalitě je profesní povinností každé laboratoře. Dosažení těchto předpokladů napomůže zavedení akreditace, tj. zavedení systému managementu kvality, podle normy ISO 15189. Akreditace, neboli úřední potvrzení kompetence laboratoře, vyžaduje zavedení pořádku do

laboratorní dokumentace, pravidelnou údržbu a kalibraci přístrojové techniky, ale především používání řádně validovaných a verifikovaných metod.

Validace je potvrzení získané prostřednictvím poskytnutí objektivních důkazů, že požadavky na specifické zamýšlené použití nebo specifickou aplikaci byly splněny. Potvrzuje, že měřící postup/systém/výrobek je schopen plnit požadavky na ně kladené. Jinak řečeno, že úroveň měření je dostatečná, postupy měření korektní a s řádně provedenou kalibrací.

Rozsah a hloubka validace musí vždy odpovídat potřebě získat dostatek údajů k rozhodnutí, zda je metoda či technologie skutečně vhodná pro zamýšlený účel. Validace nových technologií by měla být provedena před jejich zavedením do laboratorního provozu v širokém měřítku, a to nejlépe na vícero pracovištích v rámci tzv. mezilaboratorní validační studie. Důraz by měl být kladen na podrobné prozkoumání kritických parametrů a limitací metody i na identifikaci možných zdrojů interference.

Metody validované výrobcem disponující značením CE vyžadují nižší rozsah validace než metody typu „in-house“ vyvinuté samotnou laboratoří a v takovém případě provádíme pouze verifikací, tj. ověření, že měřící postup/systém/výrobek je ve shodě s hodnotami deklarovanými výrobcem a je plně funkční v konkrétní laboratoři. Předmětem verifikace je tedy schopnost realizovat měřící proces v konkrétní laboratoři v daném čase a prostoru.

Molekulárně genetické testy se řadí do kategorie testů kvalitativních, jejichž diagnostická přesnost je charakterizována dvěma komponentami: 1/ senzitivitou - pravděpodobnost pozitivního výsledku testu v případě přítomnosti hledané varianty testovaného znaku, je vyjadřována jako poměr mezi správnou pozitivitou *(True Positivity TP)* a součtem správné pozitivity a falešné negativity *(False Negativity FN)*, tj. TP/(TP+FN) a 2/ specificitou - pravděpodobnost negativního výsledku testu v případě nepřítomnosti hledané varianty testovaného znaku, je vyjadřována jako poměr mezi správnou negativitou (*True Negativity TN)* a součtem správné negativity a falešné positivity (*False Positivity FP*), tj.TN/(TN+FP). Další doporučované validační parametry jsou: 1/ opakovatelnost - preciznost měření stanovená opakováním měření za totožných podmínek v krátkém časovém úseku (stejný postup, stejný obslužný personál, stejný měřicí

systém, stejné pracovní podmínky a stejné místo), 2/ reprodukovatelnost - preciznost měření v rámci různých sérií či mezi různými laboratořemi v různém čase, 3/ robustnost - schopnost metody poskytovat přijatelné výsledky měření i v případě, že dojde k malým odchylkám od měřicího postupu či složení vzorku (např. typ izolace DNA, koncentrace DNA v reakci, změna počtu cyklů při amplifikaci atd.).

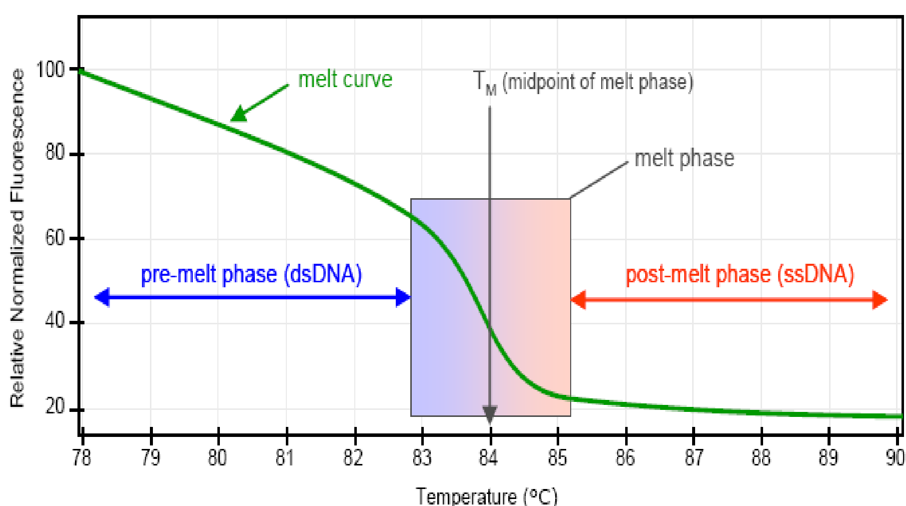
1.1.2 High Resolution Melting

Ačkoliv je Sangerovo sekvenování stále považováno za „zlatý“ standard pro záchyt neznámých mutací, je potřeba vyvíjet nové metody, které by redukovaly náklady a časovou náročnost jednotlivých vyšetření. Existuje velké množství metod, které jsou založeny na běžném laboratorním vybavení bez nutnosti nákladné počáteční investice, které zachytí změny v nukleotidové sekvenci DNA a identifikují tak oblast, ve kterém se mutace nachází, čímž se minimalizuje potřeba sekvenace. Jsou to například SSCP (single-strand conformation polymorphism analysis) (Orita et al. 1989), DGGE (denaturing gradient gel electrophoresis) (Lerman and Silverstein 1987), TGCE (temperature gradient capillary electrophoresis) (Li et al. 2002a), TTGE (temporal temperature gradient electrophoresis), dHPLC (denaturing high performance liquid chromatography) (Xiao and Oefner 2001), HA (heteroduplex analysis) (Highsmith et al. 1999) atd. Dosud bezkonkurenční alternativou na poli detekce mutací se však zdá být vysokorozlišovací analýza křivek tání (High Resolution Melting, HRM) (Wittwer et al. 2003, Erali et al. 2008), která umožňuje vysoce výkonné mutační skenování jednonukleotidových variant (SNPs, single nukleotide polymorphisms) i menších delecí a inzercí (White et al. 2006), genotypizaci (Norambuena et al. 2009) či metylační analýzu (Wojdacz and Dobrovic 2007).

Princip této metody spočívá v analýze křivek tání s vysokým rozlišením. DNA se za postupně se zvyšující teploty přeměňuje z dvouřetězcové molekuly na jednořetězcovou, kdy přítomnost mutace v heterozygotní formě v sekvenci DNA má za následek výskyt nekomplementárních bází, tj. taková molekula je méně stabilní a denaturuje při nižší teplotě. Postupným táním dvouřetězcové DNA se uvolňuje fluorescenční barvivo, jehož intenzita je snímána a zaznamenána přístrojem s citlivým fluorescenčním detektorem.

Výsledkem je tzv. křivka tání (Obr. 1), která popisuje závislost intenzity fluorescence na teplotě. Tato křivka se skládá ze třech částí. Z tzv. pre-melt fáze, kdy je v reakci přítomna DNA pouze ve dvouřetězcové formě (dsDNA) a intenzita fluorescence je nejvyšší. Postupným zvyšováním teploty dochází k denaturaci dvouřetězcových molekul, což se projeví prudkým poklesem intenzity fluorescence. Tato fáze se nazývá melt fáze, tj. fáze tání. Inflexní bod křivky je označován jako Tm (teplota tání, melting temperature). Poslední fáze, označovaná jako post-melt, je charakteristická přítomností pouze jednořetězcových molekul DNA (ssDNA) a tedy minimální fluorescencí.

Obr. 1 Křivka tání



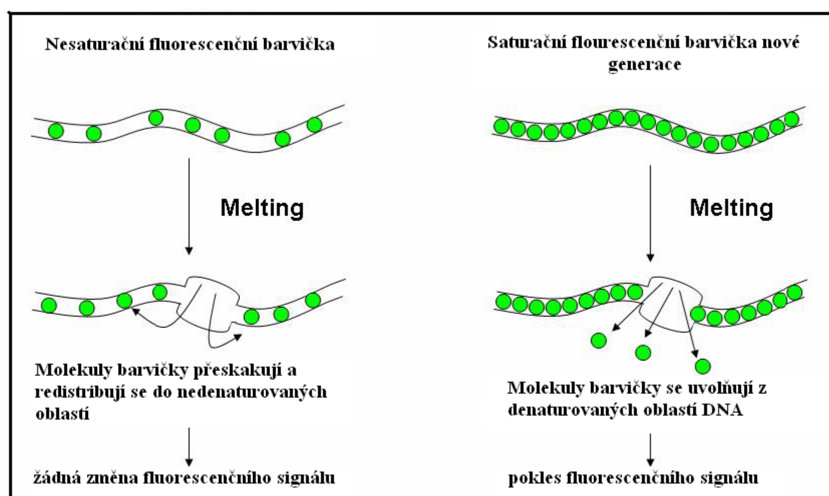
manuál CorProtocol 6000, Corbett, 2006

HRM využívá principu analýzy heteroduplexů. Po amplifikaci heterozygotního vzorku, který nese dvě alely téhož genu lišící se v jediné bázi, se provede tepelná denaturace s následnou reasociací jednotlivých řetězců. Vzniknou tak čtyři různé produkty: dva homoduplexy vykazující klasické Watson-Crickovo párování bází a dva heteroduplexy s „mismatch“ párováním bází. Všechny čtyři duplexy mají odlišné fyzikální vlastnosti, zejména se liší v teplotě, při níž dvouřetězce disociují (tj. „tají“). Vzorek se standardní „wildtype“ sekvencí či s mutací v homozygotní formě bude vytvářet pouze jeden typ homoduplexů, kde všechny báze budou párovat. Po HRM analýze bude každý duplex produkovat charakteristický disociační profil, tj. křivku tání. Přítomnost heteroduplexů v reakci se projeví snížením Tm a změnou tvaru křivky tání. Naproti tomu vzorek s mutací

v homozygotní formě se projeví pouze posunem Tm oproti křivce standardní. Tyto změny jsou malé, ale pomocí HRM přístrojů mohou být spolehlivě zachyceny (Reed and Wittwer 2004).

HRM spočívá v přípravě PCR v přítomnosti speciálního fluorescenčního barviva, které je plně saturační. Tím se redukuje redistribuce barviva během denaturace a zachytí se změny intenzity fluorescence s vysokým rozlišením. Tyto barviva lze používat v koncentracích dostatečně vysokých pro kompletní saturaci všech dvouřetězcových molekul, aniž by to inhibovalo amplifikaci (Wittwer et al. 2003) (Obr. 2).

Obr. 2 Saturační model fluorescenčního barviva používaného při HRM



White et al. 2006

HRM představuje rychlou, technicky i finančně nenáročnou, přesto vysoce výkonnou senzitivní metodu (Er and Chang 2012). Vyžaduje pouze použití standardních PCR reagencí, speciální dsDNA vázajícího fluorescenčního barviva a přístroje umožňujícího HRM. Jedná se o metodu v „uzavřené zkumavce“, tj. amplifikace i samotná analýza jsou provedeny ve stejné zkumavce bez potřeby další post-PCR manipulace, což výrazně snižuje riziko kontaminace (Gundry et al. 2003). Není třeba ani separační krok, který vyžadují ostatní skenovací metody, což snižuje časovou náročnost analýzy. Metoda je nedestruktivní, PCR produkty je možné po HRM analýze následně použít na sekvenační

analýzu. Srovnávacích studie potvrdily stejnou nebo vyšší senzitivitu HRM v porovnání se současně používanými skenovacími metodami (Chou et al. 2005, White et al. 2006).

HRM je vysoce senzitivní metoda, která umožňuje detekovat i jednonukleotidové změny. Různé SNPs však ovlivňují teplotu tání (Tm) s rozdílnou intenzitou, proto byly definovány čtyři třídy SNPs. Nejsnáze lze detekovat 1. SNP třídu, tj. substituce C>T a G>A, které způsobují největší změnu Tm – až 0,5°C. V lidském genomu se tyto změny vyskytují nejčastěji (64 %). Nejhůře se detekují substituce A>T, které způsobují velmi malou změnu Tm – méně jak 0,2°C. Naštěstí jsou tyto substituce vzácné, vyskytují se jen v 7 % případů (Venter et al. 2001).

Byl prokázán vliv délky amplikonu na senzitivitu a specificitu metody, kdy nejvhodnější pro HRM jsou produkty do 400 bp. V produktech o větší délce jsou změny v profilu tání způsobené přítomností mutací menší, což vede k falešné negativitě a ke snížení detekční citlivosti metody. Zároveň výsledky naznačily, že není prokazatelný vztah mezi pozicí SNP v amplikonu a detekční citlivostí (Reed and Wittwer 2004). Jelikož Tm amplikonu se odvíjí také od iontového složení reakční směsi, je velice důležité izolovat DNA stejným extrakčním způsobem (Liew et al. 2004). DNA vzorky různého stáří a izolované rozličným způsobem se doporučují přečistit a rozpustit ve stejném pufru. Hodnotu Tm ovlivňuje i koncentrace DNA, proto je důležité použít do výchozí reakce vzorky DNA o shodných koncentracích. Výhodou je monitorování průběhu amplifikace pomocí real-time PCR (RT-PCR), jen tak je možné se ujistit, že všechny vzorky dosáhly fáze plateau a že v analyzovaných vzorcích je stejné množství DNA bez ohledu na počáteční koncentraci v reakci. Stejně tak by měla být dodržena uniformita mezi vzorky i v dalších směrech. Je nezbytné, aby měly vzorky naprosto shodný objem a aby obsahovaly stejnou koncentraci fluorescenčního barviva. Disociační chování DNA může být ovlivněno i solemi, proto musí být v reakcích přítomné stejné množství pufru i $MgCl_2$. Pro robustní a reprodukovatelnou HRM je také nezbytné správné navržení primerů, kritickým parametrem je i optimalizace PCR. Přítomnost primer dimerů, některých sekvenčních motivů (např.vlásenek) a jiných sekundárních struktur, nespecifických produktů, oblastí s výrazně nízkým či naopak vysokým obsahem bazí G a C a sekvencí s opakujícími se

motivy výrazně ovlivňuje křivku tání a vede k falešně pozitivním výsledkům (Erali and Wittwer 2010).

HRM umožňuje, vyjma mutační skenování, kdy je nezbytné pozitivní záchyt sekvenovat, i genotypizaci známých mutací a polymorfismů, ať už metodou malých amplikonů (Liew et al. 2004) či s využitím neznačených prób (Zhou et al. 2004). Oproti běžným genotypizačním metodám má HRM řadu výhod - obejde se bez potřeby fluorescenčně značených prób, nevyžaduje monitorování amplifikace pomocí RT-PCR a pomocí jedné próby může být detekováno i více alel (Zhou et al. 2004). Genotypizace metodou malých amplikonů využívá poznatku, že čím kratší je testovaný amplikon, tím zřejmější je změna Tm mezi jednotlivými genotypy. Zároveň použití malých amplikonů do 50 bp umožní zkrácení amplifikačních časů a urychlí analýzu (Liew et al. 2004).

Mnoho publikací dokumentovalo úspěchy HRM. Herrmann et al. (2006) provedli srovnávací studii za použití různých HRM přístrojů a fluorescenčních barviv. White et al. (2006) ve své studii hodnotili senzitivitu a specificitu tří přístrojů používaných pro HRM. Chou et al. (2005) provedli srovnání detekční citlivosti HRM s dHPLC. Wittwer et al. (2003), Liew et al. (2004), Reed and Wittwer (2004), Zhou et al. (2004), Graham et al. (2005), Willmore-Payne et al. (2005), Dufresne et al. (2006), Margraf et al. (2006), Montgomery et al. (2007), Gundry et al. (2008), Audrezet et al. (2008) či Grievink and Stowell (2008) popsali na příkladu různých genů úspěšné použití HRM pro mutační skenování či genotypizaci. Zhou et al. (2005) jako první popsali možné paralelní mutační skenování a genotypizaci v jedné reakci, které je umožněné použitím asymetrické PCR. Poměr primerů 1:5 až 1:10 v reakci umožní dostatečný vznik dvouřetězcových produktů pro skenování i dostatečné množství jednořetězcových produktů pro nasednutí prób.

Žádná z publikací se však nezabývá tématikou validace HRM a její implementace do diagnostické praxe dle požadavků norem řízení kvality (ISO 15189), proto jsme se věnovali této problematice v rámci evropského projektu EuroGentest. Problematika validace HRM byla na našem pracovišti dále rozvíjena na modelu genotypizace mutací c.677 C>T (rs1801133: C>T; p.A222V) a c.1298 A>C (rs1801131: A>C; p.E429A) v genu pro methylentetrahydrofolátreduktázu (*MTHFR*) a na příkladu mutačního skenování vybraných exonů genu *CFTR* u cystické fibrózy.

1.2 Poruchy plodnosti

V posledních desetiletích dochází k nárůstu počtu párů s poruchami plodnosti. Tyto poruchy jsou Světovou zdravotnickou organizací (WHO) definovány jako stav, kdy nedojde k otěhotnění za 12 měsíců nechráněného pohlavního styku (Zegers-Hochschild et al. 2009). Udává se, že pomoc pro poruchu plodnosti vyhledá 15 % párů (De Kretser and Baker 1999), kdy se předpokládá přibližně stejný podíl ženského a mužského faktoru na příčině neplodnosti.

1.2.1 Přehled příčin poruch plodnosti

Tyto příčiny lze rozdělit do třech základních kategorií: negenetické, genetické a idiopatické.

U negenetických příčin je neplodnost způsobena fyziologickou, anatomickou, endokrinní, hematologickou či imunologickou poruchou, infekcí nebo psychosociální zátěží (Ulcova-Gallova et al. 2002, Wischmann et al. 2001, Walker 2000). U žen mezi nejdůležitější faktory ovlivňující fertilitu patří věk, jenž se odráží na zdravotním stavu ovárií, oocytů a uteru. Uplatňují se i negativní vlivy prostředí, jako například kontaminace těžkými kovy, vliv radiace, alkoholu a drog (Li et al. 2002b). I u mužů hraje věk významnou roli, kdy velké množství buněčných dělení při vzniku spermatogonií poskytuje prostor pro možné chyby v replikaci a potomci mužů nad 45 let mohou být více ohroženi autozomálně dominantními poruchami v důsledku *de novo* mutací, např. achondroplázií (Kuhnert and Nieschlag 2004). Negativní dopady na kvalitu ejakulátu a tudíž mužskou fertilitu má i zvýšený výskyt umělých estrogenů nebo estrogenům podobných látek v životním prostředí a některé virové infekce (např. příušnice).

Z genetických příčin, které mohou způsobit neschopnosti otěhotnět, jsou to především numerické a strukturální odchylky gonozomů, strukturální aberace autozomů i odchylky na úrovni genů, tj. mutace.

Správná diferenciace pohlaví, která je determinována na základě pohlavních chromozomů (X, Y), je základním krokem k dosažení fertility. Mezi nejčastější příčiny aberantní determinace pohlaví patří numerické odchylky gonozomů. Ty jsou poměrně časté, kdy mozaiky chromozomu X (45X/46XX, patologická linie do 10% všech hodnocených buněk) bývá nejčastějším cytogenetickým nálezem při vyšetřování infertilních párů. Monozomie X (45,X), tj. Turnerův syndrom, jenž je jediná monozomie slučitelná se životem, je však daleko vzácnější, protože 99% postižených plodů je spontánně potraceno. Pro fenotyp dívek s Turnerovým syndromem je typická kožní duplikatura v oblasti krku, nízká hranice vlasů, malá postava a opožděné pohlavní vyzrávání. Inteligence je normální, pacientky jsou schopné plnohodnotného života, avšak z důvodu ovariální dysgeneze jsou neplodné. I syndrom 47,XXX patří mezi klinicky významné příčiny poruch fertility. Ženy s trizomií X nebývají fenotypicky abnormální a jejich funkční gonády produkují gamety, ty však ve zvýšené míře nesou nadpočetný či naopak chybějící chromozom X, což vede ke vzniku chromozomálně abnormálního potomstva a k opakovaným spontánním potratům. Nadpočetný chromozom X v buňkách s chromozomem Y vede k vývoji chlapce postiženého Klinefelterovým syndromem (47,XXY). Ti obvykle bývají vysocí, hubení, s opožděným pohlavním dozráváním a neplodní, neboť se jim v gonádách nevytváří žádné spermie. Každý další nadpočetný chromozom X způsobuje snížení intelektu, zhoršení dysmorfických znaků a vývoje sekundárních pohlavních znaků.

Strukturní abnormality pohlavních chromozomů jsou vzácnější, avšak všechny aberace vedoucí k jejich imbalanci (především delece) ohrožují fertilitu, neboť jsou zasaženy důležité oblasti nesoucí geny, které se přímo podílejí na tvorbě gonád v mužskou či ženskou pohlavní žlázu a na její schopnosti tvořit gamety. U žen je nejčastěji nalézán izochromozom dlouhého raménka X, kdy je dlouhé raménko duplikované, zatímco krátké chybí. Pacientka je postižena sterilitou v důsledku parciální monozomie krátkého raménka tohoto chromozomu. Parciální trizomie dlouhého raménka chromozomu X se nevyznačuje

abnormálním fenotypem a gametogenezi tak neovlivňuje. U mužů bývá fertilita negativně ovlivněna delecemi genu SRY („sex determining region on chromosome Y“, tj. oblasti určující pohlaví na chromozomu Y), kdy mutace nebo delece tohoto genu vedou ke zvratu mužského pohlaví na ženské. SRY je exprimován v podpůrných buňkách nediferencované gonády a aktivuje expresi genu SOX9 („SRY [sex determining region Y]-box 9“), který umožní diferenciaci těchto buněk v Sertoliho buňky. Tyto buňky plní řadu důležitých funkcí: a/ indukují diferenciaci primordiálních zárodečných buněk ve spermatogonie, b/ produkují AMH (anti-müllerický hormon), který inhibuje tvorbu Müllerova vývodu a tak zamezuje vývoji dělohy a vaječníků, c/ indukují diferenciaci Leydigových buněk, které produkují androgeny (tj. testosteron), jenž prostřednictvím androgenního receptoru (AR) částečně řídí sestup varlete a jsou tak plně odpovědné za maskulinizaci zevního genitálu. Mutace či delece genu SOX9 tedy vedou u jedinců s karyotypem 46,XY k vývoji ženského pohlaví, naopak přítomnost tohoto genu u jedinců s karyotypem 46,XX způsobí zvrat v mužské pohlaví. Mutace v genu pro AR (lokalizovaného na X chromozomu) způsobují syndrom necitlivosti k androgenům, tj. testikulární feminizaci. Jedincům s chromozomální konstitucí XY se vyvine ženský zevní genitál, přestože mají varlata a produkují testosteron. Zároveň varlata produkují AMH, díky čemuž tito jedinci nemají dělohu. Zvláštní znak je chybějící pubické a axilární ochlupení, jehož vývoj je také závislý na androgenech. Také delece jiných částí chromozomu Y můžou být příčinou mužské neplodnosti. Jsou to oblasti důležité pro správnou tvorbu spermií, označované jako azoospermické faktory AZFa, AZFb, AZFc (Poongothai et al. 2009).

Příčinou poruch reprodukce mohou být i strukturní aberace autozomů. Ty bývají balancované, tj. genetický materiál je v buňkách přítomen v nezměněném množství, avšak je odlišně uspořádán. Tyto přestavby nemají žádný fenotypický dopad, znamenají však riziko pro další generaci z důvodu vytváření nebalancovaných gamet a zvyšují riziko opakovaných spontánních potratů, intrauterinních úmrtí atd.

Významnou roli ve vzniku neplodnosti hrají i změny na úrovni genů, např. mutace genů hormonální osy „hypotalamus-hypofýza-gonáda-zevní pohlavní ústrojí“. Gonadotropiny uvolňující hormon (gonadotropin-releasing hormon, GnRH) produkovaný hypotalamem reguluje uvolňování hypofyzárních hormonů – gonadotropinů, např.

folikulostimulačního (FSH) a luteinizačního hormonu (LH). Tyto hormony mají důležitou roli ve stimulaci gonád, sekreci steroidních hormonů (estrogenů u žen, testosteronu u mužů) a produkci gamet (Ciccone and Kaiser 2009). Mutace v LH jsou vzácné (Achard et al. 2009) a dosud bylo popsáno jen několik málo mutací v β podjednotce LH asociovaných s infertilitou (Weiss et al. 1992, Ramanujam et al. 1999, Valdes-Socin et al. 2004, Lofrano-Porto et al. 2007, Valdes-Socin et al. 2009, Mafra et al. 2010). Také variantní β podjednotka (v-LHβ), lišící se od klasické alely přítomností dvou jednonukleotidových záměn ve druhém exonu genu pro LHβ způsobující záměnu Trp8Arg a Ile15Thr, byla častěji nalézána u neplodných žen (Takahashi et al. 1999, Takahashi et al. 2004, Du et al. 2012) a u žen s ovulačními poruchami (Ramanujam et al. 1999). U chlapců je tato alela spojována s pomalejším tempem pubertálního vývoje (Raivio et al. 1996). S poruchami plodnosti byly asociovány i mutace v genech pro gonadotropinové receptory - FSHR (Simoni et al. 1999, Ahda et al. 2005) a LHR, kde aktivační mutace LHR vedou k předčasné pubertě u chlapců (Nagasaki et al. 2010), inaktivační mutace mají za následek mužský pseudohermafroditismus či mikropenis (Latronico et al. 1996, Qiao et al. 2009). Polymorfismus Ser312Asn (záměna A/G v pozici 935 v 10. exonu genu LHR) je spojován s poruchou spermatogeneze u chlapců (Simoni et al. 2008). Gen pro LHR je také považován za kandidátní gen asociovaný s předčasným ovariálním selháním (Knauff et al. 2009).

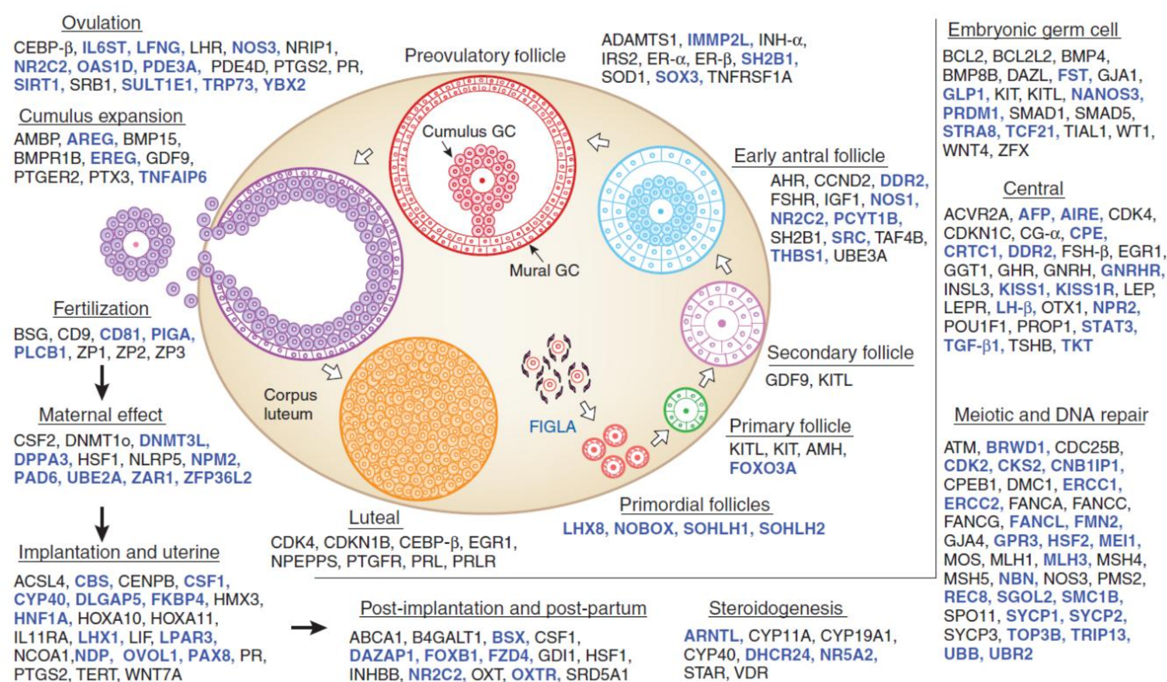
Mezi časté genetické příčiny poruch plodnosti patří i cystická fibróza, která se až u 98% nemocných mužů projevuje obstrukční azoospermií. Ta je způsobená agenezí chámovodů, v důsledku čehož je znemožněn průchod spermií z epididymis do ejakulačních vývodů (Denning et al. 1968, Ferlin et al. 2007). Cystická fibróza je dále podrobněji rozebrána v kapitole 1.2.4.

Konečně, nejen mutace ve výše uvedených genech mohou vést k reprodukčním neúspěchům, uplatňují se i mechanismy imprintingu, kdy metylace DNA určuje, zda je daný embryonální gen exprimován z maternálního či paternálního genomu a metylační profil je tak nezbytný pro správný vývoj zárodku (Emery and Carrell 2006, Kobayashi et al. 2007). Další z faktorů mužské infertility může být i aberantní mitochondriální DNA, která může způsobovat problémy s motilitou spermií (Piasecka and Kawiak 2003),

abnormální zkrácení telomer (Zalenskaya and Zalensky 2002, Hemann et al. 2001) či poruchy v kompaktaci jaderné DNA spermií (Venkatesh et al. 2011), které jsou podrobněji probrány v kapitole 1.2.3.

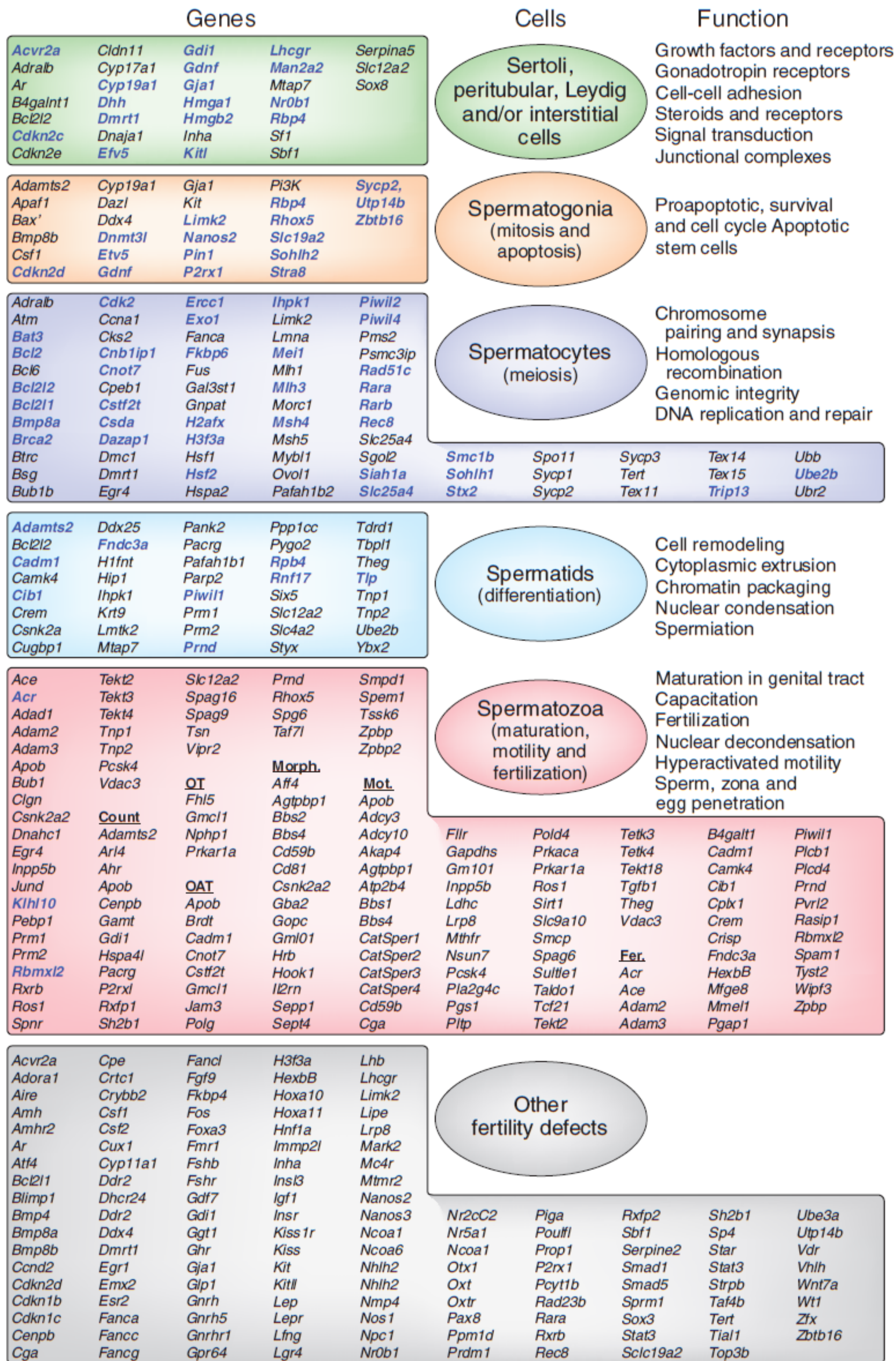
S ohledem na složitost procesů oogeneze a spermatogeneze a velkého množství genů, které se zde uplatňují (Obr. 3, Obr. 4) (Matzuk and Lamb 2008), je pochopitelné, že i přes aktivní snahu posledních let identifikovat všechny příčiny neplodnosti, stále velké procento případů zůstává neobjasněno (Nuti and Krausz 2008).

Obr. 3 Přehled genů uplatňujících se při oogenezi



Matzuk and Lamb 2008

Obr. 4 Přehled genů uplatňujících se při spermatogenezi



Matzuk and Lamb 2008

1.2.2 Spermatogeneze a hodnocení spermiogramu

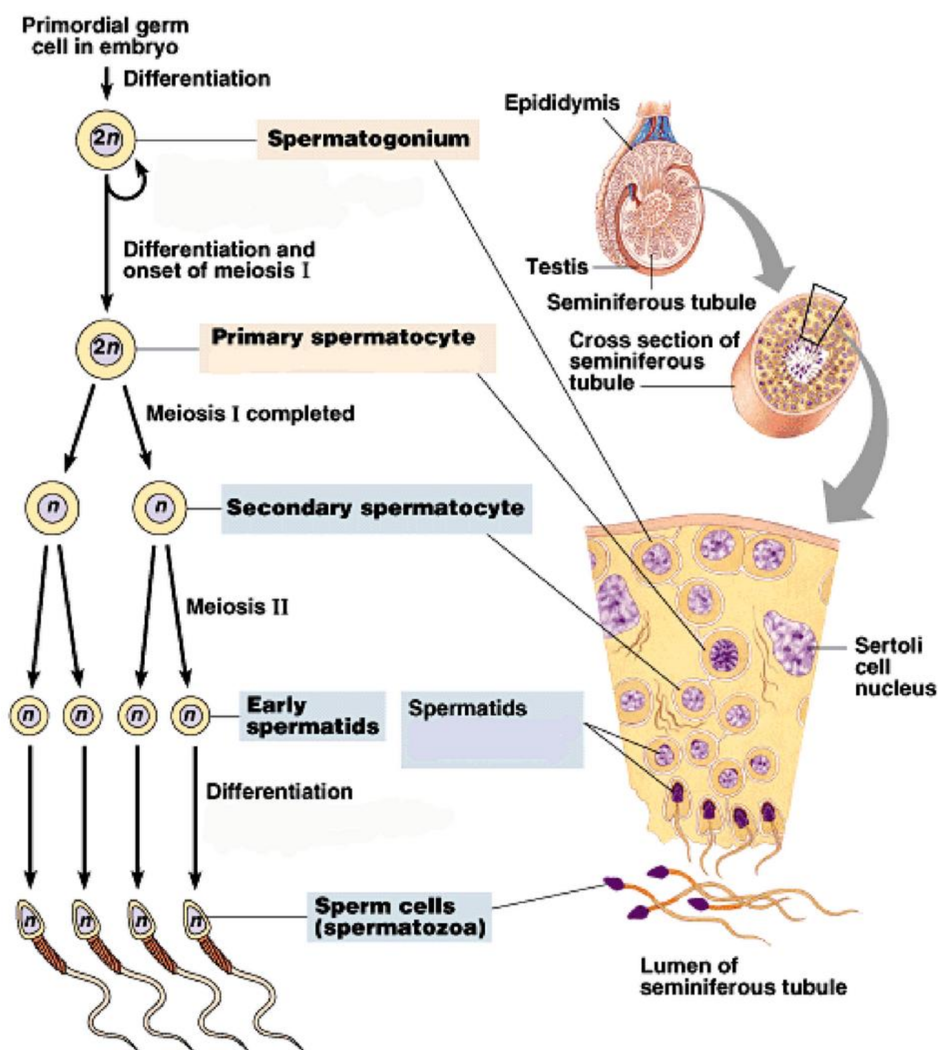
Spermatogeneze je složitý a vysoce specializovaný proces, jehož podstatou je transformace nediferencovaných pohlavních buněk (spermatogonií) ve zralé pohlavní buňky (spermie). Začíná v pubertě a za dostatečné stimulace pohlavními hormony (zejména testosteronem) probíhá po celý život muže.

Tvorba mužských pohlavních buněk probíhá za podpory Sertoliho buněk v semenotvorných kanálcích varlete mimo břišní dutinu, čímž se docílí o 2-3°C nižší teploty, která je potřebná pro správný průběh tohoto procesu. Spermie nevznikají v celém varleti naráz, ale ve vlnách v různých částech semenotvorných kanálků probíhají různé fáze vývoje (Obr. 5). Celý proces trvá přibližně 72 dní (± 2 dny).

Tvorba spermií má tři fáze: rozmnožovací, růstovou a zrání. Na počátku vývoje spermií jsou spermatogonie (kmenové buňky), které zůstávají v semenotvorném kanálku jedince po celý život. Mitoticky se dělí a vznikají primární spermatocyty (spermatocyty I. řádu). Ty jsou diploidní, avšak po prvním meiotickém dělení z nich vznikají haploidní sekundární spermatocyty (spermatocyty II. řádu). Ty mají stále zdvojené chromatidy, proto záhy dochází k druhému meiotickému dělení, jehož produktem jsou haploidní spermatidy. Po celou dobu zůstávají vyvíjející se pohlavní buňky navzájem propojené cytoplazmatickými můstky, což umožňuje synchronizaci vývoje vždy jedné populace pohlavních buněk. Spermatidy poté prochází procesem spermiogeneze, tj. postupné úpravy haploidní spermatidy na morfologicky zralou spermii, při které dochází k maximální kondenzaci chromatinu díky náhradě histonů za protaminy (viz. kapitola 1.2.3). Během tohoto procesu lze rozeznat tři vývojové kategorie spermatid: časné spermatidy s kruhovým jádrem, spermatidy s protáhlým jádrem a zralé spermatidy s jádrem kondenzovaným (Dadoune 1995). Vytvoří se bičík, kde centriola funguje jako templát pro jeho tvorbu. V jeho bázi se koncentrují mitochondrie a vytváří tzv. mitochondriální spirálu. Splynutím cisteren Golgiho aparátu vznikne akrozom a spermie se zbaví nadbytečné cytoplazmy, kterou odvrhuje ve formě reziduálních tělísek, která jsou fagocytována Sertoliho buňkami.

Spermie po dokončení spermiogeneze je sice morfologicky zralá (tj. vypadá jako „finální“ spermie), nicméně není schopná rozpoznat, navázat se a oplodnit tak vajíčko. Tyto schopnosti získává až v dalších maturačních procesech v nadvarleti. Dochází zde ke změnám ve složení a struktuře cytoplazmatické membrány, která je obohacena o cholesterol a další proteiny. Upravuje se také tvar a obsah akrozomu. Nakonec jsou spermie skladovány v koncové části nadvarlete („cauda epididymis“) do doby, než jsou ejakulovány.

Obr. 5 Spermatogeneze



http://cikgurozaini.blogspot.cz/2011/06/spermatogenesis-and-oogenesis.html

Moderní metody léčby poruch plodnosti umožňují účinně pomoci v převážné většině případů, podmínkou je však správná diagnóza. Vyšetření mužské plodnosti vychází vždy z provedení spermiogramu, tj. analýzy ejakulátu. Z hlediska vyšetřovacího algoritmu se jedná o zcela prvotní vyšetření u mužské neplodnosti.

Sperma se odebírá po dvoudenní pohlavní abstinenci (delší abstinence než 3 dny není žádoucí) při pečlivém dodržení hygieny. Kratší odstup od předchozí ejakulace může snížit objem ejakulátu a koncentraci spermií. Při delší abstinenci může být snížená motilita. Odběr je proveden do speciální jednorázové sterilní nádobky na andrologických pracovištích.

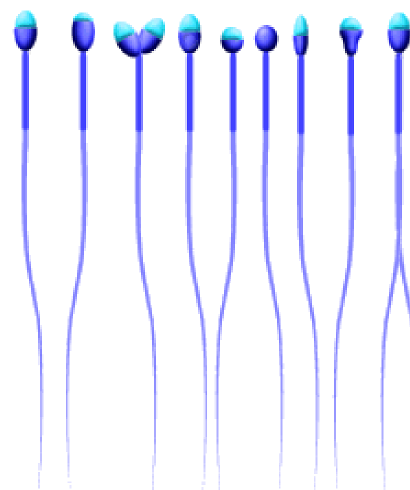
Mezi základní parametry vyšetření spermatu patří vyšetření stanovení objemu, pH, zkapalnění, dále pak počtu spermií, jejich pohyblivosti (podíl pohyblivých/nepohyblivých spermií, charakter jejich pohybu) a morfologie (podíl spermií které mají správný/špatný tvar). V rámci rozšířených vyšetření lze zjišťovat stupeň poškození DNA ve spermiích (stupeň fragmentace chromatinu) a podíl nezralých spermií.

Ke stanovení objemu ejakulátu je optimální použít váhy nebo sérologickou pipetu, která je kalibrovaná po 0,1 ml. Ke stanovení koncentrace spermií (počet spermií na mililitr) se používají speciální počítací komůrky pro vyšetření spermatu.

Zkapalnění je přirozená změna konzistence spermatu z polotekuté na tekutou. V gelovité matrix nejsou spermie schopny rovnoměrné distribuce, odebrané vzorky proto mají rozdílné parametry. Teprve po úplném zkapalnění se spermie uvolní a jejich rozptýlení se stane rovnoměrnější. Porucha zkapalnění ztěžuje spolehlivost vyšetření. Čerstvý ejakulát je charakterizován přítomností koagula, které je tvořeno sekretem semenných váčků. Jeho zkapalnění je výsledkem působení prostatických enzymů. Nepřítomnost koagula může být známkou absence semenných váčků. Porucha zkapalnění může být projevem deficitu prostatických enzymů. Viskozita ejakulátu se hodnotí po jeho zkapalnění jako schopnost tvořit kapky na špičce pipety. Pokud se tvoří drobné kapky a volně padají, je viskozita normální. Pokud se kapky netvoří nebo sperma nelze do pipety natáhnout, viskozita je zvýšena.

Pohyblivost spermií může být ovlivněna mnoha faktory včetně věku pacienta, jeho zdravotního stavu, doby od poslední ejakulace, expozice pacienta vlivům zevního prostředí jako je horko nebo toxické látky, dále metody odběru a způsobu zacházení se vzorkem v době od odběru do vyšetření. Vyšetřuje se systematicky minimálně 200 spermií v alespoň 5 zorných polí mikroskopu a stanoví se kategorie motility: a/ rychlý progresivní pohyb, b/ pomalý progresivní pohyb, c/ pohyb na místě a d/ nepohyblivé spermie.

Pro hodnocení morfologie spermií je potřeba minimálně dvou nátěrů. Při vyšetření se můžeme setkat s různými tvary spermií (Obr. 6).



Obr. 6 Morfologické typy spermií
(1) Normální spermie je hodnocena jako morfologicky normální, pokud jsou hlavička, krček, střední oddíl a bičík bez patologických změn. Tvar hlavičky je oválný, akrozom pokrývá 40 – 70 % hlavičky spermie, (2) spermie s malým akrozómem, (3) spermie a se dvěma hlavičkami, (4) spermie s vakuolou v hlavičce, (5) spermie s kulatou hlavičkou, (6) s kulatou hlavičkou bez akrozómu, (7) se zúženou hlavičkou, (8) hruškovitou hlavičkou a (9) se dvěma bičíky (www.repromeda.cz/komplexni-diagnostika-neplodnosti.html)

Vzhledem k pomalé setrvalé tendenci ke snižování kvality mužského ejakulátu WHO pravidelně aktualizuje hodnoty pro normozoospermii (WHO, 2010): objem ejakulátu minimálně 1.5 ml, pH 7.2-8.4, koncentrace spermií minimálně 15 miliónů na mililitr, počet pohyblivých spermií minimálně 40 %, z toho progresivně se pohybujících (WHO třídy a+b) minimálně 32 %. Počet morfologicky normálních spermií nad 4%.

Závěrem spermiogramu může být diagnóza a/ normozoospermie (veškeré hodnocené parametry ejakulátu jsou normální), b/ oligozoospermie (snížený počet spermií), c/ azoospermie/aspermie (v ejakulátu nejsou žádné spermie), d/ asthenozoospermie (snížený počet pohyblivých spermií či zhoršená pohyblivost spermií), e/ teratozoospermie (snížený počet spermií, které mají normální tvar) či f/ nekrozoospermie (spermie v ejakulátu jsou mrtvé).

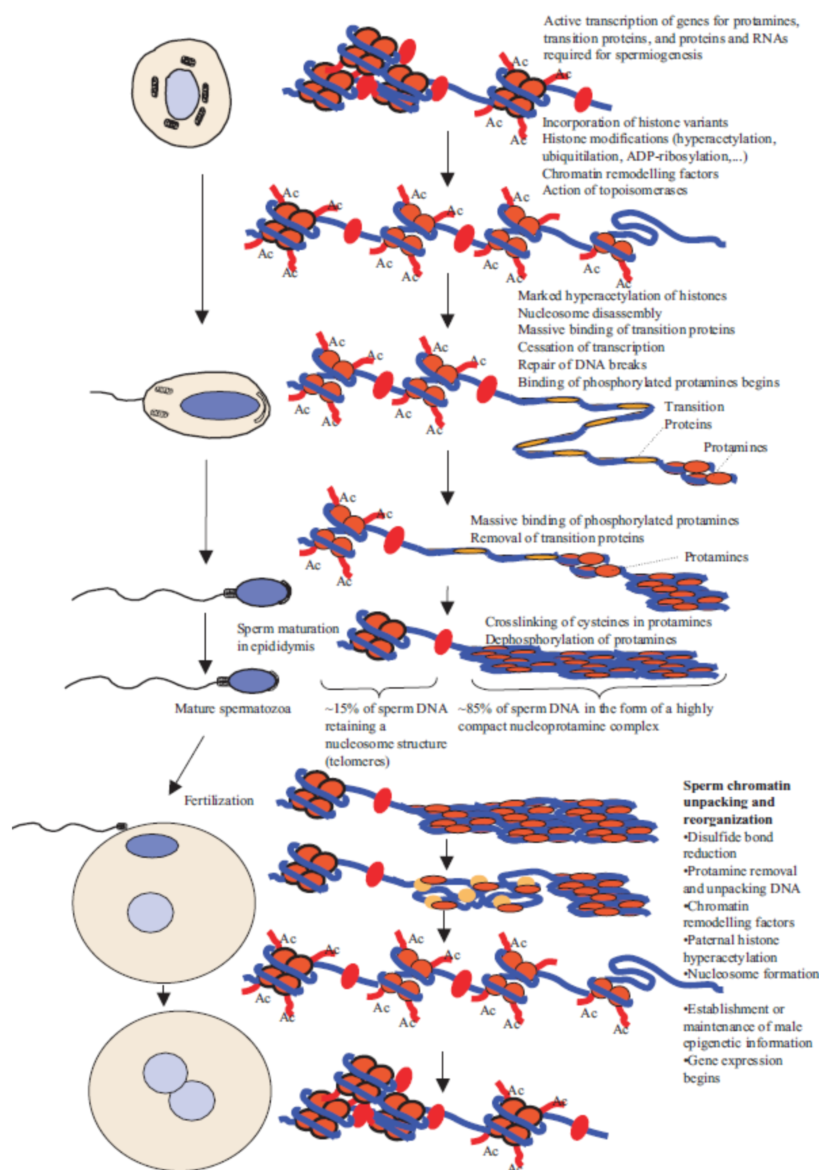
1.2.3 Protaminy a mužská neplodnost

Protaminy jsou malé bazické jaderné proteiny známé po více než století (Miescher 1874). Vyskytují se u mnoha živočišných druhů, kde hrají stěžejní roli při spermatogenezi. V hlavičkách spermií nahrazují histony a umožňují vměstnat haploidní paternální genetickou informaci do malého jádra spermií. Objem kondenzované DNA v mužských zárodečných buňkách je díky protaminům redukován na méně než 10% objemu jádra buněk somatických (Braun 2001). Tato kondenzace je nezbytná pro neporušené vyzrávání spermií, transport do oocytu a schopnost jej oplodnit (Jager 1990).

Protaminy kondenzují paternální genom a jádra spermií do kompaktního hydrodynamického tvaru, což umožní rychlejší pochyb spermií a zvýší jejich pravděpodobnost oplodnit oocyt jako první. Zvýšená kondenzace chromatinu zároveň chrání paternální genetickou informaci při transportu reprodukčním traktem před vlivem nukleáz, mutagenů či jiných genotoxických faktorů a zajišťuje tak stabilitu a integritu DNA. Dále se předpokládá vliv protaminů při epigenetickém imprintingu, kdy ovlivňují reaktivaci paternální části genomu po fertilizaci a umožňující reprogramování. Protaminy se pravděpodobně uplatňují jako jeden z kontrolních bodů při spermiogenezi (Oliva 2006).

Nahrazení histonů protaminy se odehrává v postmeiotické fázi během spermiogeneze v několika postupných krocích (Obr. 7). Nejprve je aktivována transkripce protaminových genů (*PRM1* a *PRM2*) a genů pro tranziční proteiny (*TNP1* a *TNP2*). Následně dochází k modifikaci nukleozomů. Specifické lysiny histonů H3 a H4 jsou hyperacetylavány, čímž se sníží jejich pozitivní náboj a tím jejich afinita k DNA. Tato destabilizace nukleozomů umožní výměnu histonů za tranziční proteiny. Ty jsou následně nahrazeny fosforylovanými protaminy (Dadoune 1995, Kurtz et al. 2007). K jejich fosforylaci dochází v okamžiku syntézy, kdy fosforylace je pravděpodobně nezbytná pro správnou vazbu protaminů na DNA (Oliva and Dixon 1991). Maximální kondenzace DNA a finální maturace spermií je dosaženo defosforylací protaminů a stabilizací protaminových komplexů disulfidickými můstky mezi cysteiny. Po fertilizaci dojde k dekondenzaci DNA a reorganizaci chromatinu, tj. k přestavbě nukleoprotaminů na nukleohistony, čímž jsou geny opět transkripčně aktivovány (Oliva 2006).

Obr. 7 Schematické zobrazení nukleohistonovo-nukleoprotaminové přestavby



Oliva 2006

V hlavičkách spermií je protaminy nahrazeno přibližně 85% histonů, zbytek DNA zůstává asociován s histony, nejspíše z důvodu umožnění reaktivace paternálního genomu po fertilizaci či z důvodu zachování imprintingu příslušných paternálních genů (Gatewood et al. 1990).

Protaminy se vyznačují vysokým obsahem aminokyselin s pozitivním nábojem, zejména argininu (48% v lidských protaminech). V sekvenci se dále vyskytuje cystein, jenž umožňuje tvorbu disulfidických můstků mezi přilehlými molekulami protaminů, čímž dochází k maximální stabilizaci nukloeoprotaminových komplexů (Vilfan et al. 2004).

Protaminové geny jsou lokalizované na chromozomu 16 (16p13.13) v sousedství genu TNP2, což umožňuje koordinaci exprese těchto genů při spermiogenezi (Viguie et al. 1990, Engel et al. 1992, Martins et al. 2004). Oba protaminové geny *PRM1* a *PRM2* se sestávají ze dvou exonů s jedním intronem o délce 91, resp. 163 bp (Domenjoud et al. 1990). Aminokyselinová sekvence PRM1 čítá 50 aminokyselin, z nichž 24 jsou argininy a 6 cysteiny, zatímco PRM2 je translatován jako 103 aminokyselin dlouhý protein, který na N-terminálním konci podstupuje štěpení. Finální PRM2 protein se skládá z 57 aminokyselin, z nichž 27 jsou argininy a 5 cysteiny (Ammer et al. 1986, Carrell et al. 2007).

Předpokládá se rozdílná funkce obou protaminových proteinů, ačkoliv pro správnou diferenciaci spermatid by mělo být jejich množství v hlavičkách spermií rovnocenné v poměru 1:1 (Balhorn et al. 1988). Studie na knokautovaných myších, kdy je cíleně vyřazen z činnosti přesně určený gen, a studie pacientů s poruchou kondenzace chromatinu ve spermiích prokázaly, že deficience některého z protaminových genů či jejich aberantní poměr vede k poškození DNA a snížené kvalitě semenných parametrů, ke zhoršené schopnosti fertilizce, k abnormálnímu embryonálnímu vývoji a tak ke sníženým úspěchům při IVF technikách či přímo k neplodnosti (Balhorn et al. 1988, Belokopytova et al. 1993, Chevaillier et al. 1987, de Yebra et al. 1998, de Yebra et al. 1993, Khara et al. 1997, Carrell and Liu 2001, Carrell et al. 2007, Carrell et al. 2008, Steger et al. 2003, Aoki and Carrell 2003, Aoki et al. 2005, Aoki et al. 2006b, Cho et al. 2001, Cho et al. 2003, Zhang et al. 2006).

Velké množství studií se pokoušelo prokázat vztah mutací a polymorfismů protaminových genů k mužské neplodnosti (Schlicker et al. 1994, Tanaka et al. 2003, Aoki et al. 2006a, Iguchi et al. 2006, Hammoud et al. 2007, Ravel et al. 2007, Kichine et al. 2008, Gazquez et al. 2008, Imken et al. 2009, Jodar et al. 2011, Venkatesh et al. 2011).

Výsledky jsou však nejednoznačné, protichůdné a detekované varianty jsou považovány spíše za vzácnou příčinu infertility (Tab. 1, Tab. 2).

Tab. 1 Přehled variant detekovaných v genu *PRM2* v různých studiích

Nucleotide change	Region	Amino acid change	NCBI ID	Mutation described only in patients	Statistically significant differences between control vs. patients	Comments	References
c.-512 T > G	5′ promoter region	NA		Yes	No	Rare variant	Present work
c.-392 G > A	5′ promoter region	NA		No	No	Polymorphism	Hammoud *et al.* (2007), present work
c.-389 T > C	5′ promoter region	NA	rs 376374	No	No	Polymorphism	Hammoud *et al.* (2007), present work
c.-371 G > C	5′ promoter region	NA	rs 8060767	No	No	Polymorphism	Hammoud *et al.* (2007), present work
c.-321 C > T	5′ promoter region	NA		No	No	Polymorphism	Present work
c.-226 G > A	5′ promoter region	NA	rs 74459443	No	No	Polymorphism	Hammoud *et al.* (2007), present work
c.-123 C > G	5′ promoter region	NA		Yes	No	Pathogenicity unknown	Present work
c.-67 C > T	5′ UTR	NA		Yes	No	Potential interference transcription	Imken *et al.* (2009)
c.66 T > C	Exon 1	None		No	No	Rare variant	Aoki *et al.* (2006a), present work
				Yes	–	Rare variant	Tüttelmann *et al.* (2010)
c.87 C > T	Exon 1	None		No	No	Rare variant	Imken *et al.* (2009)
c.148 C > T	Exon 1	Gln50Ter		Yes	No	Null allele	Tanaka *et al.* (2003)
c.201 C > T	Exon 1	None		No	No	Rare variant	Aoki *et al.* (2006a), Imken *et al.* (2009), present work
				Yes	–	Rare variant	Tüttelmann *et al.* (2010)
c.271 + 10 C > T	Intron	NA	rs 74007626	Yes	No	Novel donor splice site?	Aoki *et al.* (2006a)
				Yes	Yes	Novel donor splice site?	Imken *et al.* (2009)
				No	No	Rare variant	Present work
				Yes	–	Rare variant	Tüttelmann *et al.* (2010)
c.271 + 17 G > C	Intron	NA		No	No	Rare variant	Imken *et al.* (2009)
c.271 + 19 C > T	Intron	NA	rs 74007625	Yes	No	Novel donor splice site?	Aoki *et al.* (2006a)
				No	No	Rare variant	Imken *et al.* (2009), present work
				Yes	–	Rare variant	Tüttelmann *et al.* (2010)
c.271 + 27 G > C	Intron	NA	rs 1646022	No	No	Polymorphism	Tanaka *et al.* (2003), Aoki *et al.* (2006a), Imken *et al.* (2009), present work
				Yes	–	Polymorphism	Tüttelmann *et al.* (2010)
c.271 + 27 G > A	Intron	NA		No	No	Rare variant	Tanaka *et al.* (2003)
c.271 + 29 A > G	Intron	NA		Yes	–	Rare variant	Tüttelmann *et al.* (2010)
c.271 + 102 C > A	Intron	NA	rs 2070923	No	No	Polymorphism	Tanaka *et al.* (2003), Aoki *et al.* (2006a), Imken *et al.* (2009), present work
				Yes	–	Polymorphism	Tüttelmann *et al.* (2010)
c.271 + 106 C > A	Intron	NA		Yes	–	Rare variant	Tüttelmann *et al.* (2010)
c.271 + 107 G > A	Intron	NA		Yes	No	Novel donor splice site?	Imken *et al.* (2009)
c.271 + 135 C > T	Intron	NA		Yes	No	Novel donor splice site?	Aoki *et al.* (2006a), present work
				No	No	Rare variant	Imken *et al.* (2009)
c.309*61 G > C	3′ UTR	NA	rs 79674436	No	No	Abnormal P1/P2	Hammoud *et al.* (2007)
				No	No	Rare variant	Present work

Jodar et al. 2011

Tab. 2 Přehled variant detekovaných v genu *PRM1* v různých studiích

Nucleotide change	Region	Amino acid change	NCBI ID	Mutation described only in patients	Statistically significant differences between control vs. patients	Comments	References
c.-275 G > T	5′ promoter region	NA	rs 74007631	Yes	No	Rare variant	Hammoud *et al.* (2007)
c.-248 C > A	5′ promoter region	NA		Yes	No	Rare variant	Hammoud *et al.* (2007), present work
c.-238 G > A	5′ promoter region	NA		Yes	No	Rare variant	Hammoud *et al.* (2007)
c.-191 C > A	5′ promoter region	NA	rs 2301365	No	No	Polymorphism	Hammoud *et al.* (2007), Ravel *et al.* (2007), Imken *et al.* (2009)
				No	Yes	Risk factor for abnormal morphology	Gázquez *et al.* (2008), present work
c.-114 C > T	5′ promoter region	NA	rs 74007629	Yes	No	Rare variant	Hammoud *et al.* (2007)
c.-107 G > C	5′ UTR	NA		Yes	No	Rare variant	Hammoud *et al.* (2007)
				Yes	No	Create new binding site?	Ravel *et al.* (2007), Imken *et al.* (2009)
				No	No	Population-specific variant	Kichine *et al.* (2008)
c.-102 C > T	5′ UTR	NA		Yes	No	Rare variant	Hammoud *et al.* (2007)
c.42 A > G	Exon 1	None		No	No	Rare variant	Tanaka *et al.* (2003)
c.49 C > T	Exon 1	Arg17Cys		Yes	No	Pathogenicity unknown	Present work
c.54 G > A	Exon 1	None	rs 3526993	No	No	Rare variant	Aoki *et al.* (2006a), Ravel *et al.* (2007), Imken *et al.* (2009)
				Yes	–	Rare variant	Tüttelmann *et al.* (2010)
c.65 G > A	Exon 1	Ser22Asn		Yes	No	Pathogenicity unknown	Imken *et al.* (2009)
c.69 C > A	Exon 1	None		No	No	Rare variant	Tanaka *et al.* (2003)
c.93 G > C	Exon 1	Gln31His		No	No	Rare variant	Ravel *et al.* (2007)
c.102 G > T	Exon 1	Arg34Ser	rs 35576928	Yes	No	Create a new site for phosphorylation?	Iguchi *et al.* (2006), Ravel *et al.* (2007)
				No	No	Rare variant	Aoki *et al.* (2006a), Kichine *et al.* (2008), present work
				Yes	–	Rare variant	Tüttelmann *et al.* (2010)
c.112 + 40 G > A	Intron	NA		Yes	No	Pathogenicity unknown	Present work
c.113 G > T	Exon 2	Arg38Met		No	No	Rare variant	Present work
c.119 G > A	Exon 2	Cys40Tyr		Yes	No	Pathogenicity unknown	Ravel *et al.* (2007)
c.138 G > A	Exon 2	None		Yes	No	Rare variant	Tanaka *et al.* (2003)
c.139 C > A	Exon 2	None	rs 737008	No	No	Polymorphism	Tanaka *et al.* (2003), Iguchi *et al.* (2006), Aoki *et al.* (2006a), Ravel *et al.* (2007), Imken *et al.* (2009), present work
				Yes	–	Polymorphism	Tüttelmann *et al.* (2010)
c.153*4 T > A	3′ UTR	NA		Yes	No	Pathogenicity unknown	Present work
c.153*89 C > T	3′ UTR	NA		Yes	No	Rare variant	Hammoud *et al.* (2007)
c.153*96 A > G	3′ UTR	NA		No	No	Rare variant	Tanaka *et al.* (2003), Hammoud *et al.* (2007), present work

Jodar et al. 2011

1.2.4 Cystická fibróza a gen CFTR

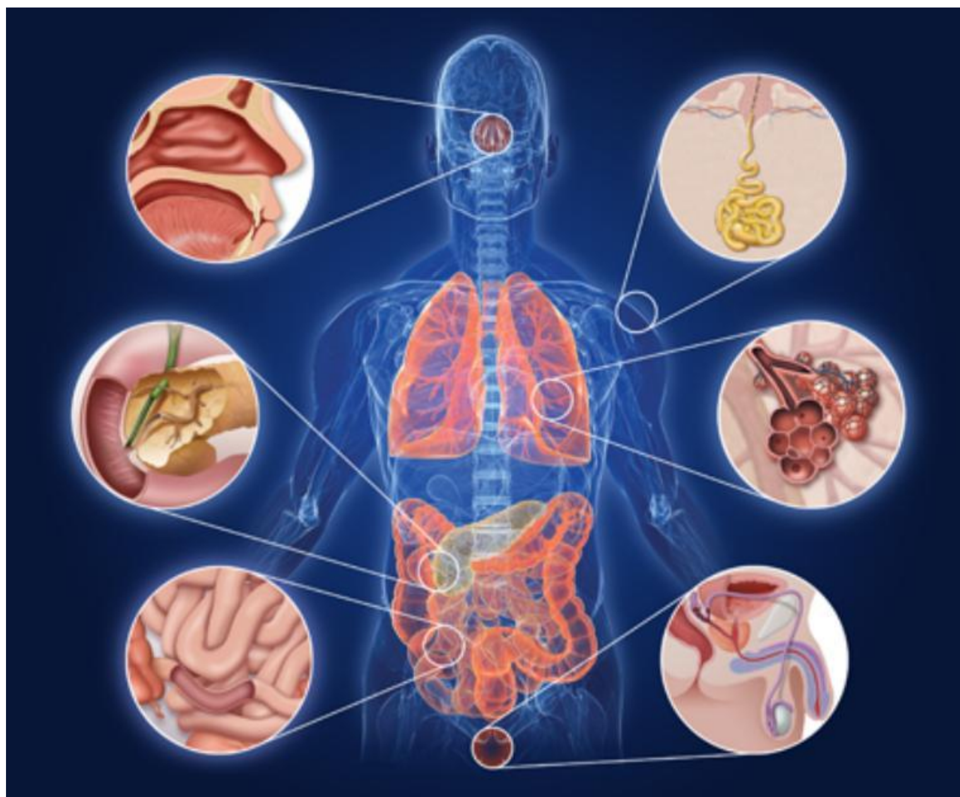
Jak již bylo uvedeno výše, mutace v genu cystické fibrózy hrají významnou roli v patogenezi mužské neplodnosti a jejich vyšetření tak bývá jedním z prvotních kroků při odhalování příčin poruch plodnosti u mužů. Tyto mutace však neovlivňují pouze reprodukci, ale u obou pohlaví i funkce celého těla - jeho růst, dýchání a trávení.

Cystická fibróza (CF) představuje jedno z nejčastějších závažných autozomálně recesivních onemocnění v evropských populacích, vyskytující se s incidencí 1:1700-7700, kde nejvyšší incidence je nalézána v Irsku, nejnižší ve Finsku (Lubamba et al. 2012). Údaje o četnosti onemocnění v České republice (ČR) se různí. Zatímco epidemiologické studie v padesátých a šedesátých letech minulého století prokázaly postižení u 1 z 2700 novorozenců (Houšťek and Vávrová 1962), nedávné studie novorozeneckého screeningu odhalily incidenci nižší – 1:4000 při započtení vlivu prenatální diagnostiky (Balascakova et al. 2009), resp. 1:5000 bez započtení tohoto vlivu (Krulisova et al. 2012). Z údajů vyplývá, že každý 26.-35. jedinec je zdravým nosičem CF a že se při průměrné roční porodnosti 110 000 novorozenců každoročně narodí v ČR 20-40 dětí s CF.

CF je závažné multisystémové onemocnění charakterizované mnohačetným orgánovým postižením respiračního, gastrointestinálního a reprodukčního systému (Obr. 8). Mezi typické klinické příznaky patří chronické sinopulmonální onemocnění projevující se chronickým kašlem s produkcí sputa. Typická je kolonizace sinobronchiálního systému bakteriálními kmeny *Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae* či *Burkholderia cepacia,* jež způsobují ireverzibilní poškození plic a následné respirační selhání. Dalšími klinickými projevy respiračního onemocnění jsou bronchiektázie, nosní polypy či rozvoj paličkovitých prstů. Onemocnění gastrointestinálního traktu je charakterizováno poruchou vstřebávání živin spojenou s následným neprospíváním. Tyto příznaky jsou vyvolány porušenou exokrinní sekrecí pankreatu, která postihuje až 90% nemocných. U zhruba 10-15% novorozenců s CF se nemoc projeví jako mekoniový ileus, který je způsoben střevní obstrukcí abnormálně vazkou smolkou (Vávrová et al. 2000). Reprodukční trakt je postižen u téměř všech mužů s CF (až 98%), kteří jsou v důsledku kongenitální bilaterální absence (ageneze) vas deferens

(CBAVD) neplodní (Denning et al. 1968). U žen může být snížena schopnost otěhotnět díky vazkému hlenu v děložním hrdle, který může bránit proniknutí spermie do děložní dutiny (Oppenheimer and Esterly 1970). Projevy onemocnění a jejich závažnost jsou však u jednotlivých pacientů odlišné. Značná variabilita se projevuje jak v průběhu onemocnění dýchacích cest, stejně tak v míře postižení pankreatu, kde může dojít ke kompletní ztrátě její exokrinní funkce, označované jako pankreatická insuficience, přes různé stupně poruchy sekrece až po plné zachování funkce slinivky břišní (Welsh et al. 2001). Společným klinickým příznakem pro všechny nemocné je postižení funkce potních žláz, kdy je porušena resorpce chloridů a sodíku ve vývodech potní žlázy. To vede k jejich „diagnosticky" zvýšené koncentraci v potu, od hraničních hodnot 30-60 mmol/l u mírných/atypických forem CF až kolem 100 mmol/l u pacientů s klasickou CF (Vávrová et al. 2001, Welsh et al. 2001). Stanovení koncentrace chloridů v potu, označované jako potní test, je tak považováno za zlatý standard diagnostiky CF.

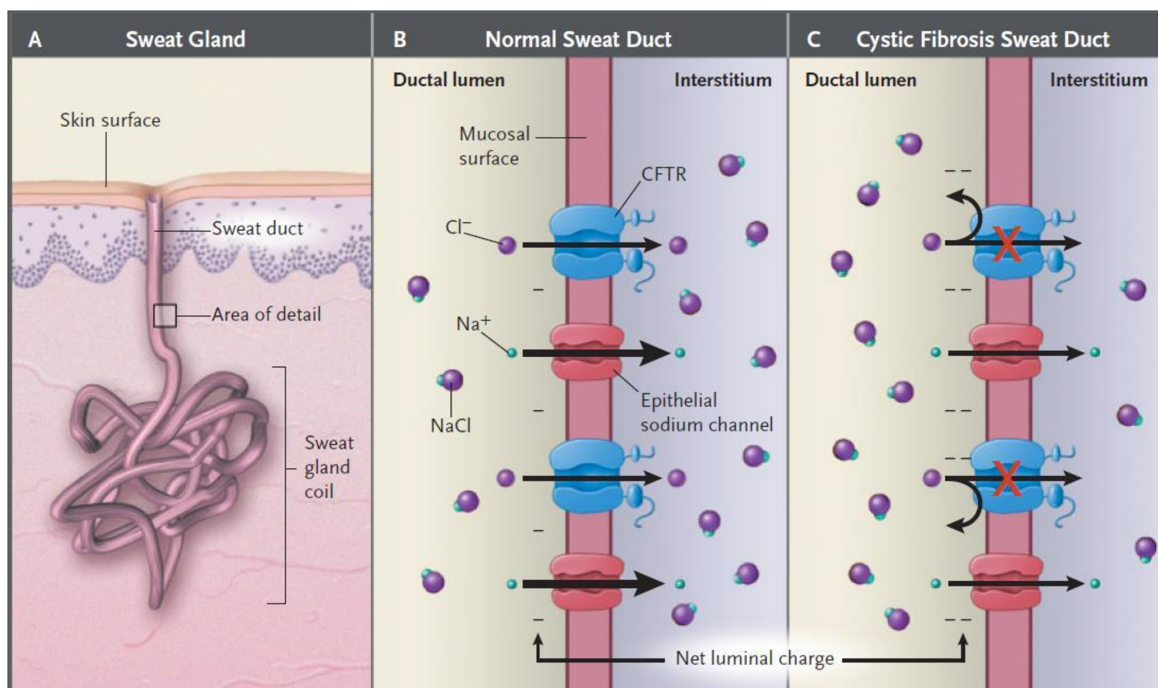
Obr. 8 Schematické znázornění rozsahu postižení u pacientů s CF



www.cftrscience.com

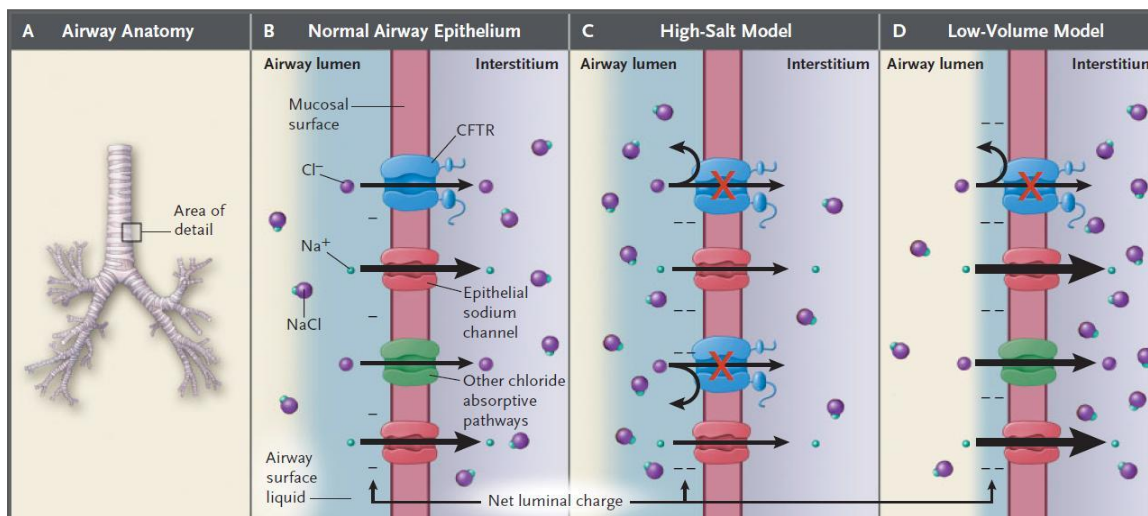
Výše popsané klinické projevy jsou důsledkem defektu v proteinu CFTR (Cystic Fibrosis Transmembrane Conductance Regulator), který má funkci chloridového kanálu a je tak nezbytný pro transport chloridových iontů, regulaci sodíkového kanálu (epithelial sodium channel - ENaC) a dalšího chloridového kanálu (outwardly rectified chloride channel - ORCC) v epiteliálních buňkách, které vystýlají sliznici respiračního traktu, pankreatické vývody a vývody potních žláz. Při absenci, redukci či aberantní funkci chloridového kanálu v apikální membráně specializovaných epiteliálních buněk je resorpce chloridů z lumen exokrinních žláz a dýchacích cest porušena. Zároveň dochází ke zvýšené resorpci sodíku, a protože jej voda následuje do buněk, vzniká tak iontová dysbalance spojená rovněž s dehydratací sliznic exokrinních žláz. Iontová nerovnováha má rovněž za následek poruchu funkce faktorů vrozené imunity (Obr. 9, Obr. 10) (Rowe et al. 2005, Simpson 2005).

Obr. 9 Model normální (B) a porušené funkce CFTR kanálu (C) v potních žlázách



Rowe et al. 2005

Obr. 10 Model normální (B) a porušené funkce CFTR kanálu (C, D) v respiračním traktu

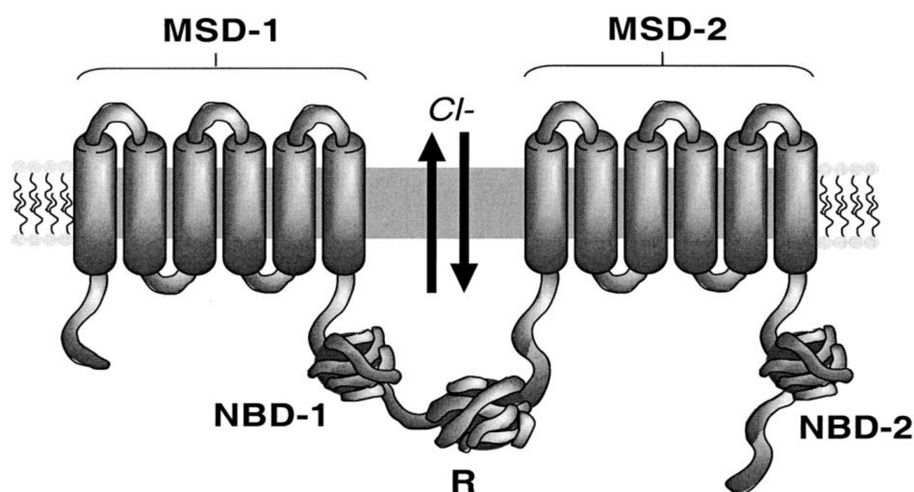


Rowe et al. 2005

Identifikace genu zodpovědného za CF je datována do roku 1989, kdy byl metodou pozičního klonování lokalizován na dlouhé raménko chromozomu 7 (7q.31.2) a nazván Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR)*, tj. transmembránový regulátor vodivosti (Kerem et al. 1989). Tento gen, zaujímající oblast zhruba 250 kb, má 27 exonů (1, 2, 3, 4, 5, 6a, 6b, 7, 8, 9, 10, 11, 12, 13, 14a, 14b, 15, 16, 17a, 17b, 18, 19, 20, 21, 22, 23 a 24, nově 1-27) (Riordan et al. 1989). Z 6,2 kb dlouhé *CFTR*-mRNA vzniká translací protein o velikosti 1480 aminokyselin a molekulární hmotnosti 168 kDa (Harris 1992).

CFTR protein se skládá z pěti domén. Ze dvou transmembránových segmentů MSD1 a MSD2 (membrane spanning domains), které jsou obě tvořeny 6 α-helixy. Ty vytvářejí vlastní chloridový kanál a ukotvují ho v apikální buněčné membráně. Ze dvou ATP-vazebných domén NBD1 a NBD2 (nucleotide-binding domains), které svými konformačními změnami regulují průtok iontů chloridovým kanálem, a z regulační domény R, která obsahuje velké množství nabitých aminokyselinových zbytků, které představují potenciální místa pro fosforylaci cAMP-dependentní proteinkinázou A (PKA) (Obr. 11)/(Gadsby et al. 2006, Lyczak et al. 2002).

Obr. 11 Schematická struktura proteinu CFTR



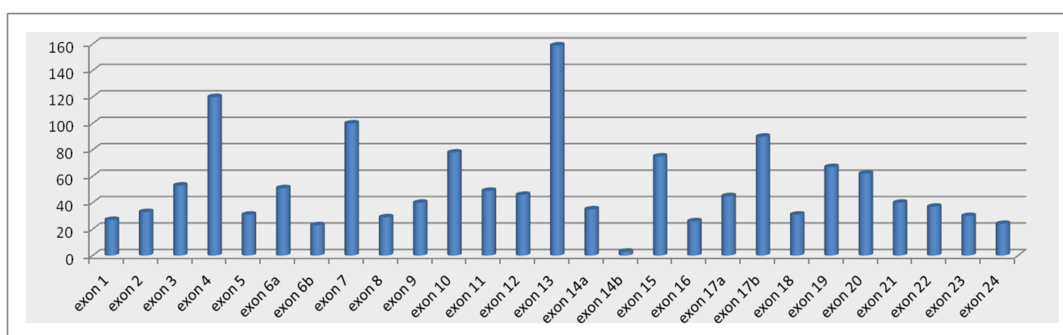
Lyczak et al. 2002

Bylo navrženo několik modelů regulace proteinu CFTR. Podle Carson et al. (1995) dochází k vazbě ATP na obě NBD domény v uzavřeném stavu. Následná hydrolýza ATP na NBD1 způsobí otevření kanálu a ionty mohou procházet pórem. Kanál je uzavřen uvolněním produktů hydrolýzy (ADP a Pi). Tento model zároveň ukazuje, že fosforylace regulační domény ovlivňuje vazbu ATP na NBD domény. Poslední výzkumy ukazují, že po navázání ATP na NBD1 a NBD2 dochází k dimerizaci těchto domén, jenž vyvolá konformační změnu transmembránových domén MSD1 a MSD2 a způsobí otevření kanálu (Gadsby et al. 2006, Hwang and Sheppard 2009). Otvírání a zavírání kanálu CFTR je pevně kontrolováno rovnováhou mezi kinázovou a fosfatázovou aktivitou a množstvím ATP uvnitř buňky. PKA je nejdůležitější kináza zodpovědná za regulaci chloridového kanálu, na jeho otvírání se ale může podílet i Ca^{2+}-independentí a Ca^{2+}-dependentní PKC (proteinkináza C) (Gadsby et al. 2006, Sheppard and Welsh 1999).

CF je charakterizována molekulární heterogenitou, o čem svědčí více než 1900 sekvenčních variant nalezených v genu *CFTR* v různých světových populacích (Cystic Fibrosis Genetic Analysis Consortium Dabatase, CFGAC, nově „Databáze CFTR1“,

www.genet.sickkids.on.ca/cftr/). Většina mutací je rozložena po celé kódující sekvenci a postihuje všechny domény proteinu CFTR (Obr. 12). Mutace v exonech 3-7 postihují funkci domény MSD1, v exonech 9-12 porušují funkci domény NBD1, regulační doménu postihují mutace v exonu 13, mutace v exonech 14-18 porušují funkci domény MSD2 a v exonech 19-22 narušují fungování NBD2 domény (Zielenski 2000).

Obr. 12 Distribuce mutací v jednotlivých exonech genu *CFTR* nahlášených v mezinárodní databázi CFGAC



www.genet.sickkids.on.ca/cftr

Mutace v genu *CFTR* jsou germinální (somatické nebyly dosud v literatuře popsány) s jednoznačně ancestrální povahou v jednotlivých rodinách (Morral et al. 1994). Jejich dlouhodobý výskyt v dané populaci svědčí pro selekční výhodu nosičů, např. vyšší odolnost k nemocem, které se historicky podílely na postnatální selekci v lidských populacích (cholera, tyfus, různá průjmová onemocnění) (Pohunek and Lebl 2008). Dosud bylo popsáno pouze omezené množství *de novo* mutací s četností přibližně 1:1200 ancestrálních alel genu *CFTR* (Girodon et al. 2008). Jejich počet je však podhodnocený, protože ne všechny diagnostické laboratoře ověřují přítomnost zachycených mutací pacienta u rodičů.

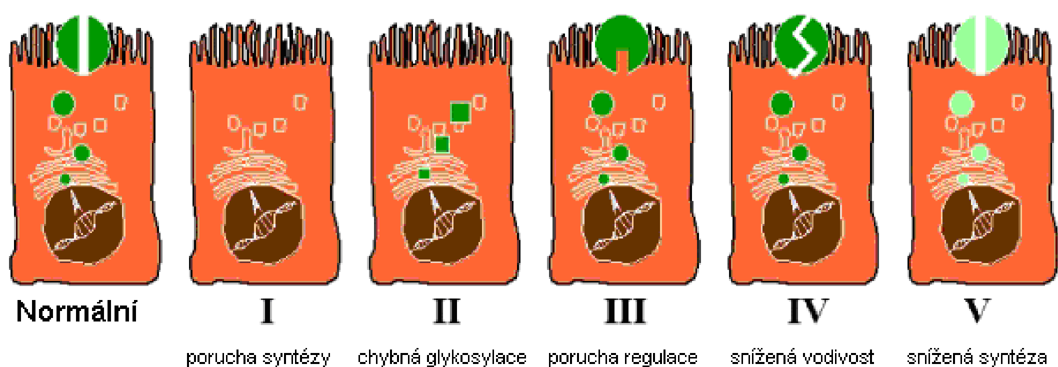
Nejčastějšími mutacemi v genu *CFTR* jsou záměny aminokyselin („missense“ mutace), které představují 40% všech dosud známých variant tohoto genu. Mutace způsobující posun čtecího rámce („frameshift“) se vyskytují v 16%, mutace způsobující abnormální sestřih exonů („splicingové“) ve 12%, nesmyslné mutace („nonsense“)

způsobující předčasné zařazení stop kodónu v 8% případů. Zhruba 3% mutací tvoří rozsáhlé intragenové delece či duplikace jednoho či více exonů. Ve 2% případů se vyskytují delece nebo inzerce násobků tři párů bazí DNA, které neporušují čtecí rámec. Typickým představitelem těchto mutací je hlavní mutace F508del, která vede ke ztrátě kodónu pro aminokyselinu fenylalanin. Zbylé alterace nukleotidové sekvence představují polymorfismy bez patogenního potenciálu (www.genet.sickkids.on.ca/cftr/).

Posouzení patogeneze jednotlivých mutací a predikce jejich klinického dopadu je velmi obtížné, proto byla navržena klasifikace pěti patogenetických tříd mutací na základě expresních studií či modelově předpověděném mechanismu jejich molekulárních dysfunkcí (Obr. 13) (Zielenski and Tsui 1995). Později došlo k rozšíření o další dvě třídy (Zielenski 2000). Třídy I-III jsou spojeny s minimální aktivitou anebo absencí proteinu CFTR, a tak s klasickou formou CF a nízkou variabilitou klinického průběhu CF mezi jednotlivými pacienty. Naopak mutace ze tříd IV–VII jsou spojeny s velkou variabilitou klinického průběhu CF a jsou více nalézány u atypických, monosymptomatických forem CF, včetně onemocnění příbuzných CF. Mutace z třídy I blokují syntézu proteinu CFTR z důvodu zařazení předčasného stop kodónu či chybného sestřihu. Důsledkem je nepřítomnost proteinu CFTR na apikální membráně epiteliálních buněk, protože zkrácené a dysfunkční formy proteinu CFTR jsou rychle degradovány. Mutace z třídy II redukují či zcela blokují správnou posttranslační glykosylaci a výslednou terciální konformaci proteinu CFTR, která je nezbytná pro následný transport na apikální membránu. Důsledkem je absence proteinu CFTR. Do III. třídy patří mutace, které porušují regulaci proteinu CFTR, který je na apikální membráně přítomný. Jedná se hlavně o mutace, které znemožňují fosforylaci R domény nebo navázání ATP na NBD1 či NBD2 podjednotky. Důsledkem je snížený transport chloridových iontů přes epiteliální membrány. Mutace třídy IV postihují MSD1 nebo MSD2 domény proteinu CFTR, které vytváří prostorově deformovaný iontový kanál, v důsledku čehož je snížená jeho propustnost. Třída V je charakterizována redukovanou syntézou, tj. sníženým množstvím plně funkčního proteinu CFTR na apikální membráně. Do třídy VI jsou řazeny mutace ovlivňující regulaci dalších iontových kanálů (ENaC nebo ORCC) a do třídy VII patří mutace způsobující sníženou stabilitu jinak plně funkčního proteinu. Jedná se zde převážně o nesmyslné a posunové mutace přítomné na konci genu *CFTR* (Zielenski and Tsui 1995, Zielenski 2000).

V poslední době došlo k systematické klasifikaci patogenetického dopadu mutací pomocí celosvětové kompilace in vitro modelací a/nebo asociovaných klinických dat v rámci mezinárodní databáze CFTR2 („Clinical and functional translation of CFTR", www.cftr2.org), která umožňuje poměrné přesné posouzení klinického dopadu mutací u nově diagnostikovaných případů onemocnění.

Obr. 13 Základní patogenetické třídy mutací v genu *CFTR*



Zielenski 2000

Ze všech známých mutací v genu *CFTR* se pouze 5 z nich celosvětově vyskytuje s četností větší než 1% – F508del (66.8%), G542X (2.6%), N1303K (1.6%), G551D (1.5%) a W1282X (1.0%) (Estivill et al. 1997). Dalších zhruba 30 mutací má celosvětovou frekvenci mezi 0.1–1%. Ostatní jsou již vzácné a vyskytují se ojediněle v jednotlivých rodinách s CF. Některé mutace mohou dosáhnout vysokých četností v izolovaných populacích v důsledku genetického driftu, např. G551D u keltských populací, či efektu zakladatele, např. W1282X u Ashkenazi Židů (Bobadilla et al. 2002). Frekvence celosvětově nejčastější mutace F508del (delece 3pb v 10. exonu) je nejvyšší v severní Evropě (87%) a klesá až na frekvenci 21% v Turecku. Je více než 50 000 let stará a odráží společný původ populací (Morral et al. 1994).

V České republice bylo dosud nalezeno 70 mutací a podařilo se dosáhnout více jak 96% populační záchytnosti (Balaščaková et al. 2008). Genetická a etnická charakteristika nejčastějších mutací u českých pacientů s CF potvrzuje, že naše země byla křižovatkou

všech hlavních historických migrací. Znalost geografické distribuce jednotlivých mutací v genu *CFTR* je důležitá nejen pro porozumění migrací historických populací, ale hlavně pro smysluplné vyšetření pacienta s podezřením na CF, neboť dle etnického původu vyšetřované osoby lze vybrat mutace, které jsou pro příslušnou populaci relevantní.

Kvůli vysokému počtu mutací v genu *CFTR*, jejich specifické distribuci a frekvenci v jednotlivých populacích je důležité mít k dispozici spolehlivá molekulárně genetická vyšetření, která stanoví genotyp probanda. To je důležité nejen pro potvrzení klinické diagnózy onemocnění, ale i pro budoucí prenatální diagnostiku v dalších graviditách rodičů probanda, jakož i genetické vyšetření pokrevních příbuzných ke stanovení možnosti přenašečství daných mutací.

Efektivní diagnostické nástroje jsou nezbytné, neboť včasná diagnostika do dvou měsíců života významně ovlivňuje průběh a prognózu tohoto onemocnění (Sims et al. 2007). Pozdní zahájení léčby vede jednoznačně k méně příznivému celoživotnímu průběhu onemocnění s horší prognózou přežití. V roce 1959 byl medián přežívání nemocných asi 6 měsíců, v současné době v zemích s kvalitní zdravotní péčí dosahuje střední doba přežití téměř 40 let (Pohunek and Lebl 2008). V České republice však bohužel došlo v letech 1999-2004 ke zhoršení klinické diagnostiky CF ze strany praktických pediatrů. Medián věku při diagnóze se přibližně zdvojnásobil a podíl pacientů zachycených do 1 roku života poklesl. Pacienti diagnostikovaní mezi 1. -10. rokem života často přicházeli ve velmi špatném nutričním stavu či s ireverzibilními změnami plic (Vávrová 2006). Tyto skutečnosti vedly v říjnu roku 2009 k zavedení plošného novorozeneckého skríningu CF v České republice (www.novorozenecky-screening.cz), který je založený na testování hladiny IRT (imunoreaktivní trypsinogen) a na případném molekulárně genetickém vyšetření (pouze u jedinců s pozitivními hodnotami IRT) (Krulisova et al. 2012).

V našich podmínkách používáme při molekulárně genetické diagnostice tzv. „kaskádový“ přístup, který umožňuje v sestupné řadě postupný záchyt od nejčastějších mutací až po ty relativně vzácné. Nejdříve jsou vyšetřeny nejčastější mutace genu *CFTR* pomocí komerčního kitu Elucigene CF-EU2v1^{Tm}, kdy se simultánně testuje 50 mutací (CFTRdele2,3/21kb/, I507del, 2789+5G>A, E60X, F508del, Q890X, P67L, 1677delTA, 3120+1G>A, G85E, V520F, 3272-26A>G, 394delTT, 1717-1G>A, R1066C, 444delA,

G542X, Y1092X(C>A), R117C, S549N, M1101K, R117H, S549R(T>G), D1152H, Y122X, G551D, R1158X, 621+1G>T, R553X, R1162X, 711+1G>T, R560T, 3659delC, L206W, 1811+1.6kbA>G, 3849+10kbC>T, 1078delT, 1898+1G>A, S1251N, R334W, 2143delT, 3905insT, R347P, 2184delA, W1282X, R347H, 2347delG, N1303K, A455E, W846X, včetně variant IVS8-T(5/7/9). Pokud se nepodaří odhalit obě kauzální mutace, ale pacient vykazuje jasné klinické a laboratorní známky onemocnění, jsou indikována další rozšiřující vyšetření. A to metoda MLPA (Multiplex Ligation-dependent Probe Amplification, multiplexní amplifikace prób závislá na ligaci) pro vyšetření rozsáhlých intragenových přestaveb, tj. delecí či duplikací jednoho nebo vícero exonů v rámci celého genu *CFTR*, které nejsou běžnými metodami založenými na PCR technice zachytitelné. Poslední studie poukazují na fakt, že velké přestavby mohou tvořit významnou část neidentifikovaných alel (cca. 2%), hlavně u případů klasické CF i u CBAVD, zejména u mužů, kteří jsou nosiči mírné mutace genu *CFTR* (Taulan et al. 2007, Hantash et al. 2006, Niel et al. 2004). Následně používáme mutačně skenovací metodu HRM, která umožňuje detekovat jedno- i vícenukleotidové změny sekvence DNA. „Pozitivní“ exony genu *CFTR* jsou sekvenovány (dle Sangera), aby byla identifikována konkrétní mutace nebo polymorfismus, který není součástí komerčních diagnostických souprav.

Při nálezu patogenní mutace je vždy důležité vyšetřit i rodiče pacienta s CF a potvrdit tak nosičství nalezených mutací u každého z nich. Tím vyloučíme záměnu vzorků, non-paternitu a/nebo přítomnost *de novo* mutace, což je důležité pro eventuální prenatální vyšetření v postižené rodině. Přiřazení mutací k rodičům pacienta umožní také cílené vyšetření příslušné mutace u jejich pokrevních příbuzných. U složených heterozygotů lze takto odhalit, zda obě mutace náhodou neleží v pozici cis, tj. na jedné alele, anebo že se jedná o novou mutaci. Naopak negativní nález při kompletním vyšetření genu *CFTR* dostupnými molekulárně genetickými metodami klinickou diagnózu CF nevylučuje, neboť současně používanými analytickými postupy nelze prokázat všechny mutace CFTR (např. intronové sestřihové mutace).

2. CÍLE DIZERTAČNÍ PRÁCE

Molekulárně genetická diagnostika (tj. DNA diagnostika) se díky rozvoji a zavádění nových výzkumných poznatků do rutinní praxe stala v posledních letech významným oborem, který prostupuje prakticky všechny medicínské obory. Podle kvalifikovaných odhadů trpí v zemích Evropské unie geneticky podmíněnou chorobou okolo 30 milionu lidí a genetická onemocnění tak představují, díky své chronické povaze, stále větší břemeno pro zdravotnické systémy. Pozornost se proto soustřeďuje na zavádění nových technologií při současném zvyšování kvality poskytovaných genetických služeb a jejich ekonomičnosti.

Na našem pracovišti a v této dizertační práci jsme se proto věnovali posouzení metody High Resolution Melting (HRM) pro její diagnostické využití na příkladu různých genů (*BRCA1, MTHFR, CFTR*), dále DNA diagnostice cystické fibrózy u středoevropských populací a odhalení dalších možných příčin poruch mužské plodnosti.

Cíle dizertační práce zahrnují:

1/ Posouzení využitelnosti metody HRM v diagnostice pomocí zhodnocení vybraných validačních parametrů na základě analýzy 170 variant v genu pro rakovinu prsu a ovárií *(BRCA1)*, včetně shrnutí zkušeností s touto metodou a vytvoření směrnic, které by usnadnily zavedení této technologie v evropských dle ISO 15189 akreditovaných laboratořích.

2/ Poskytnutí konceptu pro validaci nových metod na příkladu genotypizace vybraných mutací v genu *MTHFR* pomocí HRM.

3/ Zhodnocení přínosu a využitelnosti metody HRM pro mutační skenování neznámých variant nejen v DNA diagnostice cystické fibrózy.

4/ Charakterizaci spektra mutací u pacientů s cystickou fibrózou na západní Ukrajině a identifikaci dalších populačně specifických CF alel, které by zvýšily detekční

záchytnost, aby byla splněna kritéria pro zavedení novorozeneckého skríningu v této oblasti.

5/ Charakterizaci spektra mutací u pacientů s cystickou fibrózou v České republice a poskytnutí dat pro zlepšení DNA diagnostiky a/nebo novorozeneckého skríningu ostatním českým molekulárně genetickým laboratořím, firmám zabývajících se výrobou komerčních kitů pro diagnostické účely, pro okolní etnicky příbuzné populace postrádající vlastní populační studie a pro českou komunitu žijící v zahraničí (přes 1 milión krajanů žijících převážně v Kanadě a USA).

6/ Charakterizaci spektra variant protaminových genů na souboru německých mužů (pacienti a kontroly) a objasnění jejich vztahu k poruchám spermiogeneze.

3. VÝSLEDKY, DISKUZE A PŘILOŽENÉ PUBLIKACE

3.1. van der Stoep, et al. (2009) Diagnostic guidelines for high-resolution melting curve (HRM) analysis: an interlaboratory validation of BRCA1 mutation scanning using the 96-well LightScanner. Hum Mutat. (IF: 5.686)

V rámci své dizertační práce jsem se podílela na komplexním multicentrickém mezinárodním validačním projektu pod záštitou projektu EuroGentest, který představuje první mezilaboratorní studii zabývající se zhodnocením a validací metody HRM pro diagnostické účely dle normy ISO 15189. Tato studie byla provedena ve spolupráci našeho pracoviště s Centrem klinické genetiky v Leidenu (Holandsko) a Centrem lidské genetiky v Leuvenu (Belgie).

Pro posouzení mutačního skenování a genotypizace metodou HRM za použití platformy LightScanner^{TM} firmy Idaho Technology byl analyzován panel klinických DNA vzorků známého genotypu - 170 variant genu *BRCA1* a 197 vzorků standardních kontrol. Ty představovaly reprezentativní výběr všech dvanácti možných typů substitucí a různých ins/del, včetně mutací v homozygotní formě. Gen *BRCA1* byl zvolen s ohledem na velké množství identifikovaných mutací (přes 1600 v roce 2009), které jsou rozptýleny po celé kódující sekvenci a jejichž detekce pro diagnostické účely vyžaduje použití rychlé, spolehlivé a ekonomické pre-sekvenační (tj. mutačně skenovací) metody. Hodnocenými validačními parametry byla senzitivita, specificita, robustnost a mezilaboratorní reprodukovatelnost.

Celkem bylo ve studii testováno částečně nově navržených 66 párů primerů, z nichž bylo vybráno 58 párů vedoucí k dobré PCR a HRM. Pro optimalizaci anelační teploty byla provedena gradientová PCR. Během tohoto rozsáhlého testování byla stanovena kritéria pro softwarové nastavení a vyhodnocování HRM. Byla stanovena doporučená pozice normalizačních oblastí a optimální šíře jejich rozpětí (1-2.5°C), což má význam pro snížení falešné pozitivity. Hladina senzitivity byla pro všechny testované amplikony doporučena na 3.0 při nastavení „Auto Group“ „High“. Za optimální byla považována taková

senzitivita, při které byly všechny varianty detekovány při maximálně 5% falešné pozitivitě u vzorků se standardní alelou. Specificita HRM byla hodnocena na základě analýzy 352 reakcí se standardní alelou. Z 58 amplikonů bylo na základě výsledků a stanovených kritérií zvoleno 40 nejlepších párů primerů pokrývajících celou kódující oblast genu *BRCA1*, které vykazovaly specificitu a senzitivitu 98%.

V dalším kroku bylo testováno analytické provedení a mezilaboratorní reprodukovatelnost na analýze 10 vybraných amplikonů (lišících se velikostí, obsahem GC a typem variant) za totožných podmínek. Participující laboratoře detekovaly všechny heterozygotní varianty značící dobrou reprodukovatelnost HRM. Stejně jako v případě analýzy všech 58 amplikonů, nepodařilo se detekovat homozygotní variantu c.3113A›G.

Jelikož různé varianty mohou produkovat stejný profil křivky tání, je vždy nezbytné potvrdit nález sekvenační analýzou. Některé polymorfismy se však objevují s velkou četností, proto bylo za účelem redukce sekvenování navrženo 9 neznačených prób pro detekci často se vyskytujících heterozygotních a homozygotních nepatogenních polymorfismů genu *BRCA1.* Na analýze 19 vzorků byla ilustrována úspěšná genotypizace metodou HRM, kdy všechny próby detekovaly příslušný polymorfismus.

Robustnost HRM byla demonstrována analýzou DNA vzorků izolovaných čtyřmi různými izolačními postupy. Výsledky vyloučily vliv zvolené extrakční metody na úspěšnost HRM, DNA vzorky z různých typů izolace tak mohou být analyzovány v rámci stejného běhu. Vliv výchozí koncentrace DNA v reakci neprokázal rozdíl v úspěšnosti HRM při použití až 4x méně DNA než standardně (tj. 5 ng místo 20 ng DNA v reakci), křivky tání se odchylovaly až při 2.5x vyšší výchozí koncentraci DNA (tj. při 50 ng DNA), což může ústit v navýšení falešně pozitivních výsledků.

Závěrem byla provedena zaslepená analýza 28 DNA vzorků (1120 PCR reakcí) porovnávající výsledky HRM se zlatým standardem, tj. sekvenační analýzou podle Sangera. Mutační skenování metodou HRM detekovalo všechny heterozygotní varianty se 100% senzitivitou a genotypizace devíti častých polymorfismů detekovala všechny správně, čímž se výrazně redukovala potřeba sekvenace. Pouze 5% PCR reakcí vyžadovalo následnou sekvenační analýzu (tj. vzorky s pozitivním záchytem při mutačním skenování,

které nesly jiné varianty než oněch 9 genotypizovaných polymorfismů a 2 % falešně pozitivních vzorků).

Tato studie poukázala na parametry, které by měly být před následným testováním dalších genů metodou HRM zváženy. Bylo proto doporučeno zvolit rozsáhlý panel variant a standardních kontrol, se zaměřením na testování malých delecí, které se zdají být obtížněji detekovatelné, otestovat geny s nižším i vyšším obsahem bazí G a C (pod 30% a nad 60%), které by mohly negativně ovlivnit senzitivitu HRM a otestovat jiná plně saturační barviva.

V souhrnu, tato studie poskytla rozsáhlou validaci a zhodnocení metody HRM, včetně detailního doporučení pro správnou interpretaci získaných dat a pro usnadnění zavedení metody HRM v ostatních diagnostických laboratořích pro další geny. Dále poskytla panel primerů a prób pro kompletní skenování genu *BRCA1* a genotypizaci častých polymorfismů, včetně parametrů PCR, HRM a doporučení při vyhodnocování dat. Potvrdila vysokou senzitivitu (100%), specificitu (97-98%) i reprodukovatelnost HRM a tím její vhodnost pro diagnostické využití jakožto pre-sekvenační metody. Nevýhodou metody je pouze její snížená senzitivita při analyzování delších úseků nad 400 bp, jak již bylo dříve publikováno (Reed and Wittwer 2004). Z toho důvodu je při mutačním skenování potřeba rozdělit rozsáhlé exony na menší amplikony. Tím se sníží i pravděpodobnost výskytu více domén tání, které detekci mutací a interpretaci výsledků taktéž ztěžují. Velice výhodné je navrhnout všechny páry primerů tak, aby byly zakončeny univerzálním primerem M13 a aby bylo možné analyzovat všechny naráz za identických podmínek.

Výsledky této studie byly využity i pro obecná doporučení pro validaci a verifikaci všech DNA diagnostických metod v klinických laboratořích v rámci projektu Eurogentest (Mattocks et al. 2010), který však byl pouze mým vedlejším projektem a nebude proto blíže diskutován.

METHODS

Human Mutation

OFFICIAL JOURNAL
HGVS
HUMAN GENOME VARIATION SOCIETY
www.hgvs.org

Diagnostic Guidelines for High-Resolution Melting Curve (HRM) Analysis: An Interlaboratory Validation of *BRCA1* Mutation Scanning Using the 96-Well LightScanner™

Nienke van der Stoep,[1]* Chantal D.M. van Paridon,[1] Tom Janssens,[3] Petra Krenkova,[2] Alexandra Stambergova,[2] Milan Macek,[2] Gert Matthijs,[3] and Egbert Bakker[1]

[1]*Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands;* [2]*Institute of Biology and Medical Genetics, Charles University, Prague, Czech Republic;* [3]*Center for Human Genetics, University of Leuven, Leuven, Belgium*

For the Focus Section on HRMA Technology

Received 4 December 2008; accepted revised manuscript 10 February 2009.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/humu.21004

ABSTRACT: Genetic analysis of *BRCA1* by sequencing is often preceded by a scanning method like denaturing gradient gel electrophoresis (DGGE), protein truncation test (PTT) or DHPLC. High-resolution melting curve (HRM) analysis is a promising and economical method for high-throughput mutation scanning. The EuroGentest network (www.eurogentest.org) aims to assist with the introduction of novel technologies in the diagnostic setting. Therefore, we have performed a thorough and high-standard interlaboratory evaluation and validation of HRM, in collaboration with Idaho Technology, the manufacturer of the LightScanner™ (LS). Through this detailed study of 170 variants, we have generated guidelines for easy setup and implementation of HRM as a scanning technique for new genes, which are adaptable to the quality system of an individual diagnostic laboratory. This validation study includes the description of a *BRCA1*-specific mutation screening test using the 96-well LS. This assay comprises 40 amplicons and was evaluated using a statistically significant elaborate panel of variants and control DNA samples. All heterozygous variants were detected. Moreover, genotype analysis for nine common polymorphisms created a fast screening and detection method for these frequently occurring nonpathogenic variants. A blind study using a total of 28 patient-derived DNA samples resulted also in 100% detection and showed an average specificity of 98%, indicating a low incidence of false positives (FPs).
Hum Mutat 30:1–11, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: high-resolution melting curve analysis; HRM; *BRCA1*; diagnostic validation

Introduction

The *BRCA1* gene (MIM# 113705) is involved in susceptibility to breast and ovarian cancer with a very high penetrance rate [Castilla et al., 1994; Claes et al., 2003; Couch and Weber, 1996; Deffenbaugh et al., 2002; Easton et al., 1995; Ford et al., 1998]. Approximately 3 to 5% of breast cancers are caused by germline mutations in the breast cancer genes *BRCA1* and *BRCA2* [Beck et al., 1993; Guldberg and Guttler, 1993; Narod and Foulkes, 2004]. After the *BRCA1* and *BRCA2* (MIM# 600185) genes were identified, genetic testing became available and is now routinely offered to women from high-risk families. Over 1,590 different heterozygous mutations have been reported in the *BRCA1* gene; these mutations have been named the "Breast Cancer Information Core" (BIC) of the Breast Cancer Mutation Database, hosted by the National Human Genome Research Institute (NHGRI) (http://research.nhgri.nih.gov/projects/bic) [Ozcelik et al., 1996; Goldgar et al., 2004]. Mutations are scattered over all exons; therefore, genetic tests for this gene require a mutation scanning analysis of the entire coding region of the *BRCA1* gene. In most diagnostic laboratories, this procedure is currently performed using direct sequence analysis, often preceded by denaturing gradient gel electrophoresis (DGGE) [Beck et al., 1993; Guldberg and Guttler, 1993], protein truncation test (PTT) [den Dunnen and van Ommen, 1999; Ozcelik et al., 1996], or denaturing high-performance liquid chromatography (DHPLC) [Gross et al., 1999; Liu et al., 1998]. However, all these techniques are time-consuming and/or expensive. Moreover, considering the increasing number of requests for *BRCA1* scanning tests, the demand for a fast and reliable scanning technique is high.

High-resolution melting curve (HRM) analysis is a potentially useful new method for fast genotyping and high-throughput mutation scanning of disease-related genes in genome diagnostics [Herrmann et al., 2006; Montgomery et al., 2007; Wittwer et al., 2003]. The procedure is simple and consists of PCR, followed by a short melting step and subsequent analysis. This post-PCR analysis method scans entire amplicons and detects sequence variations using a saturating double-stranded DNA (dsDNA) binding dye, such as LCGreen Plus (Idaho Technology, Salt Lake City, UT) [Wittwer et al., 2003]. The melting profile of the PCR product depends on its GC content, length, sequence, and heterozygosity, and mutations in the sequence will give rise to heteroduplexes that change the shape of the melting curve when compared to the wild-type (wt) melt profile [Herrmann et al., 2006; Montgomery et al., 2007; Wittwer et al., 2003]. Although several tests using this new technique have been described; we present the first interlaboratory assessment study for diagnostic use that includes a thorough evaluation and validation of HRM analysis on the LightScanner™ (LS) (Idaho Technology), which employs a statistically significant large panel of more than 150 variants for *BRCA1* only. This ensures that the number of variant samples tested is large enough that, under the assumption that the sensitivity point estimate will be 100%, the lower bound of the 95% confidence interval will be at or above 98%.

Moreover, we composed a list of diagnostic guidelines that can also be applied for setting up and interpreting HRM scans for

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Nienke van der Stoep, Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands.
E-mail: nvdstoep@lumc.nl

© 2009 WILEY-LISS, INC.

other genes, and we describe a validated HRM test for the *BRCA1* gene using the 96-well LS that includes genotype analysis of nine common polymorphisms.

Materials and Methods

Interlaboratory Validation by EuroGentest

EuroGentest is a European Network of Excellence aiming at harmonizing genetic testing services throughout Europe. One key objective of EuroGentest is to set up evaluation and validation programs for new techniques and tests in diagnostics. In this respect, we performed this validation study for HRM for *BRCA1* on the 96-well LS in close collaboration with the Center for Human Genetics in Leuven (Belgium), the Institute of Biology and Medical Genetics in Prague (Czech Republic), the LS manufacturer Idaho Technology, and the LS distributor BIOKÉ (Leiden, the Netherlands). Validation of the HRM technology was performed according to international guidelines ISO15189, Medical laboratories.

DNA Samples

DNA samples used for the initial validation study were all patient-derived and isolated from whole blood using the PURE-GENE™ nucleic acid purification method on the Autopure LS robotic workstation (Gentra Systems, Minneapolis, MN) according to validated diagnostic isolation procedures; the samples were diluted to 10 ng/μl. DNA concentrations were measured using the Nanodrop® ND-1000 Spectrophotometer (Isogen Life Science, Ijsselstein, The Netherlands).

Robustness of the HRM tests was evaluated further by using DNA samples isolated by four different DNA isolation procedures, including the Autopure LS (Gentra), the Chemagen procedure (Chemagen AG, Baesweiler, Germany), manual phenol extraction, and the QIAamp® DNA Kit (Qiagen, Venlo, The Netherlands).

The panel of DNA samples tested for *BRCA1* included 170 variants and 197 wt controls, which were all verified by direct sequencing using the Big Dye Terminator method (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). Supp. Table S1 lists all variants tested and indicates the classification, type, and distribution of the mutations. Mutations are indicated according to HGVS mutation nomenclature guidelines and DNA mutation numbering system used is based on cDNA sequence. For the blind tests, 28 patient-derived DNA samples were selected, together with two wt control samples. Note that 2 of the 28 patient-derived DNA samples were also included in the evaluation of the cohort of variants.

Note: We have consent from the patients to use the DNA that remains after diagnostic testing in the framework of quality of care (re)assessment; we comply with the Federation of Biomedical Scientific Societies (FMWV) Code: "Proper for Proper Secondary Use of Human Tissue" (2002).

All gradient PCR assays for optimization of the PCR annealing temperature (Ta) were performed using a pool of four individual Autopure-derived genomic DNA samples at a concentration of 10 ng/μl. This way all PCR optimization results were obtained from a mixed DNA sample that represents an average DNA purity as obtained upon isolation by the Autopure LS.

Validation Criteria for Determining Sensitivity and Specificity of the Technique

Validation of a mutation scanning method is limited to the assessment of a selected panel of variants. It is unfeasible and technically impossible to validate all possible occurring variants that can arise; therefore, a statistically reliable number of variants needs to be tested for the validation to establish the sensitivity of the method at a chosen confidence level. According to the "rule of three" we can say with a confidence interval of 95% that the probability of detecting a false negative, given a study of n samples with no false negatives, is 3/n [Hanley and Lippman-Hand, 1983]. Consequently, if we take the conservative point of view that the lower bound of the 95% confidence interval of the test sensitivity should be at or above 98%, then we would have to achieve perfect detection of at least 150 variants.

Because all possibly occurring mutations cannot be tested, it is important to select and examine a representative assortment of variants that comprises all types of substitutions and/or small deletions and insertions at various locations in the amplicon. We included variants at various locations ranging from 2 bp away from the PCR primer (e.g., c.4837A>G in MEX16B) to the middle of the amplicon, representing all 12 possible types of substitution (75%) and different nucleotide insertions, duplications, deletions, or indels of 1 to 62 bp (25%). Moreover, although only heterozygous variants are considered to be potential pathogenic mutations for the *BRCA1* gene, we have also included a set of 14 individual homozygous variants to examine their detection efficiency.

Selection Primer Sets

A large panel of 66 primer pairs was evaluated for HRM of *BRCA1*. The primers were newly designed or derived from current primer sets in use for DGGE and sequence analysis of *BRCA1*. All primer sets contain an M13 forward and reverse sequence tail, respectively, in order to allow direct sequencing analysis, and were synthesized by Biolegio (Nijmegen, The Netherlands). The design of most new primers was performed using the LightScanner Primer Design software package, version 1.0 (PD-v1.0; Idaho Technology). Primers were completely homologous to the reported *BRCA1* gene sequence, accession L78833, NM_007294.2, NT_025965.11. The nucleotide sequence of the primers was judged according to the criteria described in Supp. Table S2A. The final selection of validated primer set is shown in Supp. Table S3.

Optimization of HRM PCR

Optimal PCR Ta for all primer sets was evaluated using a gradient PCR setup of 55°C to 67°C on the MJ-PTC 200 PCR machine (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). The PCR was performed in a 10-μl volume using 20 ng of genomic DNA, 4 μl LS Mastermix (LCGreen® Plus dye), 3 pmol forward primer, 3 pmol reverse primer, and water (molecular grade) (Supp. Table S4). Mineral oil (15 μl per reaction), necessary for the LS melt step was already added before starting the PCR. The PCR was initiated with a 10-min hold at 95°C. Thermal cycling consisted of a 20-second hold at 95°C, a 30-second hold at the indicated Ta (in Supp. Table S3), and a 40-second hold at 72°C for 40 cycles. Finally, reactions were elongated for 5 min at 72°C and heteroduplexes were generated by adding a step at 95°C for 1 min and cooling the reactions to 25°C. Ramp speed of the PCR machine was set at 2.5°C/second.

Only primer sets that fulfilled the criteria as described in Supp. Table S2B were qualified for further HRM evaluation and the most optimal Ta was selected.

Genotype Analysis of Common Polymorphisms

Nine unlabeled oligonucleotide probes were designed and examined for the detection of heterozygous and homozygous

frequently occurring polymorphisms of *BRCA1*. Probes and characteristics are listed in Supp. Table S5. Probe criteria were set as described previously and indicated in Supp. Table S2C [Montgomery et al., 2007]. All probes carried a block at the 3′ end omitting participation in the PCR reaction and 3′ exonuclease activity. We tested both the 3′ phosphate- and the 3′-C3 carbon (phosphoramidite)-spacer block. In addition we examined HPLC purified probes and compared results. All unlabeled probe-containing PCR reactions were performed using an asymmetric dilution of the forward and reverse primers of 1:5 at the same final primer concentration of 6 pmol, as used for the standard PCR and 5-pmol probe. Different numbers of PCR cycles were tested and all PCR reactions were performed as depicted in Supp. Table S4 using the optimal Ta (Supp. Table S3).

Optimization and Defining Instrument Setting Per Amplicon on the LS

PCR reactions were carried out on the Biometra thermocylcer (Westburg, Leusden, the Netherlands) as described above using 4titude Framestar plates (BIOKÉ) as recommended for HRM on the LS. Subsequently, samples were melted in the LS type HR96 (Idaho Technology and BIOKÉ) according to operating instructions using two different melt ranges of either 55°C to 98°C or 70°C to 98°C at a hold temperature of 50°C and 65°C, respectively.

The basic data analysis of the melt curves was performed using the supplied Call IT 1.5 Software according to the LS manual supplied by Idaho Technologies.

Both gene scanning and genotyping by HRM are performed by simple analysis of a single melting profile. Sequence variants are identified as groups that exhibit similar melting profiles and, if applicable, these groups can be genotyped for frequently-occurring polymorphisms. The scanning settings were assessed per amplicon by analyzing both the selected wt and all variant DNA samples (as listed in Supp. Table S1) and according to the guidelines described in this study.

Interlaboratory Testing of 10 Selected Amplicons

Interlaboratory performance of the test was evaluated in two laboratories using 10 different amplicons. Selected samples and matching amplicons are listed in Table 1. LS Mastermix, 10 primer sets and 37 DNA samples, including 22 wt samples and 27 variant samples, were sent to both laboratories indicated. Note that several variant DNA samples were used as wt control for other fragments. The Center of Human Genetics in Leuven performed additional validation experiments by testing a set of 19 DNA samples purified by Chemagen as indicated in Table 2. All PCR reactions were carried out on either the PTC200 (Bio-Rad) or the Biometra Thermocycler (Westburg) using the same conditions as defined by the diagnostic laboratory in Leiden. All plates were melted in the LS and the raw data files were evaluated by both laboratories themselves and sent to Leiden for reevaluation using the specifically defined Call IT 1.5 amplicon scanning settings of Leiden.

BRCA1 Blind Tests for 28 Patient-Derived DNA Samples

The blind tests included two complete *BRCA1* mutation scanning rounds using a selection of the 40 best performing primer sets that encompass the entire *BRCA1* gene. Two series of each 14 patient-derived DNA samples, one negative wt control and one blank control were tested. The 40 amplicon reactions were distributed over seven 96-well plates according to their Ta, in groups of six amplicons, as depicted in Supp. Figure S1.

Table 1. Interlaboratory Validation for BRCA1: EUGT Test-Set

Exon	Amplicon	GC%	Wts tested[a]	Mutation[b]	Variant classification	Length (bp)
3	MEX3	35	2	c.81–6T>A	MUT	347
8	MEX 8	39	2	c.442–34T>C	POL	320
				c.536A>G;		
				c.442–34T>C	UV+POL2	
				c.442–34T>C	POL2	
11	MEX 11A-L	39	2	c.1067A>G	POL2	625
				c.1067A>G	POL	
				c.825C>T	UV	
				c.1016delA	MUT	
7	MEX 7	34	2	c.302–3C>G	MUT	279
				c.441G>C	UV (1X)	
				c.302–41T>C	UV	
9	MEX 9	31	2	c.591C>T	POL	187
				c.548–17G>T	UV	
11	MEX 11J	36	2	c.3113A>G	POL	378
				c.3113A>G;		
				c.3119G>A	POL	
				c.3113A>G	POL2	
				c.2989_2990dupAA	MUT	
11	MEX 11-6	37	2	c.1648A>C	UV	320
				c.1621C>T	MUT	
				c.1525A>G	UV	
11	MEX 11-7	37	2	c.1865C>T	UV	272
11	MEX 11-8	40	3	c.1961delA	MUT	254
				c.2019delA	MUT	
				c.2014A>T	MUT	
11	MEX 11-5	38	3	c.1292dupT	MUT	316
				c.1456T>C	UV	
				c.1289dupA	MUT	

MUT, heterozygous pathogenic variant; UV, unclassified variant; POL heterozygous polymorphism; POL2, homozygous polymorphism.
[a]Number of wild type samples tested.
[b]Mutations are indicated according to HGVS mutation nomenclature guidelines and DNA mutation numbering system used is based on cDNA sequence NM_007294.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

The analysis was carried out applying the selected and fixed scanning settings obtained from the primary evaluations study. In addition all these plate specific settings were saved per plate as specific "Scanning Analysis" and "Genotype Analysis," including the subset annotation, localization of lower and upper normalization settings, and "Auto Group" sensitivity level for the analysis of the amplicon and, if applicable, the same was done for the genotype analysis using the unlabeled probe.

Results

Selection of *BRCA1* Primer Sets and Primary Optimization

In total we evaluated a panel of 66 primer pairs for HRM analysis of the *BRCA1* gene that often included two or more overlapping sets per region. Based on criteria mentioned in Supp. Table S2A and B, the 58 sets gave rise to good PCR and HRM results. Products that gave either low yield, additional side products, poor melting curves, or more than two melting domains were disqualified. All primer sets that were designed using the IT PD-v1.0 performed very well. Primers that performed poorly in HRM analysis were designed before applying less stringent criteria or had an amplicon length of 420 bp and longer. In addition, these primers often scored poorly when judged by the PD-v1.0 or gave rise to many hits with other genomic regions upon a NCBI BLAST search.

Table 2. Interlaboratory Validation for BRCA1: DNA Samples Leuven

Exon	Amplicon	Wts tested[a]	Mutation[b]	Variant classification	Length (bp)
3	MEX3	2	c.133A>C	UV	347
8	MEX 8	2	c.442–34T>C c.442–34T>C c.470_471delCT	POL POL2 MUT	320
11	MEX 11A-L	2	c.693G>A c.744C>G c.844_850dupTCATTAC c.1016delA	UV UV MUT MUT	625
7	MEX 7	2	c.441G>C	UV	279
9	MEX 9	2	c.591C>T	POL	187
11	MEX 11J	2	c.2920T>C c.3119G>A	UV POL	378
11	MEX 11-5	2	c.1292delT c.1418A>G c.1525A>G	UV UV UV	317
11	MEX 11-6	2	c.1487G>A	POL	320
11	MEX 11-7	2	c.1865C>T	UV	272
11	MEX 11-8	2	c.1878A>G c.2005A>T	POL UV	254

MUT, heterozygous pathogenic variant; UV, unclassified variant; POL heterozygous polymorphism; POL2, homozygous polymorphism.
[a]Number of wild-type samples tested.
[b]Mutations are indicated according to HGVS mutation nomenclature guidelines and DNA mutation numbering system used is based on cDNA sequence NM_007294.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Evaluation and Defining Guidelines for Optimal HRM Analysis Per Amplicon on the LS

In order to perform a thorough and elaborate analytical evaluation of various amplicons, we initially extensively examined all 58 well-performing primer sets for the detection of variants through HRM using the elaborate panel of 170 variants and 197 negative control (wt) patient-derived DNA samples as listed in Supp. Table S1. Some variants and many wt samples could be used for multiple amplicons resulting in a total of 248 and 352 different variant (including homozygous variants) and wt control HRM reactions, respectively. All melting curves were analyzed using the software program Call IT version 1.5. First, subsets were selected per amplicon, the "Curve Shift" setting was left at the default setting of 0.050 and the grouping of the curves was performed using the "Auto Group" option at high sensitivity. During our examination of all 58 primer sets, we established additional critical criteria for adjusting instrument and software settings to create optimal HRM analysis per amplicon on the LS. An overview is given in Supp. Table S6. This includes the positioning of normalization bars and defining relative fluorescence levels and sensitivity levels, but also criteria related to the melting curve profiles that need to be fulfilled. The following guidelines were established. First of all we tested the criteria of the lower and upper normalization bars and concluded that the optimal width per set of bars should be in a range of 1°C to 2.5°C. The location of the bars should be close around the melt domain and can be adjusted to optimal position by establishing a straight horizontal line at the start of the normalized melting curve (Fig. 1). Figure 1A illustrates the optimization of MEX11-13E, the left column shows the result of melting curves upon incorrect setting of the normalization regions. The obtained aberrant normalized melting curve profiles are indicated with a red circle in plot 2, and plot 3 shows the subsequent incorrect variant calling. The right column depicts the correct normalization setting. Variant curves and congruent mutations are called correctly and are indicated in lower right of plot 6. Note sensitivity levels were kept fixed at 3.0 ("Auto Group" high). Figure 1B shows a similar comparison of correct and incorrect normalization settings for amplicon MEX11-15; again sensitivity levels were kept fixed at 3.0.

The optimal sensitivity level was determined by analyzing all 170 different variants and 197 different wt samples for the 58 amplicons. The level of sensitivity at the "Auto Group" menu option was adjusted for each individual amplicon and all amplicons were set at sensitivity "High." The maximum sensitivity level that can be applied was established by testing a series of 5 to 10 individual wt curves and allowing a limited detection of 0 to 5% (average) false positives (FPs). We recommend designing new primers for sets that yield a maximum sensitivity level below 2.7. Subsequently, the minimum sensitivity of the amplicon was established by analyzing the available variants. Again, new primers were designed for sets that yielded a sensitivity level below 2.7. Optimal sensitivity was reached when all variants could be detected and no more than an average of 5% FP reactions were observed in the wt series (excluding reactions that fail in the PCR).

Both the "Normal" and "High" sensitivity levels were evaluated for all variants. Although the vast majority of variants could easily be detected using a "Normal" sensitivity setting at the "Grouping" standard, "Auto Group" at level "Normal" 2.7, some variants were only visible at a sensitivity level "High." Several of these less easily detectable variants were homozygous variants. However, also some heterozygous variants, such as c.135-15_135-12delCTTT and c.2898delT in the respective amplicons MEX05 and MEX11IB could only be detected using the "Auto Group" "High" sensitivity setting. Figure 2 depicts the analysis of these variants and compares the results obtained with "Normal" and "High" sensitivity. Clearly, grouping of all curves needs to be performed at high sensitivity level to be sure these variants are detected. Due to this observation we chose to select a "High" sensitivity level for all amplicons to accomplish a detection level as optimal as possible and to reduce the risk of missing important pathogenic mutations such as c.2898delT.

In general, "Auto Group" sensitivity level "High" was often set higher than 2.7 to reach the most optimal and stringent detection level. In case few variants were available, we focused mainly on the quality of the detection by using a large panel of negative control samples and selected only primer sets that yielded wt melt curves that were located closely together and gave few FPs, never more than 5%. Figure 3 shows the importance of testing a large series of wt samples. Both plots depict HRM of 15 wts; however, only amplicon B gave rise to wt melt curves that are in close range of each other and that were all correctly called by the software. In contrast, amplicon A results in wt curves that are much more broadly dispersed from one another and give rise to four FP reactions. The latter result would give rise to a low sensitivity level and subsequent potential loss in variant detection.

Evaluation Results Using All 58 Amplicons

Evaluation of HRM for all 58 amplicons applying the general guidelines described in the previous section and using the panel of variant and wt DNA samples resulted in the detection of all tested heterozygous variants representing 220 analyses (+28 homozygous variant analyses). This data was in full coherence with the results obtained by sequence analysis. According to the "rule of three" this results in a sensitivity of at least 98.6% with a confidence interval of 95%. Of the homozygous variants, only one could not be detected by two different primer sets (MEX11J and MEX11-13C), namely

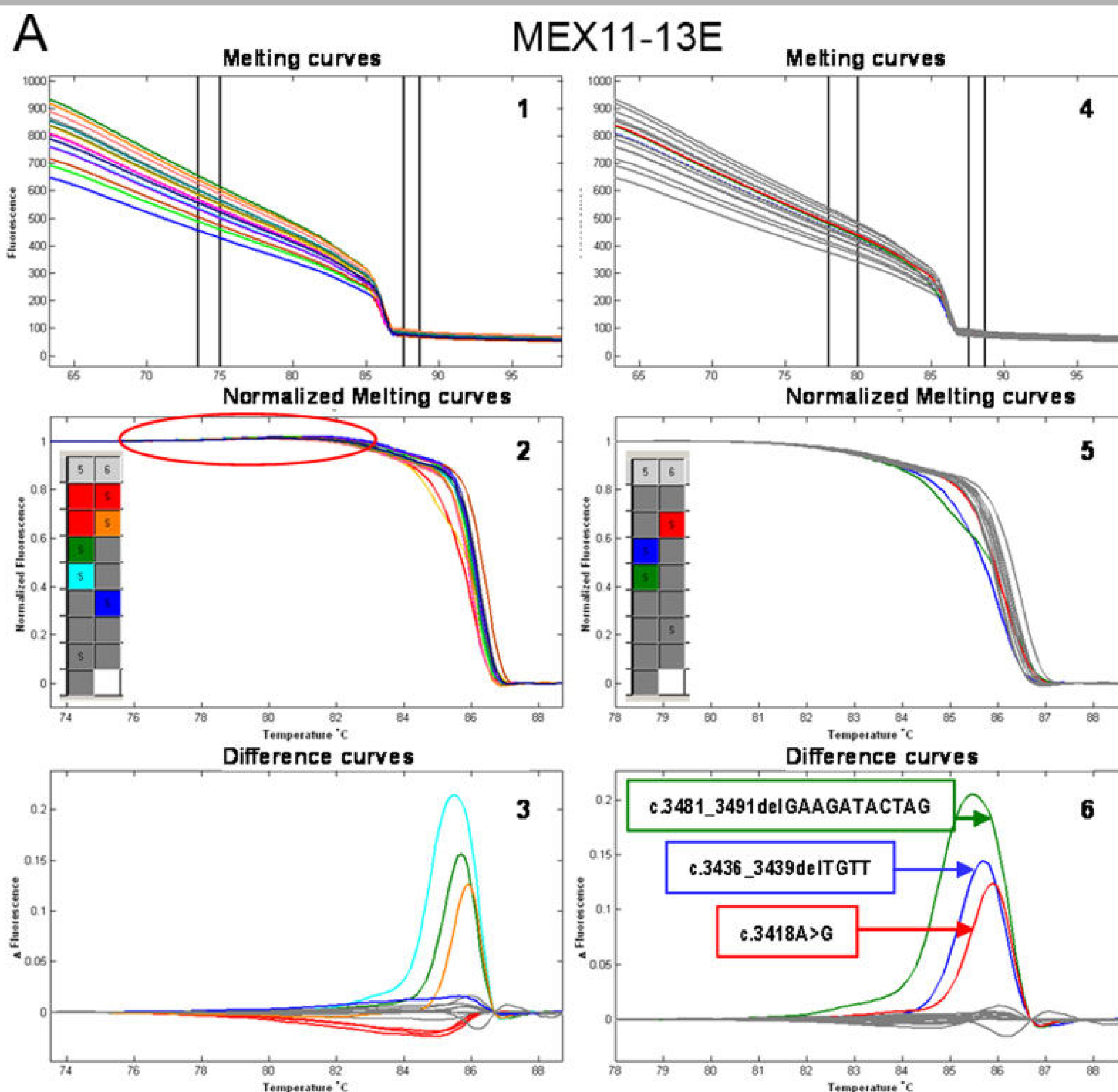


Figure 1. **A,B:** Adjusting "Normalize Setting" for optimal melt curve analysis. Evaluation of optimal adjustment of normalization bar settings is illustrated for two amplicons. A: MEX11-13E: HRM analysis of 12 wt DNA samples and three variant samples of amplicon MEX11-13E. First left column depicts results of melting curves upon incorrect setting of normalization bars, resulting in aberrant normalized melting curve profiles as indicated with a red circle (2). Lowest plot 3 shows subsequent incorrect variant calling with several FP calls. Right column depicts correct normalization setting. Variant curves and corresponding mutations are called correctly and are indicated in lower right plot (6). No FPs are detected. Note that sensitivity levels are kept fixed at 3.0. **B:** MEX11-15: HRM analysis of 14 wt DNA samples and one variant sample of amplicon MEX11-15. First left column depicts results of melting curves upon incorrect setting of normalization bars, resulting in aberrant normalized melting curve profiles as indicated with a red circle (2). Lowest plot 3 shows subsequent incorrect variant calling. Right column depicts correct normalization setting. Variant curves and congruent mutations are called correctly and are indicated in lower right plot (6). No FPs are detected. Note that sensitivity levels are kept fixed at 3.0.

c.3113A>G. In total, 26 out of 28 homozygous variants reactions were detected, representing 14 unique homozygous variants. This results in a detection ratio of 93%. In Supp. Figure S2 the variant detection by several amplicons is illustrated, including MEX07 (A), MEX08 (B), MEX11-10 (C), MEX16B (D), and MEX24 (E). For amplicon MEX11-10, seven different variants were tested, including the homozygous polymorphism c.2082C>T and several different deletions ranging from 1 to 5 nucleotides. Another homozygous polymorphism (POL2), c.4837A>G, is shown for amplicon MEX16B, of which the melt curve is relatively close to the wt curves but clearly visible and reproducibly detected. This polymorphism is located only two nucleotides from the start site of the forward primer located at c.4835. For the other amplicons the variants are shown in triplicate or duplicate to illustrate repeatability of the tests. All identical variants grouped nicely together at the indicated sensitivity levels.

The specificity of the HRM for all amplicons was addressed by evaluating a total of 197 different wt DNA samples. This resulted in 352 wt reactions for the complete evaluation of all 58 amplicons. In total, 12 FP reactions were observed (3.4%), resulting in a specificity

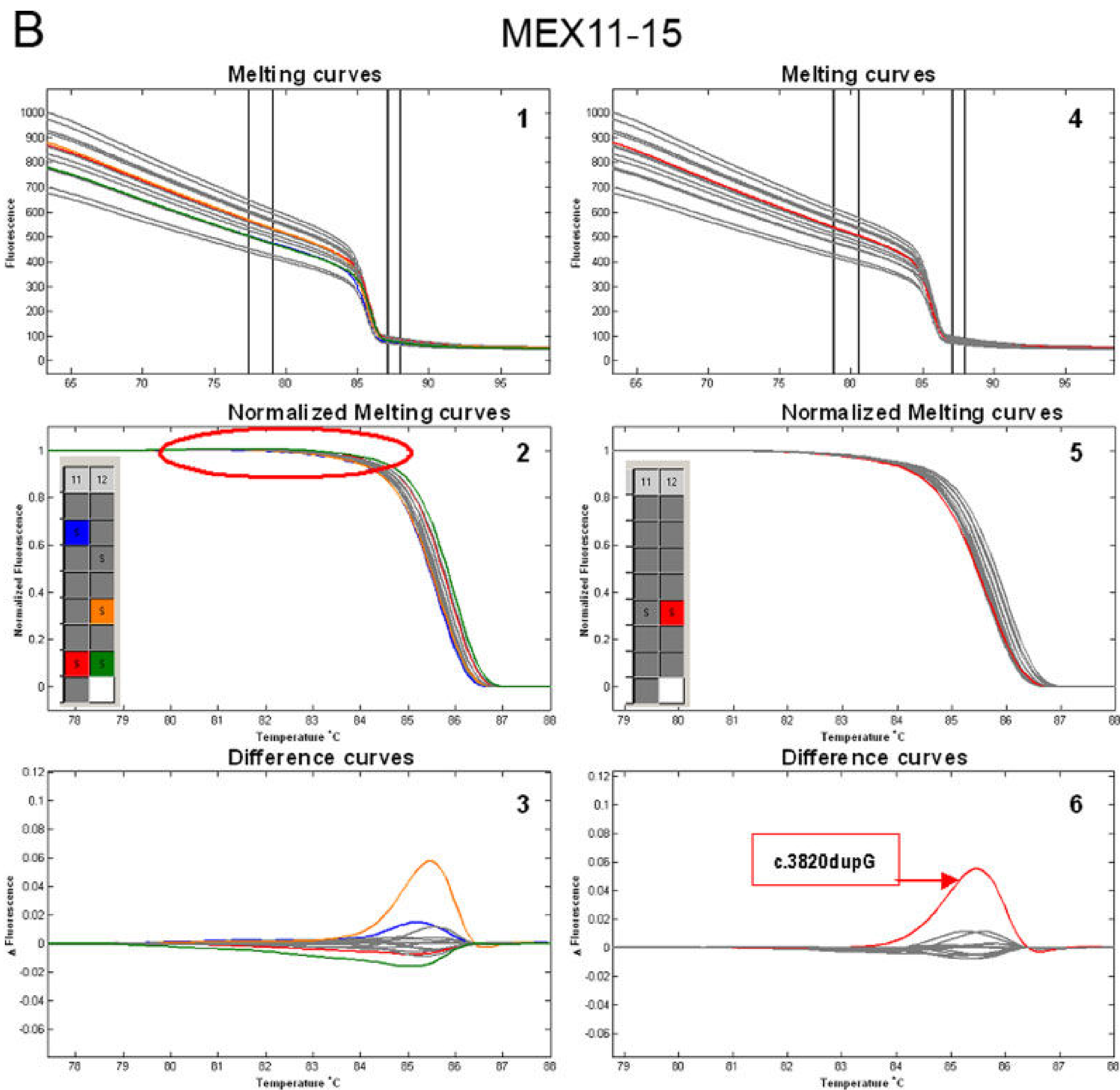


Figure 1. *Continued.*

of 96.6%. The detection of FPs varied per amplicon and overall a good reproducibility of the melt curves per amplicon was observed using this broad panel of Gentra-derived DNA isolations.

Eventually, based on our results and the described criteria for primers and HRM analysis, we subsequently selected the 40 best-performing primer sets that encompassed the entire coding region of *BRCA1*. This primer selection is depicted in Supp. Table S3 and results in an increased specificity of 97.6% and a sensitivity of at least 98.3% with a confidence interval of 95% (180/180 heterozygous variant reactions). Supp. Table S7 gives an overview of the LS software settings applied.

Interlaboratory Testing of 10 Selected Amplicons

Next, we tested the analytical performance and reproducibility of a selection of primer sets in two other laboratories (EuroGentest member). For this interlaboratory performance test we selected 10 different amplicons ranging in size, GC content, and HRM performance, including variants that gave melt profiles close to the wt control melt curve (Table 1). Apart from amplicons that harbored variants that were easily detected, we also included the largest amplicon MEX11A-L tested (625-bp-long) and the amplicon MEX11J that harbors the homozygous variant c.3113A>G, which could not be detected in the evaluation studies performed in Leiden. In both laboratories all tests were performed with identical consumables, samples, and PCR conditions as described in Materials and Methods. All HRM reactions were examined as described in Materials and Methods with “Auto Group High.” Both laboratories detected all heterozygous variants, indicating good reproducibility of the HRM tests. Again the homozygous variant c.3113A>G could not be detected by amplicon MEX11J. For the large amplicon MEX11A-L (624-bp-long) all variants were correctly detected, including the one nucleotide deletion of variant c.1016delA that displays a melt

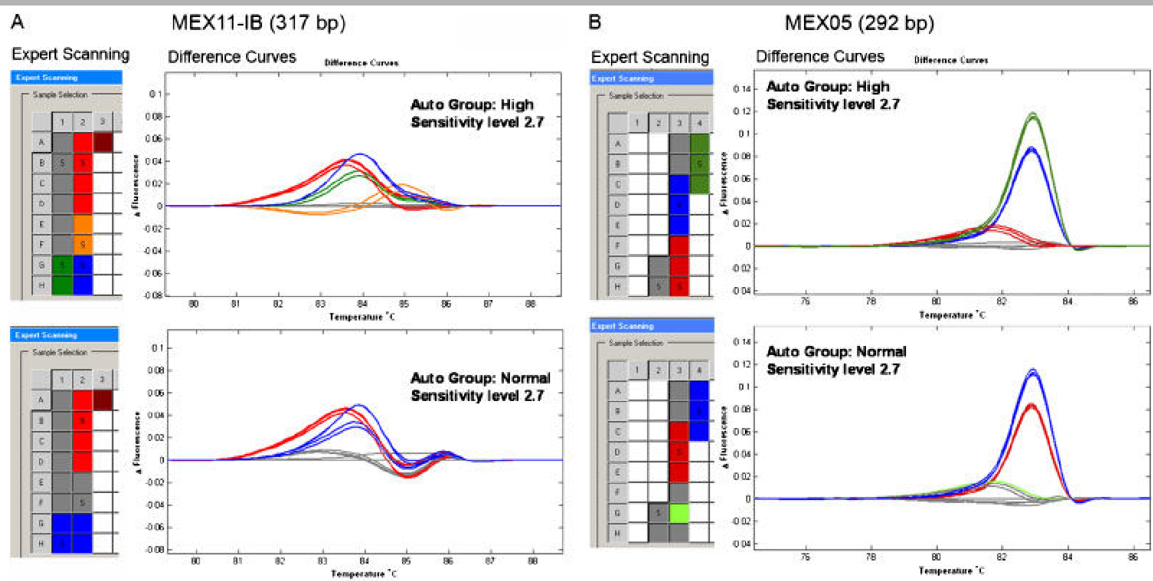


Figure 2. **A,B:** High sensitivity level is needed for detection of certain deletion variants. A comparison of mutation detection analysis is shown for two amplicons using either High sensitivity level vs. Normal sensitivity level. In (A) variant detection for of several variants of amplicon MEX11I-B at normal and high sensitivity levels is illustrated. The pathogenic mutation c.2898delT (wells E2-F2) is clearly detected at high sensitivity level (upper plot orange curve) but detection is lost at normal sensitivity level. Similarly, the mutation c.135-15_135-12delCTTT of amplicon MEX05 depicted in (B) is not consistently detected at normal sensitivity level but shows a well reproducible curve (red) at high sensitivity level. Samples and the location in the 96-well plate are indicated below the plots. WT curves are in depicted in gray.

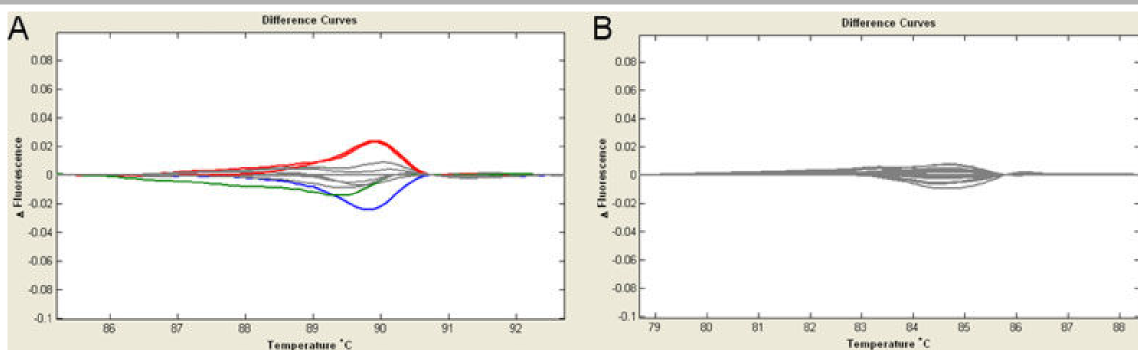


Figure 3. Curve profile of optimal wt melt curves. **A,B:** Comparison of two primer sets A and B and the examination of their HRM performance; each difference plot depicts the HRM analysis of 10 different wt samples. A: A typical bad profile of widely distributed wt curves, which indicates poor HRM quality and gives rise to FP reactions as indicated in two red, one green, and one blue curves. B: Shows the example of an optimal performing primer set for HRM; here all 10 different wt samples arrange close together and show little curve variation. Grouping is at standard ''Auto Group'' and sensitivity is set at High level 3.

curve which is close to the wt curves and that is occasionally not detected at normal sensitivity. Examination of all raw data from the LS melt files using the instrument settings defined by Leiden gave rise to identical results, indicating good reproducibility of LS performance and test results in general from all the different laboratories.

In addition to repeating the DNA samples from Leiden, Leuven performed additional validation experiments by testing a set of 19 additional DNA samples purified by Chemagen in combination with the wt control DNA samples from Leiden purified with the Gentra method (Table 2). Again all variants were detected. This implies that this DNA extraction method does not interfere with the HRM results and samples isolated by both these methods can be combined in one test.

In conclusion, these results indicate good interlaboratory reproducibility, even for more critical amplicons such as the large 625-bp fragment for exon 11, and show little influence of the DNA purification method.

Genotyping of Common *BRCA1* Polymorphisms

BRCA1 is known for harboring several common polymorphisms, resulting in the recurrent detection of variants for several amplicons. Because HRM has shown that different variants can group into the same melting curve profile, one can never conclude that variants that are detected as one group (color) all represent the same known variant and/or polymorphism. Consequently confirmation by

sequence analysis would be essential for all variants detected. However, for *BRCA1* this would result in the sequence analysis of all detected polymorphisms, which occur in around 10 to 20% of the PCRs. Therefore, we designed and tested nine unlabeled probes to identify the frequently-occurring polymorphisms of *BRCA1*, to avoid unnecessary sequence analysis upon detection of these nonpathogenic variants. In Supp. Table S5, we listed the probes and the corresponding amplicon used for this study. All probes were complementary to the reverse strand except for amplicon MEX11-4, and were designed using the criteria described by Wittwer et al. [2003] and in Supp. Table S2C. Due to the overlap of some amplicons, the presence of several polymorphisms was confirmed both directly and indirectly through probe analysis of the overlapping amplicon. For example, POL c.4837A>G occurs both in MEX16A and MEX16B, but is only confirmed by probe analysis for MEX16A.

Previous studies performed their genotype PCRs using 50 to 55 cycles; however, by using a slightly higher end concentration of primers, we obtained correct and good genotype results at 40 cycles. This made combination of amplicons with and without probes on the same plate feasible even though the unlabeled probe genotyping requires an asymmetric PCR. Finally, all probes correctly detected their specific polymorphism as illustrated for polymorphisms c.4308 T>C and c.4837A>G. of exon 13 and 16, respectively, in Supp. Figure S3A and B. Both the 3′ phosphate- and a 3′-C3 carbon-spacer block gave good and similar results. However, due to better stability (especially at 4°C) the Idaho Technology research team recommends employing the C3-block. The use of HPLC purified probes did slightly improve the grouping and subsequent detection of the polymorphisms.

Robustness: The Effect of DNA Purification, Concentration, and Sample Variation

To determine the effect of the DNA isolation method, we compared the results of several HRM tests using DNA samples purified by different methods including phenol extraction, Qiagen columns purification, and automated Chemagen and Gentra (Autopure) DNA isolations. PCR and HRM analysis criteria were identical for all tests as described above. Results showed that the DNA isolation method applied did not specifically influence the HRM results and differently-isolated DNA samples could easily be examined and compared in one test. The minor signal variation among the samples was similar to the signal variation observed in series of identically isolated DNA samples.

In addition to the variation in DNA isolation methods, we also examined the influence of different DNA concentrations for the detection of wt samples and variant samples. Three amplicons, MEX07, MEX11-13C, and MEX11-13F, were evaluated in duplex using a DNA concentration range of 50, 20, 10, and 5 ng per reaction. For each amplicon, two wt control DNA samples were tested together with one variant. We observed no deviating results when using 20, 10, or 5 ng per reaction and all curves correctly grouped together; only the reactions that contained an input of 50 ng DNA per reaction occasionally (3/18), gave rise to a slightly deviating curve (data not shown).

In conclusion, HRM is a robust assay and the detection of variant or wt samples is not hampered when HRM reactions contain four times less DNA than usual (down to 5 ng). An input of a higher amount of DNA (2.5×) can occasionally result in a deviating curve and therefore could give rise to an FP reaction. Note that if samples give rise to poor amplification results this will always result in an aberrant melt curve, indicating that further analysis for this sample is necessary.

Guidelines for Interpretation of HRM Data

The analysis of both the amplicon melt curves and, if applicable, the additional probe melt curves, results in a large set of data that requires correct interpretation. The combination of probe data and amplicon data especially needs extra attention. Based on all our observations during this elaborate evaluation study, we propose a list of guidelines that can be applied for the interpretation of the HRM data, which is described in detail in Supp. Table S8. In principle, all curves that deviate from the wt curve and appear in a different color in the difference plots using validated fixed settings potentially contain a variant in the sequence and need to be sequenced. This can be done directly on the HRM PCR sample. In case aberrant melt curves are visible for some reactions, repeating the melt step of the same plate again can improve the results (for details see Supp. Table S8). Standard rule is that once a sample is tested as a wt curve, the sample will not contain a heterozygous variant even if it shows some deviation in the other melt step.

Note that the unlabeled probe genotype analysis will only confirm the presence (heterozygous or homozygous) or absence (wt) of the examined common polymorphisms. But it does not exclude the presence of an additional variant in the total amplicon and the results should always be combined with the melt results of the complete amplicon. More general aspects are indicated in Supp. Table S8.

Final Validation Step: Blind Tests Using 28 DNA Samples and the 40 Best-Performing Primer Sets

Finally, we performed two complete *BRCA1* specific HRM mutation scans using the 40 best-performing primer sets and two series of 15 DNA samples each, including one wt control. Again, we compared the performance of the HRM method with the current "gold standard" for mutation detection; i.e., sequence analysis. PCR reactions were performed as described previously and analyzed using the same fixed scanning analysis settings that were defined during the assessment of the large cohort of variants.

All 18 heterozygous variants, also identified with sequence analysis, were detected in the blind studies and include pathogenic mutations, unclassified variants, and infrequent polymorphisms resulting in 100% detection (see Supp. Table S9A and B). In addition, genotype analysis for nine common polymorphisms detected all 154 polymorphisms correctly and omitted sequence analysis of an additional 147 (13%) polymorphism-containing reactions. Note that upon probe detection of only one homozygous polymorphism in one series of 14 samples it was essential to sequence this PCR since we can never exclude the presence of an additional mutation in such samples. We detected 1.4% (8/560) and 2.5% (14/560) of FPs and three and four negative reactions (no product) per series, respectively, resulting in an average specificity of 98%. Table 3 shows a summary of all data and demonstrates that in total (two series) only 54 out of 1,120 PCR reactions (4.8%) would require subsequent sequence analysis. Indicating the large reduction in sequence analysis realized after prescanning the samples with HRM and applying genotype analysis for nine common polymorphisms. Due to the presence of M13 tags, sequence analysis could be performed directly on the HRM PCR reactions.

In summary, we again observed good reproducibility and high specificity of the selected primer sets using the previously fixed instrument settings.

Discussion

In this study we have not only performed an extensive evaluation and validation of HRM on the IT LS, but we also

Table 3. Summary of Two Blind Studies (28 Samples)*

	Series 1 [% (n)][a]	Series 2 [% (n)][a]
New VAR	1.6 (9)	1.6 (9)
FP	1.4 (8)	2.5 (14)
N.R	0.5 (3)	0.7 (4)
FN	0 (0)	0 (0)
POL/POL2	11.8 (66)	15.7 (88)
Single POL2[b]	1.3 (7)	–
Total sequence reactions[c]	4.8 (27)	4.8 (27)

New VAR, total of variants detected excluding the common polymorphisms; FP, false positive; N.R., no reaction and/or no clear melting curve observed; FN, false negative; POL, heterozygous polymorphism; POL2, homozygous polymorphism.
*PCR reactions per series = 560.
[a]Percentage of total samples analyzed per series (number of reactions in parentheses).
[b]Probe-mediated detection of a single POL2 per set of 14 reactions.
[c]Total number of reactions that would need subsequent sequence analysis.

include an interlaboratory assessment of HRM, a genotype analysis of nine common polymorphisms, the validation of a primer set for *BRCA1* mutation scanning, and most of all a list of diagnostic guidelines that can be applied for setting up HRM for other genes.

Previous studies have compared different HRM platforms and examined many individual HRM tests for specific mutations or small sets of samples [De et al., 2008; Takano et al., 2008; Reed and Wittwer, 2004], but these studies did not include interlaboratory testing nor such an elaborate panel of selected and validated variant and wt samples for one gene that were all verified by sequence analysis. A recent literature study reviewing the quality of various scanning techniques for *BRCA1* and *2*, indicated the need for more statistically significant studies that thoroughly verify the diagnostic accuracy of new mutation scanning techniques that include confidence intervals of the results [Gerhardus et al., 2007]. Therefore we tested a panel of variants exceeding the minimal number of 150 samples in order to reach a satisfactory lower bound of the 95% confidence interval of the assay sensitivity.

In this study, we compared HRM analysis with sequence analysis, which is the current gold standard for mutation detection in most diagnostic laboratories. Our study, using 170 individual variant and 197 individual wt DNA samples, indicate that HRM analysis is a highly sensitive method that detected all heterozygous mutations (157 samples) with a sensitivity of 100%. This results in a final statistical sensitivity point estimate of 100% with a 95% confidence interval of 98.3 to 100.0% (for 180 heterozygous variant reactions) when using the 40 selected primer sets. In total 58 amplicons, were thoroughly evaluated and this resulted in 248 different variant analyses and 352 wt analyses. Using the large panel of amplicons we already observed a specificity of 96.6%. This was increased to an average of 98.1% in the final blind test, which includes large sets of wt sequence-encoding amplicons and employs the selected 40 best-performing primer sets. Common *BRCA1* polymorphism could be easily detected using nine specific unlabeled probes that harbored the SNP, omitting unnecessary sequence analysis of 10 to 20% reactions per series. Evaluation in two independent diagnostic laboratories of 10 different amplicons that vary in size, melt profiles, and GC content yielded identical results. Moreover, the raw data files from these laboratories were analyzed using settings selected by Leiden, and again equal results were obtained, indicating good reproducibility of the melt file profiles derived from the individual LS instruments.

Based on this evaluation study, we composed a list of diagnostic guidelines for setting up, analyzing, and interpreting HRM for new genes, as summarized in Supp. Tables S2, S6, S7, and S8. This way, our extensive evaluation can not only greatly facilitate the set up for *BRCA1* but also of new mutation scanning tests in diagnostic laboratories using HRM and will avoid the evaluation of very large panels of variants.

However, the following critical issues will also need to be initially addressed when setting up a new test. First, HRMC testing for a new gene is only valuable when a large part of the samples and amplicons generate wt sequences and/or harbor the repetitive detection of a common polymorphism. In case a specific exon or DNA fragment of the gene can contain many different variants it is recommended to perform direct sequence analysis for this particular gene region. Second, the current evaluation concerns a gene with amplicons that have an average GC content ranging from 31% to 54%. Previous studies have shown that high GC content (>60%) can be a critical factor in obtaining optimal PCR and HRM results (Technology Assessment on HRM as reported by Helen White, National Genetics Reference Laboratory [NGRL], Wessex, United Kingdom; http://www.ngrl.org.uk/Wessex/downloads_reports.htm). Similarly, low GC content can also limit the detection of variants and most of all reduce sensitivity. Consequently, we recommend performing a more elaborate variant analysis for such high-GC– or low-GC–containing amplicons to evaluate the sensitivity and reproducibility of the tests while using fixed scanning settings. Reducing the size of the amplicon to 200 bp or even less can be one option to increase the mutation detection sensitivity. Again, more related technical details can also be found in the study by Helen White (NGRL).

Third, although we and others have shown that many homozygous variants can be detected, it is essential to realize that not all are found. Hence the detection of these variants will always require spiking with other (wt) PCR reactions. Finally, it is important to note that changing the reagents indicated in this study can lead to poor results. The dye is an especially crucial factor and should always be saturating. Today many more dyes are available and are also being evaluated for HRM. However, although it has been shown that several dyes give rise to good reproducibility for detection of specific known variants, so far their performance for mutation scanning analysis has not been shown in an statistically valuable study. Clearly this latter type of analysis demands excellent performance quality; therefore, we recommend thorough evaluation tests when using a new fluorescent dye.

An important guideline that resulted from our study is the evaluation of large series of wt samples per amplicon. The wt melt curves should always cluster close to each other for each amplicon in the difference plot. This way high sensitivity levels can also be selected when no variants are available for evaluation, and variant curves located close to the wt curve can be detected; moreover, it circumvents the detection of frequent FP scores. We recommend reevaluating the results again after performing the first series of diagnostic scanning tests. Note that when wt samples give large variation in the individual curves and adjustment of Ta or Mg concentration give no improvement, one should always consider developing new primer sets. Do note that this applies for amplicons which fall in the indicated GC content range tested in this study.

Based on our evaluation, we selected the "Auto Grouping" at "High" sensitivity level from the software menu. Although the fast majority of variants could easily be detected using the "Normal" sensitivity setting, some variants were only visible at this high setting. Several of these undetectable variants were homozygous variants; however, two heterozygous variants, namely c.135-15_135-12delCTTT and c.2898delT could also only be detected using the "High" sensitivity setting. Due to this

observation, we choose to select this level for all amplicons to accomplish a detection level as high as possible and to reduce the risk of missing important mutations such as c.2898delT. Because we selected for amplicons that have their wt melt curves in close range, we could easily increase the sensitivity level and only observed a very moderate increase of FPs compared to "Normal" sensitivity. Correspondingly, we observed a high specificity of 98% in the final blind tests using the "High" sensitivity levels for all amplicons, which is similar to the specificity found in the study by De Leeneer et al. [2008], who applied a "Normal" sensitivity level. Unfortunately, the two critical "del" mutations that needed a high sensitivity level in our investigation were not tested in this particular study. It may be that lower sensitivities could be applied to some amplicons and that the adjustment might be amplicon-dependent. However, since both examples concern small deletions, which occur less frequently than the substitutions during mutation scans for *BRCA1*, they were present less often in our cohort and could not be tested for all amplicons. Future tests using additional high numbers of such variants will need to confirm whether this is indeed applicable and not intrinsically related to the detection of some small deletions. Do note that 26 deletion variants in our variant panel are detected at normal sensitivity level, although c.1961delA is also closer to the wt curves at normal sensitivity. Because all 130 substitutions (including all possible nucleotides at various locations) were detected as close as up to two nucleotides away from the primer, it appears that, in general, deletions are more critical to detect and it is advisable to evaluate this type of variants more extensively.

In our study we noticed that some melt profiles could be significantly improved upon repeating the melt step in the LS. Such second melts can reduce the detection of FP samples in the test, as explained extensively in point 2 of Supp. Table S8.

Note also that the software can not always discriminate different variants in the same amplicon. Therefore, common polymorphisms should never be judged only by their similarity in melt profiles, but should always be confirmed by probe or sequence analysis to exclude the presence of a mutation with an identical melt profile. Also in our study, the overlap in melt profiles was not always simply explained by similarity of the substitution and short distance in location of the two variants. For example, c.2014A>T could not be discriminated from c.2019delA in amplicon MEX11-8, and c.1067A>G resulted in a similar melt profile as c.1209dupT in amplicon MEX11-4. However, in contrast to the observation in a previous study [De et al., 2008], we were able to distinguish the SNPs c.3113A>G from c.3119G>A and the pathogenic mutations c.2934T>G and c.2989_2990dupAA (data not shown). The latter two could also be easily distinguished at normal sensitivity level. This indicates first of all the obvious influence of elevating the sensitivity level from "normal" to "high" sensitivity for better distinction of variants, but also the influence of using different primer sets for the same gene region. The latter suggests that a small shift in nucleotide composition of the amplicon tested can significantly improve the detection. Notably, the mutation c.2989_2990dupAA is located only 20 bp away from the reverse primer in MEX11I-B, whereas the other one is more or less in the middle of the amplicon, which could perhaps explain the clear difference in melt profiles.

As indicated, we used the "Primer Design" (PD-v1.0) software as supplied by Idaho Technology for the design of 10 new primer sets to replace either moderate performing primers or omit the presence of a rare SNP under the primer. All sets were indicated in green by the PD primer list (indicating a theoretical good quality) and gave very good PCR products and HRM results. Therefore, we can recommend using the PD software for the design of new primer sets. Of course primers still need to be examined for SNPs and number of hits in a BLAST or BLAT search.

In summary, we conclude that HRM is a rapid and sensitive post-PCR mutation scanning method that can easily be applied in diagnostics to scan genes for various mutations. All results were in coherence with the earlier sequence data, indicating at least a similar sensitivity level when compared to this technique. The use of M13 tags greatly facilitates the direct sequence analysis of samples that show a variant melt curve and create a fast workflow. We do recommend taking note of the critical features mentioned in this study, which should be specifically addressed when applying HRM for mutation scanning analysis.

We have summarized all recommendations and guidelines that can be considered when setting up and performing HRM for other genes in the online supporting information. Finally, we supply a validated set of PCR primers for mutation scanning analysis of the *BRCA1* gene on the LS using identical test conditions.

Acknowledgments

This study was funded through EuroGentest Network of Excellence Project 2005–EU Contract no. FP6-512148 (http://www.eurogentest.org/). P.K. and P.N. were supported by VZFNM 00064203/6112/. We thank the research team from Idaho Technology (Salt Lake City, Utah, USA), in particular Dr. Deepika del Silva, and BIOKÉ (Leiden, Netherlands), for their outstanding help, technical improvements, and advice during the entire evaluation project.

References

Beck JS, Kwitek AE, Cogen PH, Metzger AK, Duyk GM, Sheffield VC. 1993. A denaturing gradient gel electrophoresis assay for sensitive detection of p53 mutations. Hum Genet 91:25–30.

Castilla LH, Couch FJ, Erdos MR, Hoskins KF, Calzone K, Garber JE, Boyd J, Lubin MB, Deshano ML, Brody LC. 1994. Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer. Nat Genet 8:387–391.

Claes K, Poppe B, Machackova E, Coene I, Foretova L, De Paepe A, Messiaen L. 2003. Differentiating pathogenic mutations from polymorphic alterations in the splice sites of BRCA1 and BRCA2. Genes Chromosomes Cancer 37:314–320.

Couch FJ, Weber BL. 1996. Mutations and polymorphisms in the familial early-onset breast cancer (BRCA1) gene. Breast Cancer Information Core. Hum Mutat 8:8–18.

De Leeneer K, Coene I, Poppe B, De Paepe A, Claes K. 2008. Rapid and sensitive detection of BRCA1/2 mutations in a diagnostic setting: comparison of two high-resolution melting platforms. Clin Chem 54:982–989.

Deffenbaugh AM, Frank TS, Hoffman M, Cannon-Albright L, Neuhausen SL. 2002. Characterization of common BRCA1 and BRCA2 variants. Genet Test 6: 119–121.

den Dunnen JT, van Ommen GJ. 1999. The protein truncation test: a review. Hum Mutat 14:95–102.

Easton DF, Ford D, Bishop DT. 1995. Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. Am J Hum Genet 56:265–271.

Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J, Sobol H, Teare MD, Struewing J, Arason A, Scherneck S, Peto J, Rebbeck TR, Tonin P, Neuhausen S, Barkardottir R, Eyfjord J, Lynch H, Ponder BA, Gayther SA, Zelada-Hedman M. 1998. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. Am J Hum Genet 62:676–689.

Gerhardus A, Schleberger H, Schlegelberger B, Gadzicki D. 2007. Diagnostic accuracy of methods for the detection of BRCA1 and BRCA2 mutations: a systematic review 2. Eur J Hum Genet 15:619–627.

Goldgar DE, Easton DF, Deffenbaugh AM, Monteiro AN, Tavtigian SV, Couch FJ. 2004. Integrated evaluation of DNA sequence variants of unknown clinical significance: application to BRCA1 and BRCA2. Am J Hum Genet 75:535–544.

Gross E, Arnold N, Goette J, Schwarz-Boeger U, Kiechle M. 1999. A comparison of BRCA1 mutation analysis by direct sequencing, SSCP and DHPLC. Hum Genet 105:72–78.

Guldberg P, Guttler F. 1993. A simple method for identification of point mutations using denaturing gradient gel electrophoresis. Nucleic Acids Res 21:2261–2262.

Hanley JA, Lippman-Hand A. 1983. If nothing goes wrong, is everything all right? Interpreting zero numerators. JAMA 249:1743–1745.

Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. 2006. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. Clin Chem 52:494–503.

Liu W, Smith DI, Rechtzigel KJ, Thibodeau SN, James CD. 1998. Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. Nucleic Acids Res 26:1396–1400.

Montgomery J, Wittwer CT, Palais R, Zhou L. 2007. Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. Nat Protoc 2:59–66.

Narod SA, Foulkes WD. 2004. BRCA1 and BRCA2: 1994 and beyond. Nat Rev Cancer 4:665–676.

Ozcelik H, Antebi YJ, Cole DE, Andrulis IL. 1996. Heteroduplex and protein truncation analysis of the BRCA1 185delAG mutation. Hum Genet 98:310–312.

Reed GH, Wittwer CT. 2004. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis 14. Clin Chem 50:1748–1754.

Takano EA, Mitchell G, Fox SB, Dobrovic A. 2008. Rapid detection of carriers with BRCA1 and BRCA2 mutations using high resolution melting analysis. BMC Cancer 8:59.

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49:853–860.

Supp. Table S1A and B.

Supp. Table S1A. list of BRCA1 variants			
Exon	**Mutation[%]**	**classification variant [$]**	**type of variant [&]**
2	c.-7G>A	UV	Sub
	c.-19-22A>G	UV	Sub
	c.34C>A	MUT	Sub
	c.68_69delAG	MUT	Del
	c.68dupA	MUT	Dup
3	c.81-6T>A	MUT	Sub
	c.133A>C	UV	Sub
5	c.135-15_135-12delCTTT	UV	Del
	c.211A>G	UV	Sub
	c.212+5A>G	UV	Sub
6	c.213-26 G>A	UV	Sub
	c.213-12A>G	MUT	Sub
	c.288C>T	POL	Sub
	c.220C>T	MUT	Sub
	c.232delA	MUT	Del
7	c.302-41T>C	UV	Sub
	c.302-3C>G	MUT	Sub
	c.314A>G	UV	Sub
	c.396C>A	UV	Sub
	c.441G>C	UV	Sub
8	c.442-34C>T	POL	Sub
	c.442-34C>T	POL2	Sub
	c.470_471delCT	MUT	Del
	c.547+14delG	UV	Del
	c.536A>G + c.442-34C>T	UV + POL2	Sub
9	c.548-17G>T	UV	Sub
	c.557C>A	UV	Sub
	c.591C>T	POL	Sub
	c.591C>T	POL2	Sub
	c.593+25A>T	UV	Sub
10	c.594-34T>C	UV	Sub
	c.594-2A>C+ c.641A>G	UV + POL	Sub
11	c.693G>A	UV	Sub
	c.736T>G	POL	Sub
	c.744C>G	UV	Sub
	c.825C>T	UV	Sub
	c.981A>G	UV	Sub
	c.1065G>A	UV	Sub
	c.1067A>G	POL2	Sub
	c.1067A>G	POL	Sub
	c.1016delA	MUT	Del
	c.1209dupT	MUT	Dup
	c.1289dupA	MUT	Dup
	c.1292delT	MUT	Del
	c.1292dupT	MUT	Dup
	c.1456T>C	UV	Sub

	c.1411dupC	MUT	Dup
	c.1418A>G	UV	Sub
	c.1487G>A	POL	Sub
	c.1525A>G	UV	Sub
	c.1621C>T	MUT	Sub
	c.1648A>C	UV	Sub
	c.1865C>T	UV	Sub
	c.1878A>G	POL	Sub
	c.1961delA	MUT	Del
	c.2005A>T	UV	Sub
	c.2014A>T	MUT	Sub
	c.2019delA	MUT	Del
	c.2014A>T + c.2082C>T	POL + POL	Sub
	c.2077G>A + c.2082C>T	POL + POL	Sub
	c.2077G>A + c.2082C>T	POL + POL2	Sub
	c.2077G>A + c.2082C>T	POL2 + POL2	Sub
	c.2082C>T	POL	Sub
	c.2082C>T	POL2	Sub
	c.2156_2157delAA	MUT	Del
	c.2197_2201delGAGAA	MUT	Del
	c.2311T>C + c.2612C>T	POL+ POL	Sub
	c.2311T>C + c.2612C>T	POL2 + POL2	Sub
	c.2338C>T	MUT	Sub
	c.2338C>T + c.2612C>T	MUT + POL	Sub
	c.2338C>T + c.2311T>C + c.2612C>T	MUT + POL2 + POL2	Sub
	c.2338C>T + c.2311T>C + c.2612C>T	MUT + POL + POL	Sub
	c.2359dupG	MUT	Dup
	c.2521C>T	POL	Sub
	c.2522G>A	UV	Sub
	c.2612C>T	POL	Sub
	c.2612C>T	POL2	Sub
	c.2599C>T + c.2612C>T	MUT	Sub
	c.2685_2686delAA	MUT	Del
	c.2733A>G	UV	Sub
	c.2898delT	MUT	Del
	c.2920T>C	UV	Sub
	c.2934T>G	MUT	Sub
	c.2989_2990dupAA	MUT	Dup
	c.3024G>A	UV	Sub
	c.3113A>G	POL	Sub
	c.3113A>G	POL2	Sub
	c.3113A>G + c.3119G>A	POL + POL	Sub
	c.3119G>A	POL	Sub
	c.3328_3330delAAG	UV	Del
	c.3418A>G	UV	Sub
	c.3436_3439delTGTT	MUT	Del
	c.3481_3491delGAAGATACTAG	MUT	Del
	c.3485delA	MUT	Del
	c.3485delA + c.3548A>G	MUT + POL	Del + Sub

	c.3548A>G	POL	Sub
	c.3548A>G	POL2	Sub
	c.3627A>G c.3548A>G	UV + POL2	Sub
	c.3627A>G	UV	Sub
	c.3640G>A	UV	Sub
	c.3640G>T	MUT	Sub
	c.3748G>T	MUT	Sub
	c.3770_3771delAG	MUT	Del
	c.3820_3821insG	MUT	Ins
	c.4039A>G	POL	Sub
	c.4039A>G	POL2	Sub
	c.4065_4068delTCAA	MUT	Del
12	c.4183C>T	MUT	Sub
	c.4165_4166delAG	MUT	Del
13	c.4186C>T + c.4308T>C	MUT+ POL	Sub
	c.4308T>C	POL	Sub
	c.4308T>C	POL2	Sub
	c.4327C>T + c.4308T>C	MUT + POL	Sub
14	c.4358-11T>C	UV	Sub
	c.4391delCinsTT	MUT	Del/Ins
	c.4416_4417delTTinsG	MUT	Del/Ins
	c.4483delA	MUT	Del
15	c.4535G>T	POL	Sub
	c.4599T>C	UV	Sub
	c.4600G>A	UV	Sub
	c.4644G>A	POL	Sub
16	c.4689 C>G	MUT	Sub
	c.4812A>G	UV	Sub
	c.4837A>G	POL	Sub
	c.4837A>G	POL2	Sub
	c.4951T>C + c.4837A>G	UV + POL	Sub
	c.4951T>C	UV	Sub
	c.4956G>A + c.4837A>G	POL + POL	Sub
	c.4986+5G>T	UV	Sub
	c.4986+5G>T+ c.4837A>G	UV+POL	Sub
	c.4691T>C	UV	Sub
17	c.4987-92A>G + c.4987-68A>G*	POL	Sub
	c.4987-92A>G + c.4987-68A>G*	POL2	Sub
	c.4987-20A>G	POL	Sub
	c.5030_5033delCTAA	MUT	Del
	c.4987-75A>G + c.4987-90A>G*	POL + POL	Sub
18	c.5075-53C>T + c.5152+66G>A*	POL + POL	Sub
	c.5075-53C>T + c.5152+66G>A*	POL+ POL2	Sub
	c.5095C>T	MUT	Sub
	c.5096G>A + c.5075-53C>T + c.5152+66*	UV + POL + POL	Sub
	c.5096G>A	UV	Sub
	c.5152+20T>A	UV	Sub
	c.5152+66G>A*	POL	Sub
	c.5152+66G>A*	POL2	Sub

	c.5152+73A>G*	POL	Sub
19	c.5153-45C>T	UV	Sub
	c.5158A>G	UV	Sub
	c.5177_5180delGAAA	MUT	Del
	c.5177_5178delGA	MUT	Del
20	c.5239C>T	MUT	Sub
	c.5251C>T	MUT	Sub
	c.5266dupC	MUT	Dup
	c.5277+1G>A	MUT	Sub
21	c.5300G>C	UV	Sub
	c.5430dupG	MUT	Dup
22	c.5341G>T	MUT	Sub
	c.5406+5G>A	MUT	Sub
	c.5406+8T>C	UV	Sub
23	c.5407-25T>A	UV	Sub
	c.5467G>A	UV	Sub
	c.5449G>T	MUT	Sub
	c.5467+21dupT	UV	Dup
24	c.5468-10C>A	UV	Sub
	c.5485dupG	UV	Dup
	c.5503C>T	MUT	Sub
	c.5503_5564del	MUT	Del
	c.5513T>G	UV	Sub
	c.5559_5560insA	MUT	Ins
	c.5585A>T	UV	Sub
	c.*36C>G	UV	Sub

% Mutations are indicated according to HGVS mutation nomenclature guidelines and DNA mutation numbering system used is based on cDNA sequence. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.
$ MUT is pathogenic variant, UV is unclassified variant, POL is heterozygous polymorphism and POL2 is homozygous polymorphism.
& Sub is nucleotide substitution, Del is deletion, Dup is duplication, Ins is insertion
* For these amplicons the indicated POLs were tested using two sets of primers, one that generate a large amplicon including the POLs and one final selected set that amplifies an amplicon excuding the POLs. The first primer set for the large amplicon detected all POLs and the second set only those POLs that are included in the amplicon.

Supp. Table S1B. distribution of types of mutations of BRCA1

Summary+	Total Subs	Total Dels	Total Dup/Ins
Amount+	130	28	15
Percentage+	75%	16%	9%

+ Note that in this summary the 2 variants that have a combination of Del and Dup/Ins occurring in one sample were counted double at both the Dels and the Dup/Ins, similar the combination of Del and Sub is counted double.

Supp. Table S2. Criteria for set up and optimization HRM PCR

Supp. Table S2A. Primer design criteria:	
1.	Primers have to be specific for the target as judged by a genome sequence database analysis program such as the BLAST analysis of the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).
2.	Primers should not contain common/frequent Single Nucleotide Polymorphisms (SNPs) and SNPs should be avoided in primer sets that amplify a single exon-region and do not have an overlap with other primer sets. All SNPs were evaluated using the program 'Diagnostic SNP Check' supplied by the National Genetics Reference Laboratory (NGRL) Website (Manchester) which employs the latest build dbSNP (build 127 at http://www.ncbi.nlm.nih.gov/SNP/index.html) of the human genome from NCBI.
3.	The primer length should be no longer than 30 nucleotides excluding the length of the M13 tail.
4.	The region amplified should be preferentially 250-350 bp and not exceed 420 bp.

Supp. Table S2B. PCR-product and basic HRM criteria	
1.	Primer sets should give an optimal PCR product (see point 2) and reproducible melt curves that group together (same auto group/colour curve in Group menu) at a broad range of annealing temperatures as judges by the LightScanner software. Select optimal Ta within this region.
2.	PCR reaction should be at plateau phase/saturated, and result in a high fluorescent product (> 500) as measured by HRM
3.	PCR product should give no more than two melting domains in the melting curve upon HRM analysis.
4.	Purity of the PCR product needs to be examined upon optimization on agarose gel and solely reactions that generate the correct size product and do not contain additional bands, such as primer dimers, are acceptable.

Supp. Table S2C. Criteria for probe design for Genotype analysis of common polymorphisms	
1.	The Tm was kept below 70°C reducing interference with the polymerase extension step,
2.	Length of probe was around 30 bp.
3.	GC content of probe was in a range of 45 to 55%.
4.	The frequent occurring SNP was included in the probe to create most optimal detection of the polymorphism (POL) and potential other variants under the probe.
5.	All probes must carry a block at the 3' end omitting participation in the PCR reaction and 3' exonuclease activity. A phosphoramidite 3'-C3 carbon -spacer block is recommended, due to better long term stability.
6.	The use of HPLC purified probes is recommended.

Supp. Table S3

Supp. Table S3. HRM primer list for BRCA1

Exon	**Primer name**	**primer sequence 5'-3'***	Ta/cycli$	Amplicon Length#	GC %
MEX2	BR1EX2F-M13-2	**TGTAAAACGACGGCCAGT** gaagttgtcattttataaaccttt	61/40	294	32
	BR1EX2R-M13-2	**CAGGAAACAGCTATGACC** tgtgtcttttcttccctagtatgt			
MEX3	BR1EX3F-M13	**TGTAAAACGACGGCCAGT** ttgaggccttatgttgactcag	66/40	347	35
	BR1EX3R2-M13	**CAGGAAACAGCTATGACC** tgaaatggagttggatttttcg			
MEX5	BR1EX5F-M13	**TGTAAAACGACGGCCAGT** ttcatggctatttgccttttg	61/40	292	31
	BR1EX5R-M13	**CAGGAAACAGCTATGACC** tgatgaatggttttataggaacg			
MEX6	BR1EX6F-M13	**TGTAAAACGACGGCCAGT** ggttttctactgttgctgcatct	59/40	318	35
	BR1EX6R2-M13	**CAGGAAACAGCTATGACC** gaaagtaattgtgcaaacttcctg			
MEX7	BR1EX7F-M13	**TGTAAAACGACGGCCAGT** gggtttctcttggtttctttga	59/40	279	34
	BR1EX7R3-M13	**CAGGAAACAGCTATGACC** agaagaagaaaacaaatggttt			
MEX8	BR1EX8F-M13	**TGTAAAACGACGGCCAGT** ttcaggaggaaaagcacagaa	66/40	320	39
	BR1EX8R-M13	**CAGGAAACAGCTATGACC** cacttcccaaagctgcctac			
MEX9*	BR1EX9F3-M13	**TGTAAAACGACGGCCAGT** acccttttaattaagaaaacttttat	63/40	217	30
	BR1EX9R3-M13	**CAGGAAACAGCTATGACC** aaagagagaaacatcaatcct			
MEX10	BR1EX10F-M13	**TGTAAAACGACGGCCAGT** tggtcagctttctgtaatcgaa	61/40	318	39
	BR1EX10R-M13	**CAGGAAACAGCTATGACC** aaggtcccaaatggtcttca			
MEX11A*	BR1EX11AF2-M13	**TGTAAAACGACGGCCAGT** gttgatttccacctccaagg	63/40	384	37
	BR1EX11A2-M13R4	**CAGGAAACAGCTATGACC** gtttgcttttattacagaattcagcc			
MEX11C	BR1EX11C-M13F	**TGTAAAACGACGGCCAGT** tcatgccagctcattacagc	63/40	414	41
	BR1EX11C-M13R	**CAGGAAACAGCTATGACC** tcagactccccatcatgtga			
MEX11-4	BR1EX11-4F-M13	**TGTAAAACGACGGCCAGT** gtgtgagagaaaagaatgg	59/40	257	40
	BR1EX11-4R-M13	**CAGGAAACAGCTATGACC** catctacctcatttagaacg			

Exon	Primer name	primer sequence 5'-3'*	Ta/cycli$	Amplicon Length#	GC %
MEX11-5	BR1EX11-5F-M13	**TGTAAAACGACGGCCAGT** gaatcaaatgccaaagtagc	59/40	316	38
	BR1EX11-5R-M13	**CAGGAAACAGCTATGACC** ggacgctcttgtattatctg			
MEX11-6	BR1EX11-6F-M13	**TGTAAAACGACGGCCAGT** attataggagcatttgttac	59/40	320	37
	BR1EX11-6R-M13	**CAGGAAACAGCTATGACC** ttttcgagtgattctattgg			
MEX11-7	BR1EX11-7F-M13	**TGTAAAACGACGGCCAGT** caaaaggtgattctattcag	59/40	272	37
	BR1EX11-7R-M13	**CAGGAAACAGCTATGACC** attaggtgggcttagatttc			
MEX11-8	BR1EX11-8F-M13	**TGTAAAACGACGGCCAGT** caggcatattcatgcgcttg	59/40	254	40
	BR1EX11-8R-M13	**CAGGAAACAGCTATGACC** tacttgtctgttcatttggc			
MEX11-10*	BR1EX11-10F2-M13	**TGTAAAACGACGGCCAGT** aggtaaagaacctgcaac	61/40	333	38
	BR1EX11-10R2-M13	**CAGGAAACAGCTATGACC** atactgctactctctacagat			
MEX11H	BR1EX11H-M13F	**TGTAAAACGACGGCCAGT** gtggagaaagggttttgcaa	66/40	420	40
	BR1EX11H-M13R2	**CAGGAAACAGCTATGACC** cctcttctgcatttcctggat			
MEX11I-A*	BR1EX11I-AF-M13	**TGTAAAACGACGGCCAGT** gtatccattgggacatgaag	66/40	347	41
	BR1EX11I-AR-M13	**CAGGAAACAGCTATGACC** gaccaaccacaggaaagcct			
MEX11I-B*	BR1EX11I-BF-M13	**TGTAAAACGACGGCCAGT** caagcctgtacagacagtta	66/40	317	38
	BR1EX11-13BR-M13	**CAGGAAACAGCTATGACC** caggtgacattgaatgttcc			
MEX11-13C	BR1EX11-13CF-M13	**TGTAAAACGACGGCCAGT** aaaatctgctagaggaaaac	59/40	246	37
	BR1EX11-13CR-M13	**CAGGAAACAGCTATGACC** catcactggaacctatttc			
MEX11-13D	BR1EX11-13DF2-M13	**TGTAAAACGACGGCCAGT** gttccagtactaatgaagtgggct	59/40	285	37
	BR1EX11-13DR-M13	**CAGGAAACAGCTATGACC** ctgaaatcagatatggagag			
MEX11-13E	BR1EX11-13EF-M13	**TGTAAAACGACGGCCAGT** tgaagaagtagttcagactg	59/40	276	39
	BR1EX11-13ER-M13	**CAGGAAACAGCTATGACC** aaagggctaggactcctgct			
MEX11-13F	BR1EX11-13FF-M13	**TGTAAAACGACGGCCAGT** agtcatgcatctcaggtttg	59/40	268	43
	BR1EX11-13FR-M13	**CAGGAAACAGCTATGACC** ataagttctcttctgaggac			
MEX11-14	BR1EX11-14F-M13	**TGTAAAACGACGGCCAGT** ctttcacccatacacatttg	59/40	273	41
	BR1EX11-14R-M13	**CAGGAAACAGCTATGACC** tgcagtcatttaagctattc			
MEX11-15	BR1EX11-15F-M13	**TGTAAAACGACGGCCAGT** gagtgtctgtctaagaacac	59/40	217	38
	BR1EX11-15R-M13	**CAGGAAACAGCTATGACC** tatttgcagtcaagtcttcc			
MEX11N	BR1EX11N-M13F	**TGTAAAACGACGGCCAGT** taatattggcaaaggcatctca	61/40	387	41
	BR1EX11N-M13R	**CAGGAAACAGCTATGACC** gctccccaaaagcataaaca			
MEX12*	BR1EX12F3-M13	**TGTAAAACGACGGCCAGT** cagcaagttgcagcgtt	63/40	251	44
	BR1EX12R3-M13	**CAGGAAACAGCTATGACC** atacatactactgaatgcaaaggac			
MEX13*	BR1EX13F3-M13	**TGTAAAACGACGGCCAGT** aatggaaagcttctcaaagtatt	63/40	341	41
	BR1EX13R2-M13	**CAGGAAACAGCTATGACC** ccttactcttcagaaggagat			
MEX14*	BR1EX14F2-M13	**TGTAAAACGACGGCCAGT** ctaacctgaattatcactatc	63/40	348	34
	BR1EX14R2-M13	**CAGGAAACAGCTATGACC** gtgtataaatgcctgtatgca			
MEX15*	BR1EX15F5-M13	**TGTAAAACGACGGCCAGT** ctttcacaattggtggcg	63/40	346	42
	BR1EX15R4-M13	**CAGGAAACAGCTATGACC** ccagaatatctttatgtaggattcag			
MEX16A	BR1EX16AF-M13	**TGTAAAACGACGGCCAGT** gaccagaactttgtaattc	59/40	296	46
	BR1EX16AR-M13	**CAGGAAACAGCTATGACC** cccagcagtatcagtagtat			
MEX16B	BR1EX16BF-M13	**TGTAAAACGACGGCCAGT** aaagttgcagaatctgccc	63/40	273	43
	BR1EX16R2-M13	**CAGGAAACAGCTATGACC** cataaaactctttccagaatgttg			
MEX17*	BR1EX17F4-M13	**TGTAAAACGACGGCCAGT** actagtattctgagctgtgtgc	66/40	249	37
	BR1EX17R3-M13	**CAGGAAACAGCTATGACC** cctcgcctcatgtggtt			

Exon	Primer name	primer sequence 5'-3'*	Ta/cycli$	Amplicon Length#	GC %
MEX18*	BR1EX18F2-M13	**TGTAAAACGACGGCCAGT** ctttagcttcttaggacagca	63/40	242	38
	BR1EX18R2-M13	**CAGGAAACAGCTATGACC** aaatgcaattctgaggtgtta			
MEX19	BR1EX19F-M13	**TGTAAAACGACGGCCAGT** ttgtgaatcgctgacctctct	66/40	247	37
	BR1EX19R-M13	**CAGGAAACAGCTATGACC** ggtgcattgatggaaggaag			
MEX20	BR1EX20F2-M13	**TGTAAAACGACGGCCAGT** ctggcctgaatgccttaaat	63/40	266	46
	BR1EX20R2-M13	**CAGGAAACAGCTATGACC** cagagtggtggggtgagatt			
MEX21	BR1EX21F2-M13	**TGTAAAACGACGGCCAGT** agattttccttctctccattcc	63/40	227	50
	BR1EX21R2-M13	**CAGGAAACAGCTATGACC** ccatcgtgggatcttgctta			
MEX22*	BR1EX22F3-M13	**TGTAAAACGACGGCCAGT** tcccattgagaggtcttgct	66/40	333	48
	BR1EX22R3-M13	**CAGGAAACAGCTATGACC** gagaagacttctgaggctac			
MEX23	BR1Ex23-M13F2	**TGTAAAACGACGGCCAGT** gatgaagtgacagttccagtag	59/40	225	50
	BR1EX23R-M13	**CAGGAAACAGCTATGACC** gtgataaaccaaacccatgc			
MEX24	BR1EX24F3-M13	**TGTAAAACGACGGCCAGT** cctagtccaggagaatgaattga	63/40	282	54
	BR1EX24R2-M13	**CAGGAAACAGCTATGACC** ctggaaaggccactttgtaa			

* Primers 'redesigned' using Primer Design Software (IT)
$ Ta/Cycli is optimal PCR annealing temperature (Ta) and number of PCR cycles
# amplicon length in bp includes primer sequence and M13 tails.

Supp. Table S4

Supp. Table S4. Composition of HR-MC PCR reactions		
Number of reactions:	no probe (µl)	with probe (µl)
LS mastermix	4	4
Mol. grade water	3.4	3.4
Primer F (10 pmol/ µl)	0.3	0.1#
Primer R (10 pmol/ µl)	0.3	0.5#
Probe (10 pmol/ µl)	-	0.5
DNA (10 ng / µl)	2	2
Total volume	10	10
Mineral oil	15	15

NB. The genotype analysis of c.1067T>C for amplicon MEX11-4 was performed using the opposite ratio of forward and reverse primers (5:1), because this probe is targeted to the forward strand.

Supp. Table S5

Supp. Table S5. List of BRCA1 probes used for Genotyping frequent polymorphisms

amplicon	Probe	sequence	SNP	alleles	location	length	GC%	Tm °C
MEX08	BR1EX08_442-34-B*	GCCAA**T**AATTGCTTGACTGTTCTTTACC	rs799923	C>T	c.442-34	28	39	66
MEX11-04#	BR1EX11_1067-B*	CTGAGCATGGCAGTTTC**C**GCTTATTCC	rs1799950	T>C	c.1067	26	50	68
MEX11-10	BR1EX11_2082-B*	GACATGACAG**T**GATACTTTCCCAGAG	rs1799949	C>T	c.2082	26	46	66
MEX11H	BR1EX11_2311-B*	GTAGCAGTATTTCA**C**TGGTACCTGGTAC	rs16940	T>C	c.2311	28	46	68
MEX11IA	BR1EX11_2612-B*	GCGCCAGTCATTTGCTC**T**GTTTTCA	rs799917	C>T	c.2612	25	48	66
MEX11-13C	BR1EX11-13c_3113-B*	GAAAATGTTTTTAAAG**G**AGCCAGCTCAAGC	rs16941	A>G	c.3113	30	40	68
MEX11-13E	BR1EX11_3548-B*	TAGCAAAAGCGTCCAGA**G**AGGAGAGCT	rs16942	A>G	c.3548	27	48	68
MEX13	BR1EX13_4308-B*	CATAAGTGACTC**C**TCTGCCCTTGAGGA	rs1060915	T>C	c.4308	27	52	69
MEX16A	BR1EX16A_4837-B*	CCCAG**G**GTCCAGCTGCTGCTCATA	rs1799966	A>G	c.4837	24	63	71

*B is C3 carbon (phosphoramidite)-spacer block as described in Material and Methods
Note this probe is specific for the forward strand.

Supp. Table S6.

Supp. Table S6. Guidelines for HRM analysis for detecting heterozygous variants$ (LS software program: Call IT version 1.5)

1.	Subset menu: Select subsets per amplicon.
2.	Negative Filter menu: Check fluorescent signal of products (>500) and no template control (≤ the end fluorescent value of the melted PCR products at 98^{0}C)
3.A	**Normalize menu:** A) The optimal width per set of the lower and upper normalization bars should be in a range of 1 to 2.5 degrees Celsius.
3.B	B) The location of the bars should be close around the melt domain and can be adjusted to optimal position by establishing a straight horizontal line at the start of the normalized melting curve (see study Figure 1)
4.	Curve Shift menu: Leave 'Curve Shift' setting at the default setting of 0.050.
5.A	**Grouping menu:** A) Group the curves for each individual amplicon using the standard 'Auto Group' option and select Sensitivity High.

5.B	B) The optimal sensitivity level can be examined by analyzing at least 5-10 wt control DNA samples and various variant DNA samples that preferentially include variants that harbour 1 nucleotide deletion.
5.C	C) The maximum sensitivity level that can be applied was established by testing the series of 5-10 individual wt curves and allowing a limited detection of 0 to 5 % (average) false positives. We recommend designing new primers for sets that yield a maximum grouping-sensitivity level below 2.7.
5.D	D) It is essential that Wt melt curves appear closely grouped together in the difference plot (see also Figure 2). This will allow selection of high sensitivity levels and subsequent detection of modest deviating DNA variants.
5.E	E) The minimum sensitivity of the amplicon can be established by analyzing the available variants. Again new primers were designed for sets that yielded a sensitivity level below 2.7. In case of AT rich amplicons, splitting up fragments and thus choosing a smaller sized amplicon can significantly increase the sensitivity value and subsequently improve the detection level of variants.
5.F	F) Based on the evaluation of wt's and variants, we recommend using the highest sensitivity value possible. The optimal sensitivity is reached when al variants are detected and no more than 0-5 % false positive reactions are observed in the wt series (excluding reactions that fail in the PCR). Again make sure wt curves are closely grouped in difference plot.

$Note these guidelines are based on the described evaluation and validation study of the indicated amplicon set for the BRCA1 gene using the LightScanner mastermix for PCR and the LightScanner for melting the plates. More GC rich and especially AT rich amplicons may require more stringent sensitivity settings.

Supp. Table S7.

Supp. Table S7. Overview of LightScanner software settings of validation study

	Lower Normalize settings*		Upper Normalize settings*			
Amplicon	**Lower Min**	**Lower Max**	**Upper Min**	**Upper Max**	**Ta/cycli**	**Sensitivity$**
MEX02	74.1	77.7	86.6	88.8	61/40	3.5
MEX03	78.2	80.2	87.3	88.5	63/40	3,0
MEX05	74.4	77.7	85.5	86.5	61/40	3.2
MEX06	78.7	81.1	87.4	88.4	59/40	3,0
MEX07	78.1	80.4	86.5	87.5	59/40	3.1
MEX08	77.6	79.6	89.2	90.2	66/40	3.3
MEX09	75.2	77.6	84.9	85.9	63/40	3.5
MEX10	78.1	81.1	88,0	89,0	61/40	3.3
MEX11A	78.2	80.2	89.3	90.3	63/40	3,0
MEX11C	80.5	83,0	89,0	90.4	63/40	3.3
MEX11-4	80.3	82.2	87.9	88.9	59/40	3,0
MEX11-5	78.5	81,0	86.7	88.5	59/40	2.9
MEX11-6	77.3	79.8	87.5	88.5	59/40	3.5
MEX11-7	79.6	81.2	87.6	89.1	59/40	3,0
MEX11-8	79.1	81.3	88.5	90,0	59/40	3.5
MEX11-10	78.7	80.7	87.3	88.3	61/40	3.0
MEX11H	80.0	82.0	88.5	89.5	66/40	3.1
MEX11IA	80.1	82.1	88.6	89.6	66/40	3.4
MEX11IB	79.3	80.3	87.7	88.7	66/40	3.2
MEX11-13c	78.4	80.4	86.3	88.8	59/40	3.2
MEX11-13d	77.2	80.1	87.4	88.4	59/40	3.0
MEX11-13e	78,0	80,0	88.4	89.9	59/40	3.0
MEX11-13f	80.9	82.7	89.5	90.5	59/40	3.1
MEX11-14	79.1	80.8	88.8	89.8	59/40	3.5
MEX11-15	78.8	80.6	87.1	88,0	59/40	3,0
MEX11N	79.9	82.6	88.7	90.4	61/40	3.1
MEX12	81,0	82.4	90,0	91,0	63/40	3.1
MEX13	79.8	81.9	89.9	90.9	63/40	3.0
MEX14	78.4	79.8	86.6	87.8	63/40	3,0
MEX15	78.6	79.9	89.7	90.7	63/40	3.8
MEX16A	81.3	83.3	90.7	91.7	59/40	2.9
MEX16B	77.6	80.2	90.4	91.4	63/40	3,0
MEX17	77.8	79.8	86.2	87.2	66/40	3.3
MEX18	78.4	80.9	87,0	88.4	63/40	3.1
MEX19	76,0	79.4	87.9	89,0	66/40	3.2
MEX20	83.5	84.8	89.8	90.7	63/40	3,0
MEX21	82.5	83.9	91.2	92.2	63/40	2.9
MEX22	82.6	84.2	90.7	91.7	66/40	2.9
MEX23	82.3	84.1	91.2	92.2	59/40	3,0
MEX24	83.5	84.8	93.8	94.8	63/40	2.9

***** Note that all normalization settings were good reproducible and applicable for all tests performed during this evaluation and validation study in Leiden and also using the raw data

obtained from Leuven and Prague. However, after replacing the LightScanner in Leiden with a new machine in 2008 all Melt temperatures appeared to make a shift of ~0.3 (ranging from 0.2-0.5) ^{o}C, requiring a overall shift in normalization settings of the same average value. Most amplicons were not affected by this change, but some like MEX11-10 appeared to have settings that were more sensitive for this change. Therefore we recommend verifying and if necessary adjusting all suggested normalization bar settings per amplicon.

$ Advised sensitivity based on evaluation of reported variants and large series of wt DNA samples.

NB. All probes were analyzed using Normal sensitivity 1.0

Supp. Table S8.

Supp. Table S8. Guidelines for interpretation of HRM data[$]	
1	In principle all curves that deviate from the wt curve and appear in a different color in the difference plots using selected fixed settings, potentially contain a variant in the sequence and need to be sequenced. (This can be done directly on the HRM PCR sample).
2[#]	In case aberrant melt curves are visible in the plots, for example due to air bubbles in reaction, it is recommended to repeat the melt step. Often this will improve the melt curve of the aberrant reaction and confirm whether this sample is wt after all or contains a variant. When performing a second melt for the same plate, melting curves can deviate from wt-curves in one melt and be wt in the second melt (or vice versa). Standard rule is that once a sample is tested as a wt curve, the sample will not contain a heterozygous variant even if it shows some deviation in a new melt step.
3	Common polymorphisms can be confirmed by using a genotype reaction and analysis. This avoids repeatedly sequencing of know polymorphisms. The genotype analysis will distinguish wt from heterozygous polymorphism (POL) and homozygous polymorphism (POL2).
4	Note that the homozygous polymorphism are not always detected in the complete amplicon melt-analysis by the software tool 'expert scanning' and can appear in this analysis step as a wt curve profile.
5	Analysis of polymorphisms in the unlabeled probe melt can only confirm the presence of the common POL/POL2, but does not display, and thus not exclude, the presence of other additional variants in the amplicon, unless the variant is under the probe (this will be clearly visible as a deviating curve). Therefore it is essential to check the 'expert scanning' melt-data of the complete amplicon to see if there is a deviating curve among the POL/POL2 curves.
6	In general (assuring no family members are tested in the same scanning round) the presence of solely the common POL(2) and no additional variants within the fragment can only be confirmed as follows: A) The unlabeled probe analysis confirms the POL (or POL2). B) The 'expert scanning' result of the complete amplicon groups all the POL containing samples in the same 'Auto Group' (same color curve). C) The analysis of the complete amplicon melt ('expert scanning') of all samples examined, there are at least 2 or more POL-curves detected. In case the 'expert scanning' analysis locates one of the POL containing curves in a different autogroup the sample needs to be sequenced to check for additional variants.
7	The same POL can be present in overlapping amplicons but only needs to be confirmed in one of the amplicons/subsets. In this case the probe analysis of one amplicon can be used for both (overlapping) amplicons to confirm the presence of the common POL.

$ Note these guidelines only apply assuming that the chosen fixed scanning settings used to analyze the HRM data have been well evaluated during a primary validation of the selected primer sets, using various wt and variant samples, and the described HRM indicated criteria are all meet.
# Do note that although in some cases the initial melt can be clearly disturbed for technical reasons and give rise to a completely aberrant melting curve that has to be excluded from the analysis, in other cases the deviation was more subtle and could be due to a lower heteroduplex formation or other factors. Based on our analysis of the large panel of wt samples (often in duplicate) we noticed that in all cases where FP samples gave deviating curve in one melt and showed a wt curve in the second melt step, the reaction represented a wt DNA sequence.

Supp. Table S9A and B

Supp. Table S9A. Overview variants& found in BRCA1 scan serie 1			
DNA code	**amplicon**	**classification$**	**variant%**
D1.06.09146	MEX08	UV + POL	c.536A>G, p.Tyr179Cys + c.442-34T>C
D1.06.09111	MEX11C*	POL	c.1067A>G, p.Gln356Arg
D1.06.09111	MEX11-4*	POL	c.1067A>G, p.Gln356Arg
D1.06.09146	MEX11-5	POL	c.1456T>C, p.Phe486Leu
D1.06.09146	MEX11-6	POL	c.1648A>C, p.Asn550His
D1.06.09052	MEX11-10	POL+ POL	c.2077G>A, p.Asp693Asn + c.2082C>T, p.Ser694Ser,
D1.06.08830	MEX11-10	POL+ POL	c.2077G>A, p.Asp693Asn + c.2082C>T, p.Ser694Ser
D1.06.08794	MEX11-10	POL+ POL2	c.2077G>A, p.Asp693Asn + c.2082C>T (pol2), p.Ser694Ser
D1.06.08794	MEX11-13E	UV	c.3418A>G p.Ser1140Gly

Supp. Table S9B. Overview variants& found in BRCA1 scan serie 2			
DNA code	**amplicon**	**classification$**	**variant%**
D1.06.09559	MEX09	UV	c.557C>A p.Ser186Tyr
D1.06.09150	MEX11C*	POL	c.1067A>G, p.Gln356Arg
D1.06.09150	MEX11-4*	POL	c.1067A>G, p.Gln356Arg
D1.06.09512	MEX11-10	POL + POL	c.2077G>A, p.Asp693Asn+ c.2082C>T, p.Ser694Ser
D1.06.09431	MEX11-13C@	POL	c.3113A>G, p.Glu1038Gly
D1.06.09396	MEX11N	POL	c.4039A>G, p.Arg1347Gly
D1.06.09559	MEX22	UV	c.5406+8T>C
D1.06.09559	MEX24	UV	c.*36C>G
D1.06.09598	MEX24	MUT	c.5503_5564del62, p.Arg1835fs

% Mutations are indicated according to HGVS mutation nomenclature guidelines and DNA mutation numbering system used is based on cDNA sequence. Nucleotide numbering reflects cDNA numbering

with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

$ MUT is pathogenic variant, UV is unclassified variant, POL is heterozygous polymorphism and POL2 is homozygous polymorphism.

& Variants represent the melt curves that deviate form the wt melt curves in the difference plots and do not fall into the frequent polymorphism groups.

* This POL was also identified by probe in MEX11-4, but because no second reaction with a POL curve was detected the presence of an extra variant could not be excluded and therefore is considered a VAR and demands to be verified by sequence analysis.

@ This melt curve, although close to c.3113A>G-pol curves, didn't group with these pol containing meltcurves and therefore was considered as a variant, however sequence analysis revealed it did only contain the polymorphism.

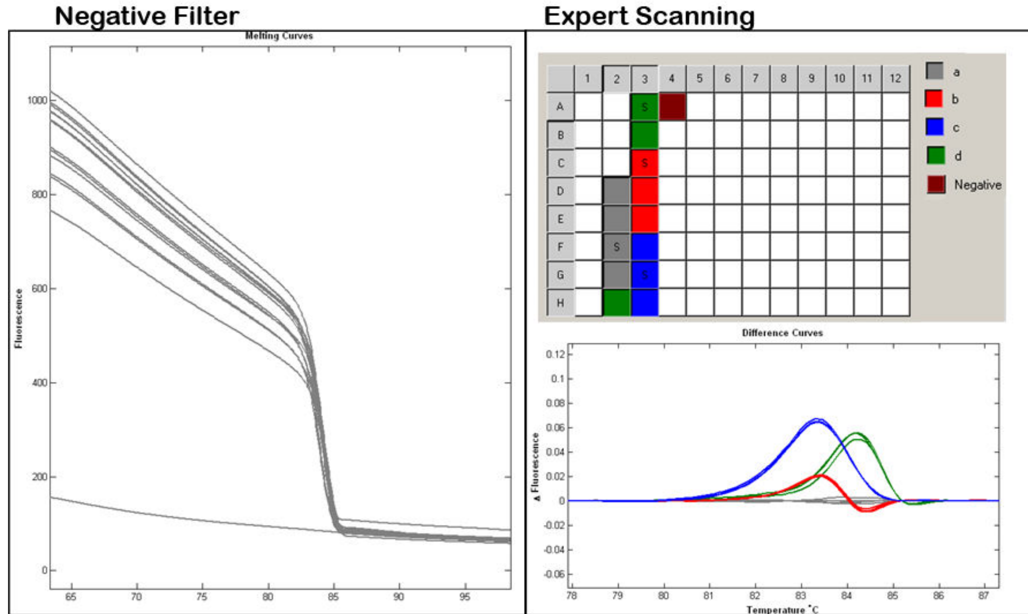
	1	2	3	4	5	6	7	8	9	10	11	12
A	WT A	8	WT A	8	WT A	8	WT A	8	WT A	8	WT A	8
B	1	9	1	9	1	9	1	9	1	9	1	9
C	2	10	2	10	2	10	2	10	2	10	2	10
D	3	11	3	11	3	11	3	11	3	11	3	11
E	4	12	4	12	4	12	4	12	4	12	4	12
F	5	13	5	13	5	13	5	13	5	13	5	13
G	6	14	6	14	6	14	6	14	6	14	6	14
H	7	N.T.	7	BL	7	N.T.	7	N.T.	7	N.T.	7	N.T.
	ampl. A		ampl. B		ampl. C		ampl. D		ampl. E		ampl. F	

Supp. Figure S1. Generic 96-well plate set up for blind study of complete BRCA1 scan
Overall 96 well plate layout is shown for the BRCA1 blind tests. Each plate contains the analysis of 6 amplicons for 14 DNA samples and includes a wt and a water control. Abbreviations; wt, wild type; N.T., no template (or Blank); BR1 ampl., BRCA1 specific amplicon/primer set.

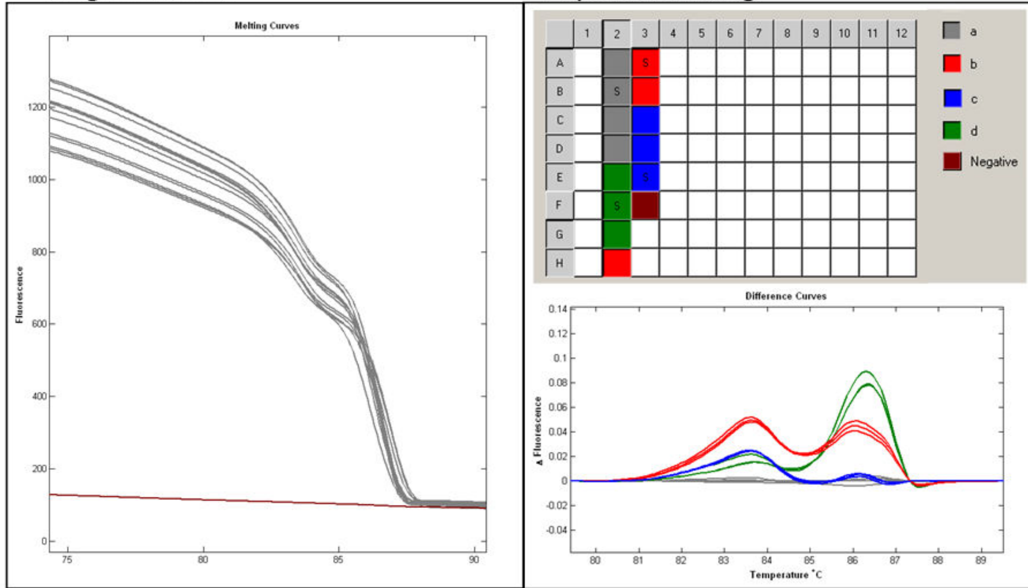
Supp. Figure S2A-E. HRM illustrated for amplicons MEX07, MEX08, MEX11-10, MEX16B and MEX24.

The mutation detection analysis for amplicons MEX07, MEX08, MEX11-10, MEX16B and MEX24 is illustrated in plots A, B, C, D and E respectively. For each amplicon the raw melting data before normalization as depicted in the Negative Filter menu is shown on the left. The meltcurve Grouping results and sample annotation (a to h) after normalization as obtained with Expert Scanning are shown on the right and include the 96-well sample selection plot and the corresponding difference plot with all the meltcurves. All variants tested are also listed in Supp. Table S1. As shown all variants are clearly detected and no false positive curves were observed. All grey curves labeled a are in each plot the wt samples. The following variants are displayed: For MEX07, b= c.302-41C>T ; c= c.302-3G>C ; d= c.441G>C; MEX08, b= [c. 536A>G] + [c. 442-34T>C] (UV, pol2) ; c= c. 442-34T>C (pol2) ; d= c. 442-34T>C (pol); MEX11-10, b=[c.2077G>A] + [c.2082C>T]; c= c.2197_2201delGAGAA (well C1 and well C2); d= c.2082C>T (pol2); e= c.2156_2157delAA; f= [c.2082C>T] + [c.2014A>T]; g= c.2019delA; h= c.2077G>A; MEX16B, b= [c.4951T>C] +[4837A>G]; c= c.4837A>G (POL2); d= [c.4956G>A] + [4837A>G]; e= c.4837A>G (POL); f= [c.4986+5G>T]+ [4837A>G] and MEX24, b= c.5559_5560insA; c= c.5585A>T; d= c.5503C>T; e= c.5503_5564del62. Grouping standards were all at Auto Group High Sensitivity and sensitivity levels are indicated in Supp. Table S7.

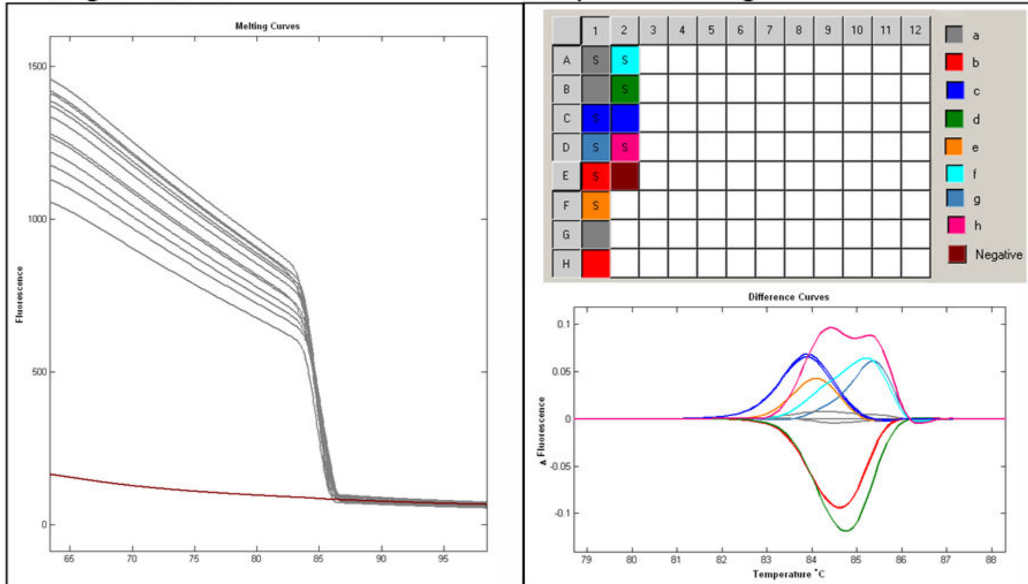
Supp Figure S2A.



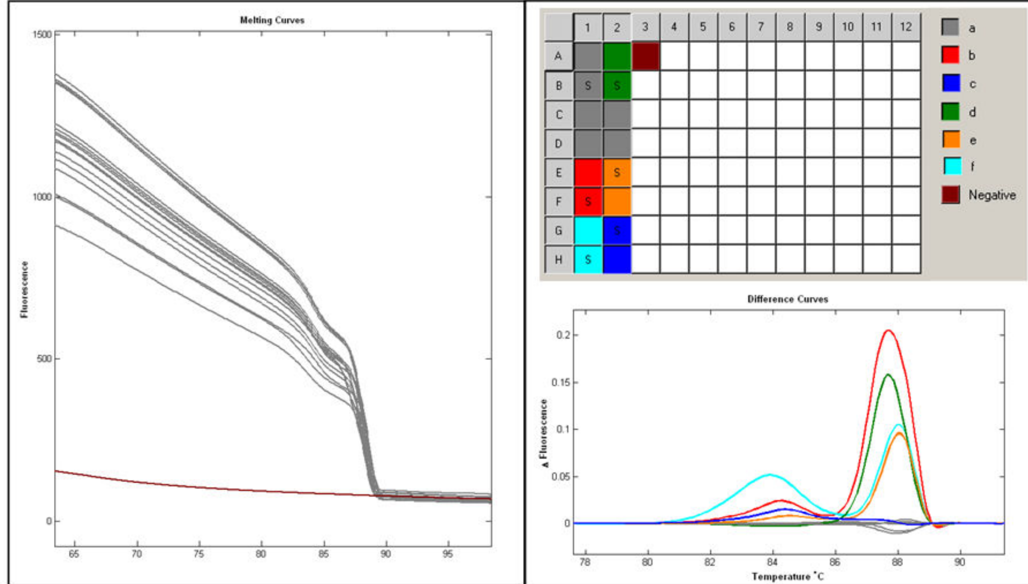
Supp. Figure S2B



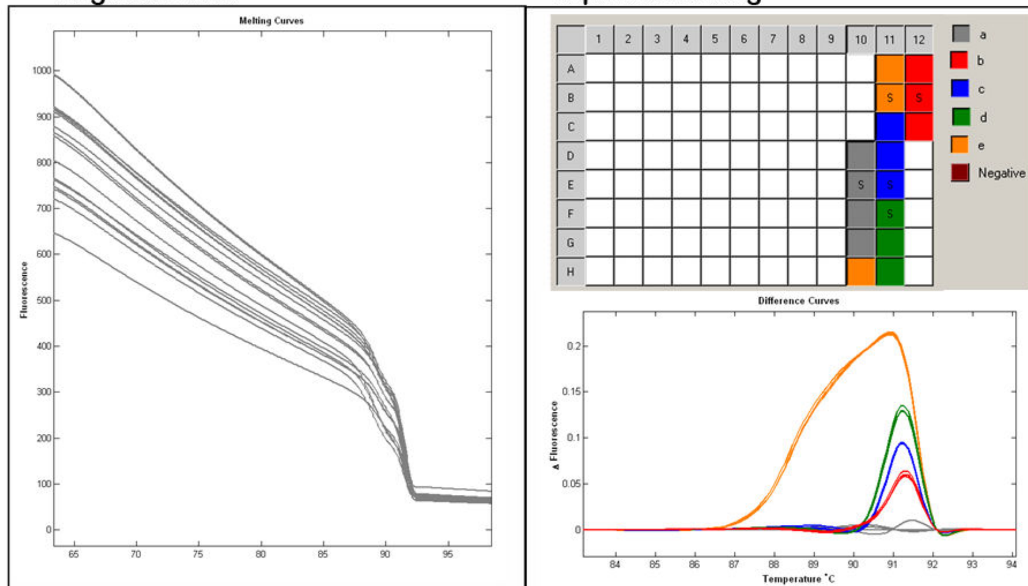
Supp. Figure S2C



Supp. Figure S2D



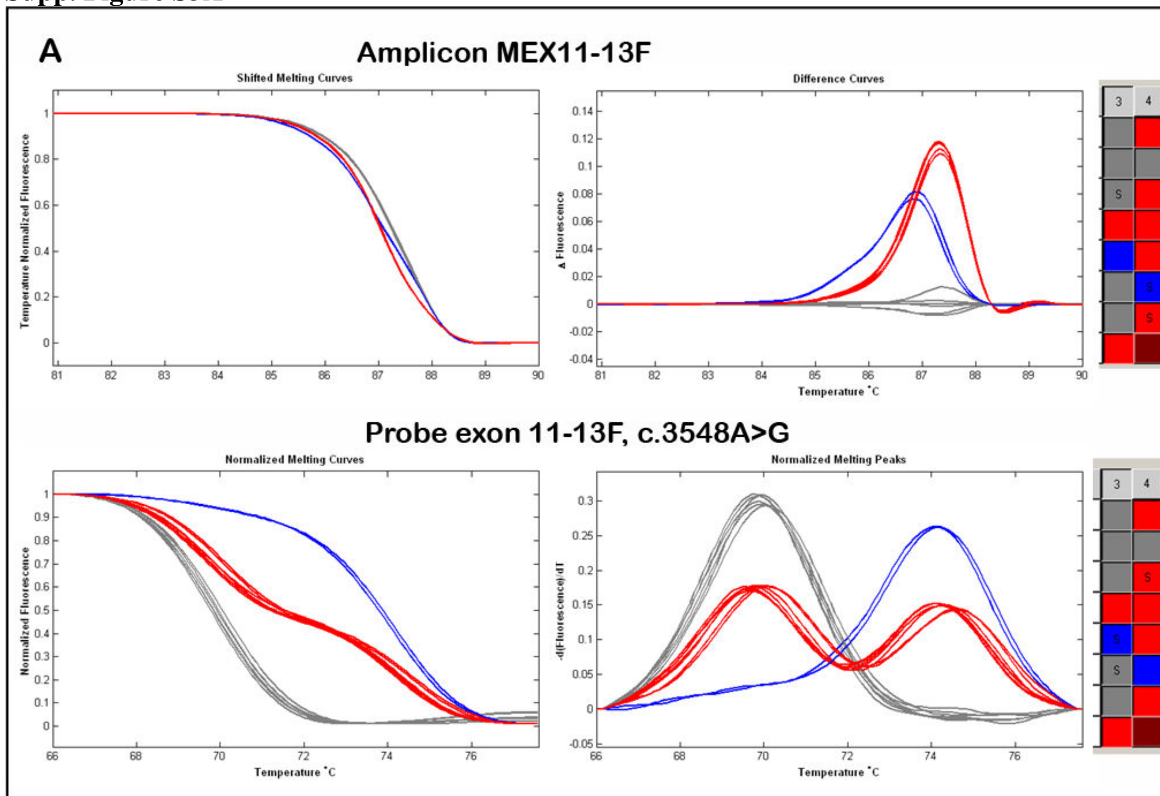
Supp. Figure S2E



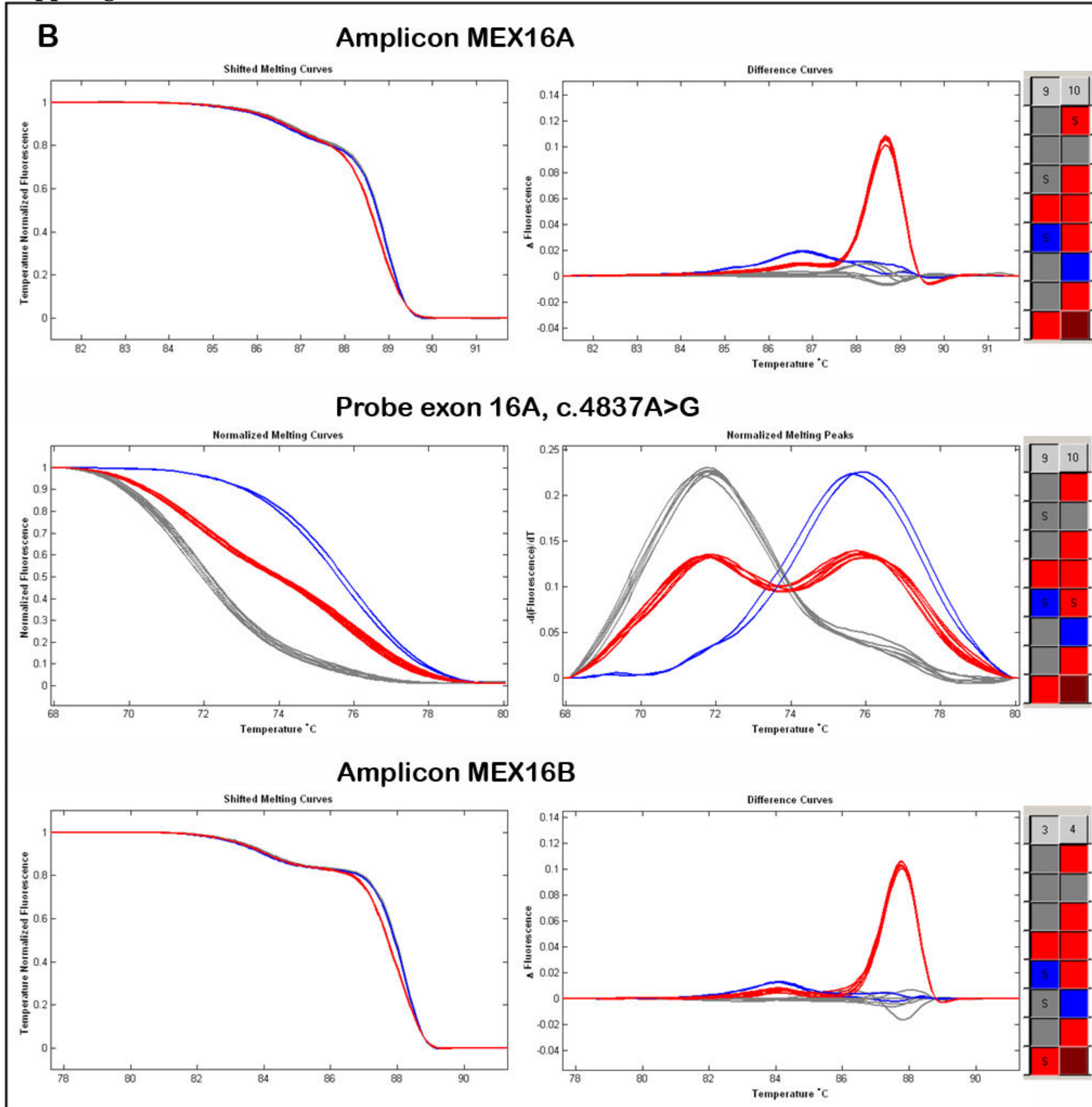
Supp. Figure S3A and B. Probe analysis of exon 11-13F, for POL c.3548A>G and exon 16 for POL c.4837A>G.

HRM was performed using 14 DNA samples and 1 wt control located at the upper left well in the first column. The dark well located at bottom of the right column contains blank control and was excluded from analysis (had no product). Shifted melting curves and Difference plots are shown for the 'Expert Scanning' analysis of A, amplicon MEX11-13F and B, amplicons MEX16A and MEX16B. Normalized melting curves and Normalized melting peaks are shown for the 'Unlabeled Probe' analysis of A, probe BR1EX11_3548-B and B, Probe BR1EX16A_4837-B. As shown all heterozygous (POL) and homozygous (POL2) containing samples were clearly detected by both 'expert scan' and the 'Unlabeled Probe' analysis and are depicted in red and blue respectively. WT samples are depicted in grey. Allocation of DNA samples is similar for all amplicons shown and the 'grouping' (clustering) of the melt curves is shown for both the 'Expert Scanning' and the 'Unlabeled Probe' analysis on the right of the plots.

Supp. Figure S3A



Supp. Figure S3B



3.2. Norambuena, et al. (2009) Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene. Clin Biochem. (IF: 2.076)

Tato práce demonstruje úspěšné využití HRM pro genotypizaci známých variant metodou malých amplikonů za použití platformy LightCycler 480 firmy Roche a poskytuje podrobné zhodnocení validačních parametrů vyžadované pro zavedení nových diagnostických metod dle normy ISO 15189. Na klinických DNA vzorcích známého genotypu, na příkladu vybraných variant v genu pro methylentetrahydrofolátreduktázu (*MTHFR*) c.677 C>T (rs1801133: C>T; p.A222V) a c.1298 A>C (rs1801131: A>C; p.E429A), byla v rámci validace testována senzitivita a specificita HRM, dále její reprodukovatelnost, opakovatelnost a robustnost.

Mnoho publikací popsalo úspěšnou detekcí heterozygotních variant pomocí HRM (Er and Chang 2012), avšak detekce homozygotních vzorků je komplikovanější, neboť profil křivky tání se od standardních „wildtype" vzorků neliší svým tvarem, pouze mírným posunem v teplotě tání, což může vést k falešně negativním výsledkům. Řešením je při mutačním skenování tvorba sekundárních heteroduplexů, kdy jsou testované vzorky smíchány s „wildtype" vzorkem v poměru 1:1 a znovu zanalyzovány. V případě genotypizace navrhli elegantní řešení Grievink a Stowell (2008) použitím malých amplikonů, tj. bez potřeby přidávat exogenní DNA do reakce. Zmenšením délky analyzovaného amplikonu se zvětší rozdíl mezi profily křivek tání, což zvýší senzitivitu metody a umožní bez problému detekovat i homozygotní varianty, což se podařilo i v našem případě. Limitací genotypizace metodou malých amplikonů je možnost ovlivnění analýzy interferencí jinou neočekávanou variantou, proto je nezbytné navrhnout co možná nejkratší amplikony.

Velmi detailně se práce věnuje posouzení robustnosti metody (vlivu koncentrace DNA, změny anelační teploty a počtu cyklů PCR či vlivu operátora), jež je důležitá pro možnou implementaci metody v ostatních laboratořích, které disponují odlišným zázemím a podmínkami.

Vyjma precizního zhodnocení analytických parametrů HRM a popsání metodologie validace práce shrnula výhody této metody, která se vyznačuje vysokou detekční spolehlivostí, jednoduchostí a finanční nenáročností. To ji predisponuje k širokému rozšíření a uplatnění v rutinním provozu mnoha diagnostických laboratoří.

Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Clinical Biochemistry 42 (2009) 1308–1316
CLINICAL BIOCHEMISTRY

Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the *MTHFR* gene

Patricia A. Norambuena, Joshua A. Copeland, Petra Křenková, Alexandra Štambergová, Milan Macek Jr.*

Department of Biology and Medical Genetics, Charles University 2nd Medical School and University Hospital Motol, V Úvalu 84, 150 06 Praha 5, Czech Republic

Received 15 December 2008; received in revised form 29 March 2009; accepted 24 April 2009
Available online 8 May 2009

Abstract

Objectives: According to OECD guidelines methods implemented in a diagnostic laboratory should be properly validated prior their implementation. For this purpose we selected genotyping by High Resolution Melting (HRM) of small amplicons using common variants in *MTHFR* as a model.

Design and methods: We selected previously typed samples on which selected analytical validation-related parameters relevant to DNA diagnostics — specificity, sensitivity, precision, robustness and ability to perform reliable calls were evaluated.

Results: Correct genotype was assigned in 375/381 (98.4%) for c.677 C>T (rs1801133: C>T; p.A222 V) and in 102/104 (98.1%) for c.1298 A>C (rs1801131: A>C; p.E429A) of all cases. Low analytical failure rate and very high specificity/sensitivity were achieved. Similarly, precision and robustness were consistent.

Conclusions: We have successfully validated HRM of small amplicons using common *MTHFR* variants as a model. We proved that this technique is highly reliable for routine diagnostics and our diagnostic validation strategy can serve as a model for other applications.
© 2009 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

Keywords: DNA diagnostics; Genotyping; High resolution melting (HRM); Diagnostic validation; Methylenetetrahydrofolate reductase gene (*MTHFR*); Organization for Economic Cooperation and Development (OECD) guidelines; Small amplicons

Introduction

Although direct DNA sequencing is considered as a "gold standard" for genotyping of known or unknown mutations, it still remains relatively expensive, laborious and time consuming. Different methods have been developed to simplify the detection of novel mutations, with the most common techniques being based on restriction enzyme analysis [1], allele/specific amplification [2], ligation based assays [3], single-base extension [4], fluorogenic ASO hybridization probes [5,6] and pyrosequencing [7].

High Resolution Melting (HRM) is a simple, rapid and low-cost mutation scanning method [8–11]. Its advantage is the fact that PCR amplification and melting curve analysis are performed within the same tube or plate, without any post-PCR processing. This feature is particularly important for a routine diagnostic setting. HRM is based on computer analysis of DNA melting transitions, whereby it is possible to record more than 25 readings per 1 °C [12], via monitoring of the change in fluorescence that results from gradual temperature-dependent release of a saturating ds-DNA binding dye [8,9].

Since HRM is based on thermodynamic differences between DNA fragments, it has been used in particular for scanning of heterozygous sequences. However, in its original form, discrimination between homozygous genotypes is more difficult, because the difference between homozygous sequence melting profiles is usually merely represented by a slight shift in the melting temperature (T_m), but not by a change of the melting curve profile [13]. Therefore, HRM has been adapted for analysis of polymorphic SNPs via PCR amplification of small amplicons. Such a reduction of the amplicon length results in a broader divergence between melting profiles and increases the sensitivity of the technique, which then could be used not only

* Corresponding author. Fax: +420 2 2443 3520.
E-mail addresses: patynorambuena@gmail.com (P.A. Norambuena), milan.macek.jr@lfmotol.cuni.cz (M. Macek Jr.).

0009-9120/$ - see front matter © 2009 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.clinbiochem.2009.04.015

for scanning, but also for accurate genotyping. Moreover, differences between homozygous wild type and homozygous mutant DNA fragments are thus more apparent [14].

Due to the advantageous features of the HRM of small amplicons this technique is currently being rapidly introduced into diagnostic laboratories for genotyping of disease-associated genes. According to the "OECD guidelines for Quality Assurance in Molecular Genetic Testing" [15], there is an obligation for diagnostic laboratories to provide high quality results. Therefore, all methods implemented within a routine setting must be duly validated prior to their diagnostic use.

In this study, we utilized suggested methodology and evaluated selected analytical validation-related parameters as stipulated by ISO15189 [16], QSOP 23 [17], the American College of Medical Genetics [18], the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [19], recommendations of the Czech Clinical Biochemistry and Medical Genetics societies [20] or as published elsewhere [21]. Parameters mostly relevant for the diagnostic setting comprise sensitivity, specificity, precision (reproducibility and repeatability) and robustness [18–21].

Although reviews and reports on the use of HRM for mutation scanning and genotyping were published previously [8,10,11,22–25], there is no report on diagnostic validation of this technique as required by OECD and/or ISO guidelines [15,16]. Furthermore, we feel that there is a particular need to provide examples of proper validation strategies for genotyping of SNPs by HRM of small amplicons due to its increasing use in diagnostics [11].

Therefore, the aim of this work was diagnostic validation of small amplicon genotyping by HRM using as model the examination of common variants of the *m*ethylene*t*etra*h*ydro *f*olate *r*eductase (*MTHFR*) gene: c.677 C>T (rs1801133: C>T; p.A222V) and c.1298 A>C (rs1801131: A>C; p. E429A). These variants are associated with a measurable reduction of enzymatic activity of *MTHFR*, in particular in the case of the c.677 C>T variant, and have been proposed to be in association with several disorders related to impaired folate metabolism such as neural tube defects, infertility, thrombosis or some types of cancer [26–30].

We hope that our study would serve as an example for diagnostic validations of other molecular genetic techniques applied in routine practice.

Materials and methods

DNA template preparation

Since the main objective of our study was to validate HRM genotyping we have randomly selected DNA aliquots from patients examined at our Department for inherited thrombophilia prior to assisted reproduction [31]. All patients or their legal representatives signed an informed consent approving general/anonymous research use of respective specimens and this study was approved by the Internal Ethics Committee of University Hospital Motol. Genomic DNA was extracted from blood using Puregene™ "Genomic DNA Purification Kit" (Gentra Systems, MN, U.S.A; currently distributed by Qiagen, Germany) and diluted to 10 ng/μL using the "DNA Hydratation Solution" provided by the manufacturer.

All samples used in this study were previously genotyped for respective *MTHFR* variants by an alternative technique and were selected retrospectively with the aim to have enough samples for each genotype. The rs1801133: C>T variant was genotyped utilizing the RHA Kit Thrombo™ (Labo Biomedical Products, The Netherlands) and/or by RFLP-based (Restriction Fragment Length Polymorphism) typing [32], while the rs1801131: A>C variant, only RFLP-based analysis was used [33].

Primer design and annealing temperature

Primers were designed to amplify a small fragment surrounding the polymorphisms and avoid the presence of other sequence variations in the primer region (Table 1). Primer T_m and general suitability were calculated using FastPCR software, version 4.0.27 [34]. In order to select the optimal annealing temperature (T_a) for our assay, we performed a gradient PCR within the range of 10 °C using the median temperature from the T_a range proposed by the FastPCR software as the starting point. Gradient PCR was performed using the PTC-220 thermocycler (MJ Research, MA, U.S.A.) under the PCR cycling and HRM conditions described in the next section.

After the gradient PCR reaction and for the purpose of the optimization of T_a, we initially performed HRM on the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Germany) followed by 4% agarose gel electrophoresis in order to detect spurious bands. The T_a with an optimal melting profile and associated with no unspecific amplification products, and which could be used for amplification of both SNPs, was selected.

PCR conditions and HRM acquisition

PCR amplification for rs1801133: C>T and rs1801131: A>C variants were performed under the same conditions in a 96-well plate in the LightCycler® 480 Real-Time PCR System. Reaction volume was 10 μL; 2 μL of genomic DNA (10 ng/μL) was added to 8 μL of reaction master mix consisting of 1× LightCycler® 480 High Resolution Melting Master (containing the proprietary ds-DNA saturating binding dye), with 2.5 mM

Table 1
Primer sequences and amplicon sizes used for rs1801133: C>T and rs1801131: A>C variants HRM small amplicon genotyping.

SNP	Primer sequences	Amplicon size (bp)
rs1801133: C>T	5′-GAAGGAGAAGGTGTCTGCG 5′-AGCTGCGTGATGATGAAATC	45
rs1801131: A>C	5′-GGGAGGAGCTGACCAGTGAA 5′-GTAAAGAACGAAGACTTCAAAGACACT	50

Table 2
Settings for the analysis of *MTHFR* variant genotyping.

SNP	Pre-melting normalization (°C)	Post-melting normalization (°C)	Temperature shift	Sensitivity
rs1801133: C>T	73.0–75.0	82.0–84.0	5	Auto-group: 0.7 In-run standards: 1.0
rs1801131: A>C	70.0–72.0	79.7–81.7	5	Auto-group: 0.7 In-run standards: 1.0

Legend: "Auto-group" calculation was used for the comparison of the control sample replicates for the three genotypes when reproducibility, repeatability and robustness parameters were evaluated. "In-run standards" calculation was used to analyze blinded samples using control samples for the three genotypes analyzed as melting standards.

$MgCl_2$ (Roche Diagnostics, Germany) and 0.5 μM of forward and reverse primers. For this study only one batch of the commercial master mix was used. The PCR program started with an initial denaturation of 10 min at 95 °C, continued with 40 cycles of 10 s at 95 °C, 15 s at 60 °C and 10 s at 72 °C. This program also allows one step for heteroduplex formation by heating to 95 °C for 30 s and cooling down to 40 °C for 1 min. For HRM, the plate was heated from 65 °C to 95 °C performing 25 acquisitions per 1 °C.

HRM analysis

Melting curve analysis was performed using the LightCycler® 480 Gene Scanning software version 1.2 (Roche Diagnostics, Germany). All the samples with a late amplification, as monitored by real-time PCR or associated with fluorescence of less than the 60% of the maximum, were excluded from the analysis. According to manufacturer's recommendations these could generate unreliable melting profiles. The

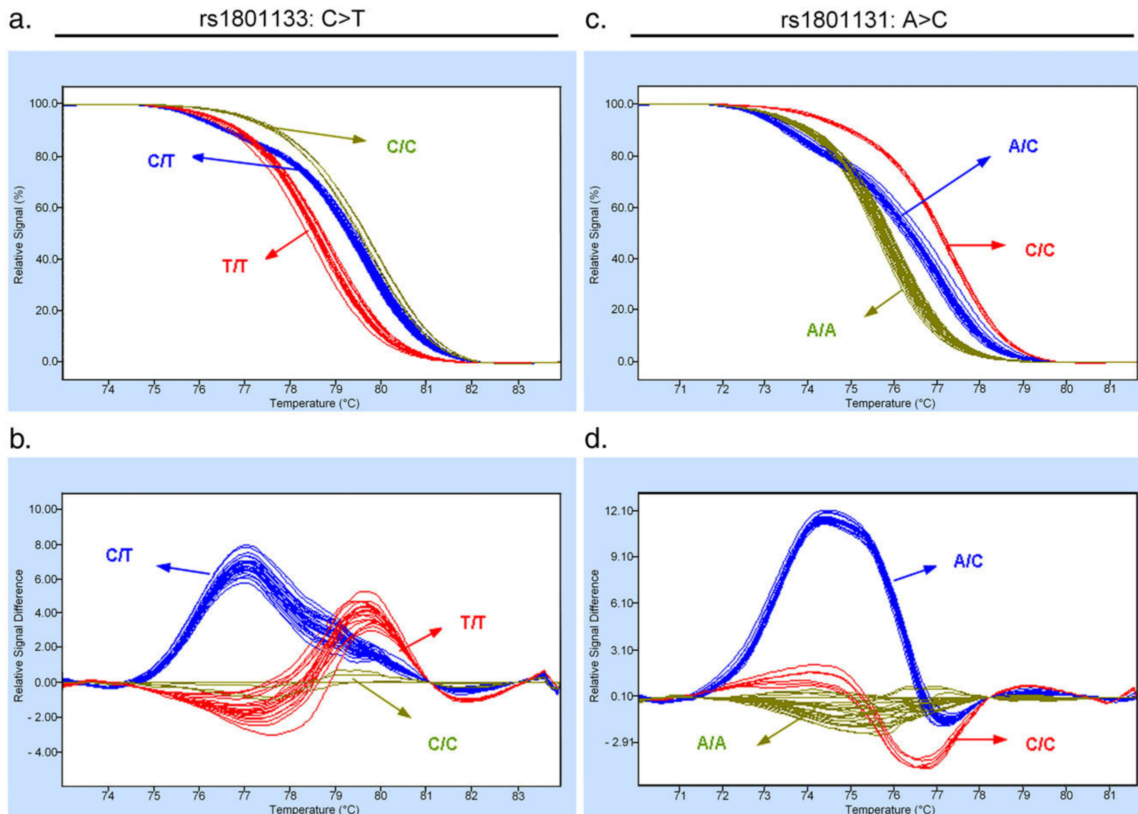


Fig. 1. Normalized plots, and normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C variants. In order to calculate sensitivity and specificity, samples were blinded. From the normalized melting curves for rs1801133: C>T and rs1801131: A>C (panels "a" and "c", respectively) it is possible to distinguish both homozygous groups by their T_m variation (C/C wild type homozygous and T/T homozygous for the variation for rs1801133: C>T and A/A wild type homozygous and C/C homozygous for the variation for rs1801131: A>C), heterozygous samples (C/T and A/C, respectively) have a different melting curve shape.

normalization settings were exactly the same for each experiment performed (Table 2).

Validation parameters

Sensitivity and specificity: sensitivity is the probability of a positive test result in the presence of a risk allele (heterozygous and homozygous for risk allele samples) expressed as the ratio between true positivity (TP) and the sum of true positivity and false negativity (FN): TP/(TP+FN), while specificity is the probability of a negative test result of the test in the absence of risk alleles (homozygous wild type samples), expressed as a ratio between true negativity (TN) and the sum of true negativity and false positivity (FP): TN/(TN+FP) [18–21]. To evaluate sensitivity and specificity we ran two replicates (i.e. same sample ran two times within the same run) of each genotype as melting control standards and one replicate of tested samples. Altogether we tested 381 samples for rs1801133: C>T and 104 samples for rs1801131: A>C, proportionate to their availability in our laboratory.

For the validation of HRM of small amplicons, these samples were organized into blinded groups. For specificity evaluation we examined 178 negative samples for rs1801133: C>T variant, while for rs1801131: A>C, 46 negative samples were analyzed. To determine sensitivity, we analyzed 203 positive samples for rs1801133: C>T variant and 58 positive samples for rs1801131: A>C.

Intra-run precision–repeatability: is the comparison of results within a single series in parallel within a single day performed by one analyst [20]. To test repeatability a single analyst ran 10 replicates of each genotype control sample within the same run.

Inter-run precision–reproducibility: is the comparison of results between the series — on different days (day to day reproducibility) [20]. For reproducibility (inter-run precision) parameter test, a single analyst prepared one sample from each genotype in triplicate, with that same analyst repeating the procedure on three different days.

Robustness: is the ability of a method to remain unaffected by minor modifications [18–21]. To evaluate robustness we ran one sample from each genotype in triplicate. The tested parameters were DNA template amount, annealing temperature, cycle number, analyst variation and pipetted volume variation. We added 10 ng, 20 ng, 50 ng and 100 ng of DNA into the reaction, respectively, for the evaluation of DNA template amount variation. Annealing temperature was modified within the range of ±1 °C. Cycle number variation was assessed adding or decreasing by two cycles in the PCR program. Three

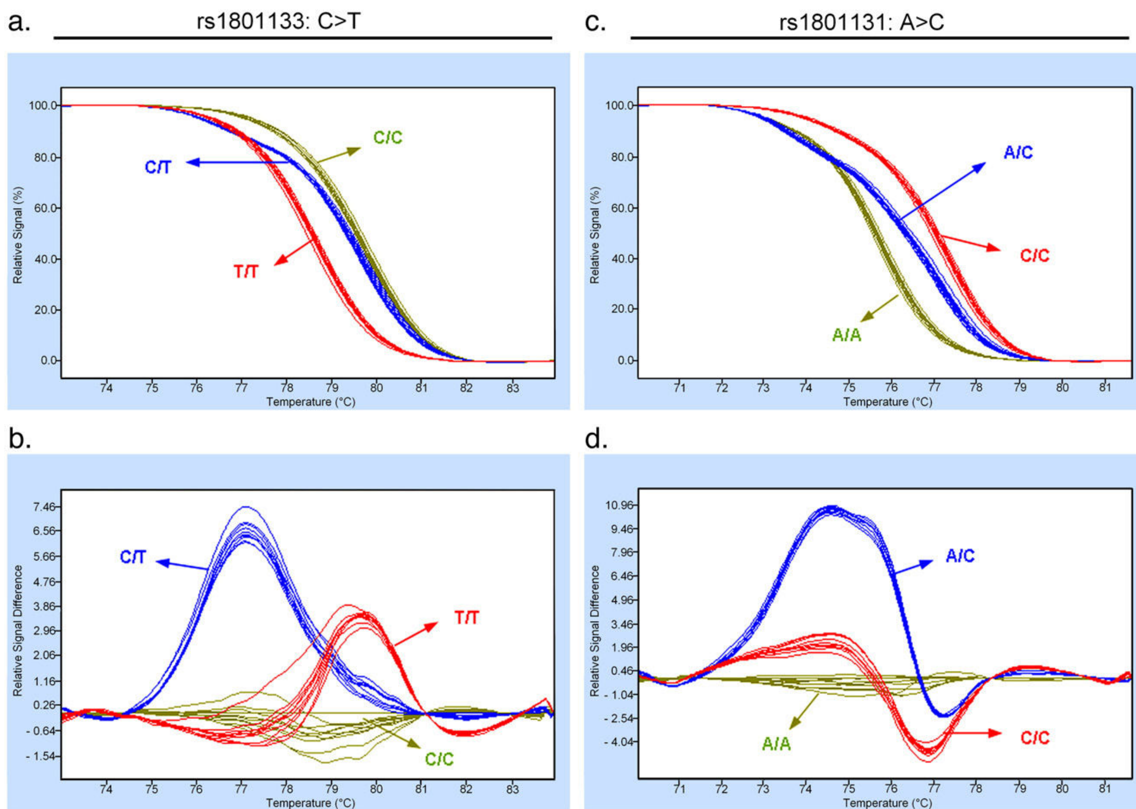


Fig. 2. Repeatability. Normalized plots, and normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C variants. The same control sample for a genotype was analyzed in 10 replicates. Panels “a” and “c” represent normalized melting curves, while panels “b” and “d” depict difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C, respectively.

different analysts repeated the same PCR and HRM procedure in order to assay analyst variation. For examining the influence of reaction master mix and DNA volume variation during pipetting, we modified respective volume in both instances by ±0.5 μL and 1.0 μL.

Results

HRM genotyping

The analytical failure rate was 6 out of 381 for rs1801133: C>T and in 102 out of 104 calls for rs1801131: A>C, while proper software-based genotype assignments ("calls") were obtained in the remaining cases. In aggregate, from the normalized melting curves and difference plots we were able to clearly distinguish three genotype melting profiles (Fig. 1). Individually, all calls corresponded to previous genotype assignments.

Sensitivity and specificity

Since we did not have any errors (no false calls) in genotyping assignments made by the software, we reached 100% sensitivity and specificity for both tested SNPs.

Intra-run precision–repeatability

All genotype replicates grouped together with regard to their melting pattern in both tested SNPs. Additionally, melting profiles were the same for each sample in all 10 replicates (Fig. 2).

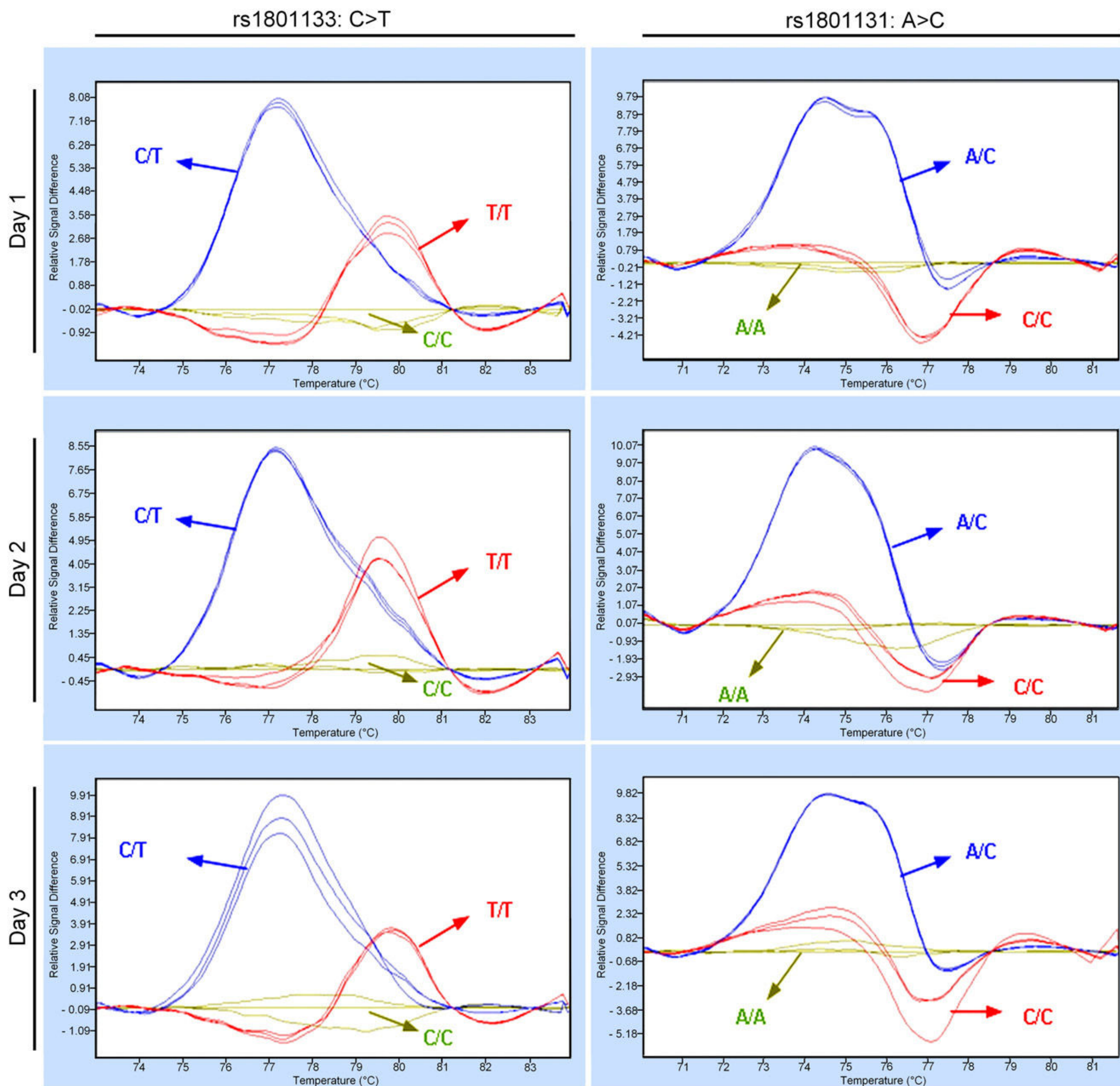


Fig. 3. Day-to-day reproducibility. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C performed on three different days by the same analyst are shown. Control samples for each genotype were run in triplicates. Each genotype group is indicated by arrows.

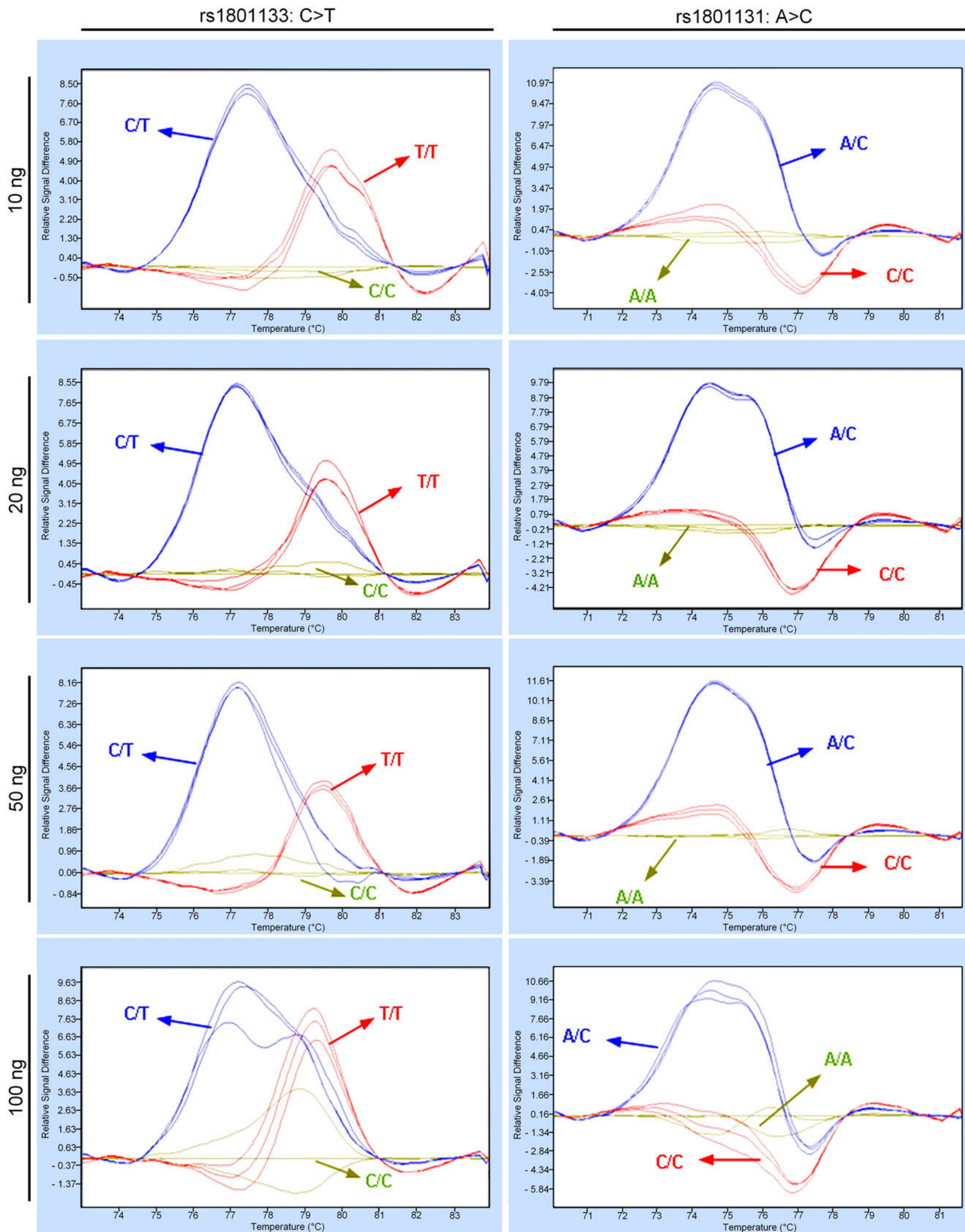


Fig. 4. DNA template amount variation. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C are shown. 10 ng, 20 ng, 50 ng and 100 ng of gDNA template per 10 μL of reaction were tested. Each genotype group is indicated by arrows.

Inter-run precision–reproducibility

There was no difference in the calls and difference plots when the same procedure was repeated on different days (Fig. 3).

Robustness

Template DNA amounts ranging from 10 ng to 50 ng provided correct grouping within the three different genotype groups (Fig. 4). Only when 100 ng of DNA was used in the reaction, melting profiles became unreliable. An annealing temperature variation of ±1 °C did not change the ability of the method to render correct grouping (data not shown). An increase in the cycle number, for both tested SNPs, did not modify the correct grouping nor did influence the melting profile. There was no detectable difference when we decreased the PCR program by two cycles for rs1801131: A>C, whereas a decrease in two cycles for rs1801133: C>T modified the melting profiles (Fig. 5).

Three different analysts repeated the same procedure in order to group same control samples (in three replicates) within the different genotype groups. With regard to the grouping ability, neither the software calls, nor the melting profiles varied (data not shown).

A difference in the melting profile was observed only when the same sample was analyzed by adding 7.0 µL of reaction master mix instead of 8.0 µL, and when template DNA volume added was 3.0 µL instead of 2.0 µL (data not shown).

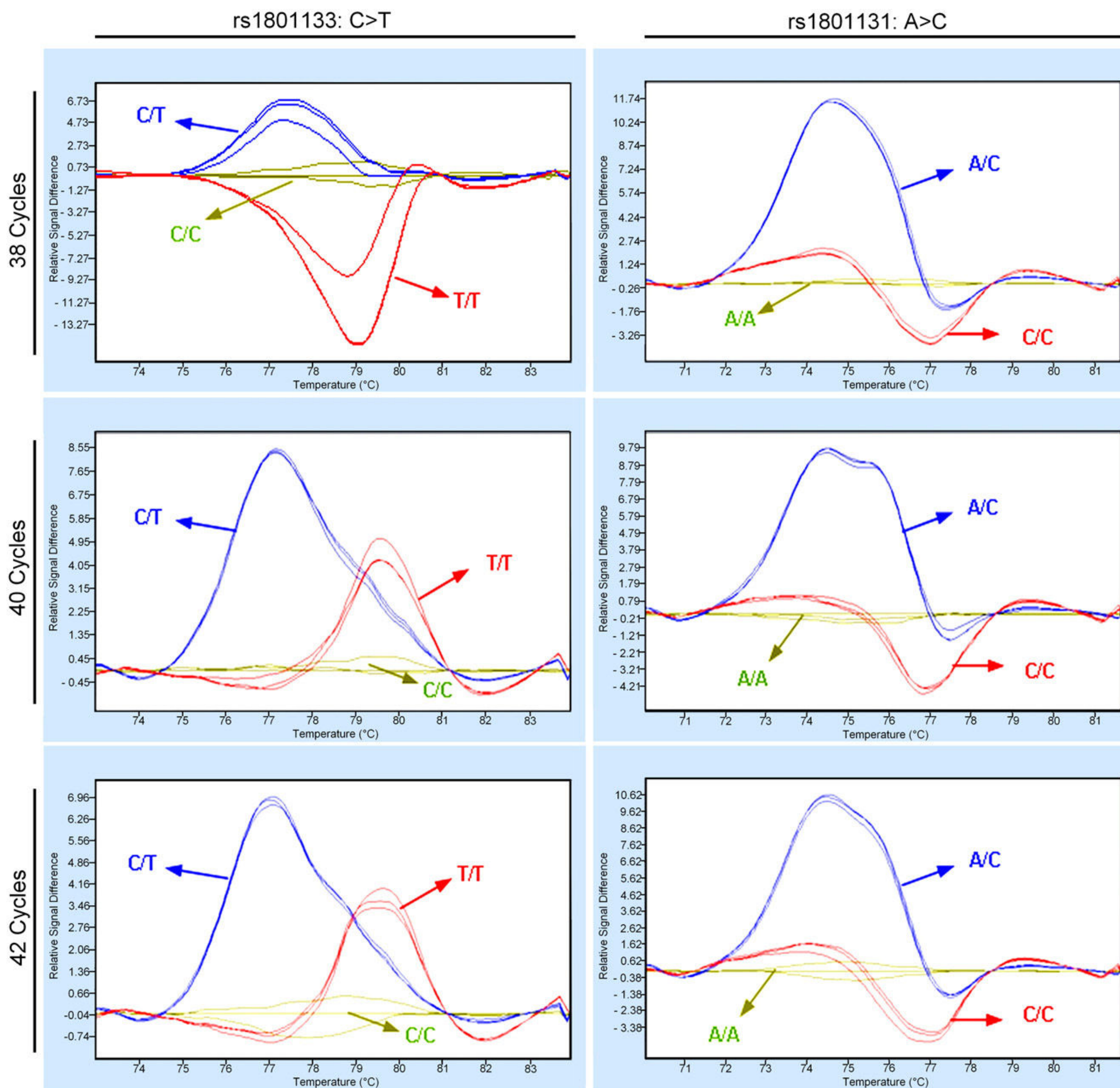


Fig. 5. Cycle number variation. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C with ±2 cycles variation from the normal PCR program (40 cycles) are shown. One sample from each genotype was run in triplicate; each genotype group is indicated by arrows.

Discussion

Genotyping by HRM of small amplicons is a technique associated with high sensitivity and specificity [14,35–37]. We were able to prove these observations by discriminating between homozygous wild type and homozygous mutant melting profiles on the model of two common *MTHFR* variants. Moreover, the advantage of this approach is that it is carried out in a closed tube environment. There is no need to add an extra oligonucleotide probe for genotyping as in the "original" form of HRM gene scanning [38,39] and even after a T_m shift we can still clearly distinguish both homozygous genotypes. We only used two flanking primers, a proprietary saturating DNA fluorescence dye and ready-to-use commercial master mix. Costs per analysis could be further decreased by preparing an "in house" master mix. These features make this technique very simple, customizable and fast, thus useful for a routine diagnostic setting [10,11,35].

Although previous studies analyzed all types of HRM from the point of view of sensitivity and specificity [8,10,11,22–25], none of them subjected HRM of small amplicons to diagnostic validation as required by OECD guidelines or ISO 151989 [15,16]. In this study, we utilized suggested methodology and evaluated selected analytical validation-related parameters which are particularly relevant in DNA diagnostics [18–21]. Assessment of sensitivity and specificity alone is not sufficient enough to cover all aspects of the diagnostic use of a given technique [40], since these do not account for possible variability of scenarios encountered at DNA diagnostic laboratories (i.e. uneven DNA template quality, imprecise DNA template concentration, change of personnel, different laboratory devices, variations in ambient temperature etc). In addition, due to the small master mix volume (10 μL) this method is particularly prone to pipetting inaccuracies. Therefore, it is necessary to include additional parameters, such as precision and robustness [18–21].

Monitoring of the progress of amplification by real-time PCR enabled us to exclude poor quality or insufficiently amplified template DNA samples. These quality measures contributed to high sensitivity and specificity [41] and our observations are in accordance with other studies [8,10,11,20–25].

Nevertheless, despite strictly applied quality measures the analytical failure rate was within the range of 1.6 to 1.9% for the tested variants. These samples cannot be assessed as "false negatives" since the assessor cannot infer any conclusions when the proprietary software discards unreliable data acquisitions due to its internal, quality-based algorithms, as is the case in LightCycler® 480 Real-Time PCR System. The only plausible technical explanation is that failure could likely result from inaccurate pipette handling of very small reaction master mix volumes, demonstrating that there is still a space for further improvements. When we repeated the analysis for the second time correct genotyping was achieved.

Since we were using the same reagents and DNA dilution conditions (i.e. same reaction chemistry), the precision (repeatability and reproducibility) of HRM was very high and we are able to use the same normalization settings between runs made on different days. This observation supports the high value of this technique for reliable genotyping in routine diagnostics.

Minor modifications of the technique do not generally affect its performance. Nevertheless, a shortening of the PCR by 2 cycles, as was the case in rs1801133: C>T, might produce different melting profiles in instances when PCR has not reached its plateau. This can be avoided by monitoring amplification in real-time and stopping the PCR after its plateau has been reached, as is possible when using the LightCycler 480 system. This step can be done manually, but we recommend to evaluate this issue at the optimization stage to set up the correct number of PCR cycles prior to future analyses. The use of larger template DNA amounts impedes HRM due to altering ds-DNA/amplicon/proprietary saturating dye ratio, thereby leading to less precise melting curves [42].

In conclusion, we have successfully performed diagnostic validation of High Resolution Melting of small amplicons for the genotyping of rs1801133: C>T and rs1801131: A>C *MTHFR* variants according to OECD and ISO guidelines [15,16] by using parameters and approaches pertinent to a diagnostic setting. In addition to accurate genotyping HRM of small amplicons, altered melting profiles could signal another mutation within the analyzed sequence. As is the case with all mutation scanning-based techniques direct sequencing will elucidate the reason for altered melting profiles. Shortening of amplicons in HRM further decreases the false negativity rate due to poor discrimination of homozygous sequences. This feature substantially decreases the necessity to implement sequencing in samples where the estimated clinical risk is highly discordant with a "negative" test result.

Finally, we believe that our approach could be of general use for diagnostic validations of other methods.

Acknowledgments

We thank I. Anguelová, M. Fialová, A. Nestorovic and E. Raušová for sample preparation and technical assistance. We acknowledge helpful consultations with Dr. N. van der Stoep (Leiden University Medical Centre) and Dr. M. Morris (Geneva University Hospital). Supported by VZFNM 00064203(6112) and IGA NR9448-3 from the Czech Ministry of Health and EuroGentest (FP6-512148).

References

[1] Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 1980;32(3):314–31.
[2] Newton CR, Graham A, Heptinstall LE, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 1989;17(7):2503–16.
[3] Baron H, Fung S, Aydin A, et al. Oligonucleotide ligation assay for detection of apolipoprotein E polymorphisms. Clin Chem 1997;43(10):1984–6.
[4] Li J, Butler JM, Tan Y, et al. Single nucleotide polymorphism determination using primer extension and time-of-flight mass spectrometry. Electrophoresis 1999;20(6):1258–65.
[5] Schaeffeler E, Schwab M, Eichelbaum M, Zanger UM. CYP2D6 genotyping strategy based on gene copy number determination by TaqMan real-time PCR. Hum Mutat 2003;22(6):476–85.

[6] Murugesan G, Aboudola S, Szpurka H, et al. Identification of the JAK2 V617F mutation in chronic myeloproliferative disorders using FRET probes and melting curve analysis. Am J Clin Pathol 2006;125(4):625–33.
[7] Ahmadian A, Gharizadeh B, Gustafsson AC, et al. Single-nucleotide polymorphism analysis by pyrosequencing. Anal Biochem 2000;280(1): 103–10.
[8] Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clin Chem 2004;50:1748–54.
[9] Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 2003;49:853–60.
[10] Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics 2007;8(6): 597–608.
[11] Erali M, Voelkerding KV, Wittwer CT. High resolution melting applications for clinical laboratory medicine. Exp Mol Pathol 2008;85(1):50–8.
[12] Roche Diagnostics GmbH and Roche Applied Science. LightCycler® 480 Operator's Manual. Gene Scanning Software. Germany, 2007.
[13] Palais RA, Liew MA, Wittwer CT. Quantitative heteroduplex analysis for single nucleotide polymorphism genotyping. Anal Biochem 2005;346(1): 167–75.
[14] Liew M, Pryor R, Palais R, *et al*. Genotyping of Single-Nucleotide Polymorphisms by High-Resolution Melting of Small Amplicons. Clin Chem 2004; 50: 1156–1164.
[15] www.oecd.org/sti/biotechnology.
[16] www.iso.org.
[17] www.evaluations-standards.org.uk.
[18] American College of Medical Genetics. Standards and Guidelines for Clinical Genetics Laboratories. Edition 2006. (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/c.htm).
[19] ICH Harmonized Tripartite Guideline. Validation of analytical procedures: text and methodology. Q2 (R1). 1994. (www.ich.org).
[20] Brdička R, Vraná M, Otáhalová E, Štambergová A, Čamajová J. Analytical Validation of Molecular Genetic Methods. (http://www.uhkt.cz/files/nrl-dna/blp/Analyticka_validace_en.pdf).
[21] Prence EM. A practical guide for the validation of genetic tests. Genet Test 1999;3:201–5.
[22] Nomoto K, Tsuta K, Takano T, et al. Detection of EGFR mutations in archived cytologic specimens of non-small cell lung cancer using high-resolution melting analysis. Am J Clin Pathol 2006;126(4):608–15.
[23] Hung CC, Lee CN, Chang CH, et al. Genotyping of the G1138A mutation of the FGFR3 gene in patients with achondroplasia using high-resolution melting analysis. Clin Biochem 2008;41(3):162–6.
[24] Bastien R, Lewis TB, Hawkes JE, et al. High-throughput amplicon scanning of the *TP53* gene in breast cancer using high-resolution fluorescent melting curve analyses and automatic mutation calling. Hum Mutat 2008;29(5):757–64.
[25] Audrezet MP, Dabricot A, Le Marechal C, Ferec C. Validation of high-resolution DNA melting analysis for mutation scanning of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. J Mol Diagn 2008;10(5):424–34.
[26] Schwahn B, Rozen R. Polymorphisms in the methylenetetrahydrofolate reductase gene: clinical consequences. Am J Pharmacogenomics 2001;1 (3):189–201.
[27] Blom HJ, Shaw GM, den Heijer M, Finnell RH. Neural tube defects and folate: case far from closed. Nat Rev Neurosci 2006;7(9):724–31.
[28] den Heijer M. Hyperhomocysteinaemia as a risk factor for venous thrombosis: an update of the current evidence. Clin Chem Lab Med 2003; 41(11):1404–7.
[29] Kono S, Chen K. Genetic polymorphisms of methylenetetrahydrofolate reductase and colorectal cancer and adenoma. Cancer Sci 2005;96(9): 535–42.
[30] Bolufer P, Barragan E, Collado M, Cervera J, López JA, Sanz MA. Influence of genetic polymorphisms on the risk of developing leukemia and on disease progression. Leuk Res 2006;30(12):1471–91.
[31] James AH. Thromboembolism in pregnancy: recurrence risks, prevention and management. Curr Opin Obstet Gynecol 2008;20(6):550–6.
[32] Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10(1):111–3.
[33] Hanson NQ, Aras O, Yang F, Tsai MY. C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase gene: incidence and effect of combined genotypes on plasma fasting and post-methionine load homocysteine in vascular disease. Clin Chem 2001;47(4):661–6.
[34] Kalendar R. FastPCR: a PCR primer and probe design and repeat sequence searching software with additional tools for the manipulation and analysis of DNA and protein. (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm) 2007.
[35] Grievink H, Stowell KM. Identification of ryanodine receptor 1 single-nucleotide polymorphisms by high-resolution melting using the LightCycler 480 System. Anal Biochem 2008;374(2):396–404.
[36] Gundry CN, Dobrowolski SF, Martin YR, et al. Base-pair neutral homozygotes can be discriminated by calibrated high-resolution melting of small amplicons. Nucleic Acids Res 2008;36(10):3401–8.
[37] Kristensen LS, Dobrovic A. Direct genotyping of single nucleotide polymorphisms in methyl metabolism genes using probe-free high-resolution melting analysis. Cancer Epidemiol Biomarkers Prev 2008;17(5):1240–7.
[38] Zhou L, Wang L, Palais R, Pryor R, Wittwer CT. High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. Clin Chem 2005;51(10):1770–7.
[39] Montgomery J, Wittwer CT, Palais R, Zhou L. Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. Nat Protoc 2007;2(1):59–66.
[40] Camajova J, Berwouts S, Matthijs G, Macek Jr M, Dequeker E. Variability in the use of CE-marked assays for in vitro diagnostics of CFTR gene mutations in European genetic testing laboratories. Eur J Hum Genet 2009;17(4):537–40.
[41] National Genetics Reference Laboratory (Wessex). Mutation scanning by high resolution melt analysis. Evaluation of Rotor-Gene™ 6000 (Corbett Life Science), HR-1™ and 384 well LightScanner™ (Idaho Technology). Report NGRLW_HRM_1.0. Salisbury, UK, 2006.
[42] Fortini D, Ciammaruconi A, De Santis R, et al. Optimization of high-resolution melting analysis for low-cost and rapid screening of allelic variants of *Bacillus anthracis* by multiple-locus variable-number tandem repeat analysis. Clin Chem 2007;53(7):1377–80.

3.3. Křenková, et al. (2009) Evaluation of high-resolution melting (HRM) for mutation scanning of selected exons of the CFTR gene. Folia Biol (Praha). (IF: 1.151)

V současné době je v genu cystické fibrózy známo více jak 1900 variant, které jsou rovnoměrně rozmístěny po celé kódující sekvenci, což při 27 exonech tohoto genu ztěžuje a prodražuje detekci vzácných mutací. V minulosti se na našem pracovišti využívaly pre-sekvenační metody, např. DGGE, jejíž nevýhodou byla především pracnost a manipulace s nebezpečnými chemikáliemi (formamid). Později byla nahrazena metodou TTGE, která byla rovněž technicky i časově náročná na přípravu a průběh, a tak se z tohoto důvodu v běžné laboratorní diagnostice téměř nevyužívala. Velký problém představovala i optimalizace podmínek elektroforézy, které byly pro jednotlivé exony genu *CFTR* rozdílné. Ani následné zavedení metody dHPLC, která umožňovala analyzovat delší fragmenty, nepřineslo výrazné zefektivnění detekce neznámých mutací v genu *CFTR*. Průlom mezi skenovacími metodami znamenal až objev nové generace plně saturačních fluorescenčních barviv, které umožnily analýzu křivek tání s vysokým rozlišením – HRM. Ta byla představena jako jednoduchá, levná a vysoce senzitivní metoda pro mutační skenování a genotypizaci (Gundry et al. 2003). Naším cílem tedy bylo ověřit spolehlivost této metody v podmínkách naší laboratoře a posoudit její využití v rutinní DNA diagnostice pro účely detekce neznámých mutací.

Pro posouzení senzitivity, specificity a opakovatelnosti metody HRM bylo za použití přístroje RotorGene6000 (Corbett Life Science) a barviva LCGreen plus (Idaho Technology) testováno devatenáct různých genotypů v šesti vybraných exonech genu *CFTR* (4, 7, 10, 11, 14b a 22). Mutace zahrnovaly všechny SNP třídy, jedno- a třínukleotidové delece a představovaly více jak 76,5% všech CF alel detekovaných u české populace. Všechny testované heterozygotní varianty byly jednoznačně odlišeny od standardních kontrol, některé mutace však vytvářely totožný profil křivky tání (G551D a R553X nebo L1335F a L1335P) způsobený proximitou těchto variant. To znamená, že ačkoliv časté polymorfismy jsou rozpoznány svým charakteristickým profilem tání, bez simultánního použití genotypizačních prób v reakci je nutné každý pozitivní nález sekvenovat. Vzorek s homozygotním genotypem pro varianty F508del/M470V vykazoval

stejný profil tání jako standardní kontrola, pouze s nepatrným posunem teploty tání. Přítomnost homozygotních variant byla potvrzena až po sekundárním vytvoření heteroduplexů, kdy byl PCR produktu smícháním s „wildtype“ PCR produktem v poměru 1:1, denaturován a opětovně zanalyzován. Poté byl profil křivky tání původně homozygotního vzorku stejný jako profil vzorku s heterozygotním genotypem F508del/M470V.

Celkem bylo pro exon 4 skenováno devět vzorků (3 heterozygotní, 6 standardních kontrol), pro exon 7 osm vzorků (4 heterozygotní, 4 standardní kontroly), pro exon 10 devět vzorků (4 heterozygotní, 1 homozygotní a 4 standardní kontroly), pro exon 11 dvanáct vzorků (7 heterozygotních, 5 standardních kontrol), pro exon 14b sedm vzorků (2 heterozygotní, 5 standardních kontrol) a pro exon 22 sedm vzorků (3 heterozygotní, 4 standardní kontroly). Ke stanovení opakovatelnosti HRM byl každý vzorek analyzován pětkrát. Celkově bylo tedy analyzováno 120 standardních křivek tání a 140 křivek tání s mutací. Bylo dosaženo 100% senzitivity a 96% specificity.

Tato studie potvrdila optimální využitelnost HRM v detekci mutací v genu *CFTR*, zároveň zhodnotila využití ne příliš rozšířené platformy RotorGene 6000, která je založena na odlišném systému, kdy místo 96-ti jamkových platíček využívá rotor se zkumavkami, který poskytuje větší teplotní a optickou uniformitu.

Konečně zkušenosti získané v rámci těchto validačních projektů byly rovněž využity i u následných populačně specifických analýz na mém domovském pracovišti (viz. dále).

Short Communication

Evaluation of High-Resolution Melting (HRM) for Mutation Scanning of Selected Exons of the *CFTR* Gene

(*CFTR* / high-resolution melting / HRM / mutation detection)

P. KŘENKOVÁ, P. NORAMBUENA, A. ŠTAMBERGOVÁ, M. MACEK JR.

Charles University in Prague, 2nd Faculty of Medicine and University Hospital Motol, Department of Biology and Medical Genetics, Cystic Fibrosis Centre, Prague, Czech Republic

Abstract. Hereby we present evaluation of high-resolution melting for mutation scanning applied to the cystic fibrosis transmembrane conductance regulator gene. High resolution melting was used for mutation scanning of selected samples derived from cystic fibrosis patients with a known cystic fibrosis transmembrane conductance regulator genotype. We tested 19 different disease-causing cystic fibrosis transmembrane conductance regulator mutant genotypes located within six exons of the cystic fibrosis transmembrane conductance regulator gene (4, 7, 10, 11, 14b and 22). Normalized melting curves of tested samples were compared to sequenced-verified wild-type samples. Determined mutations are as follows: p.F508del, p.I507del, p.G551D, p.R347P, c.1717-1G>A, c.621+1G>T, p.Y122X, p.I336K, p.R553X, c.2789+5G>A, c.574delA, c.1811+1G>C, p.L1335F, p.L1335P, p.L1324P and p.M470V and represent minimally 76.5 % of all cystic fibrosis alleles detected in the Czech cystic fibrosis population. All analysed samples with mutant genotypes were unambiguously distinguished from wild-type samples. High-resolution melting analysis enabled reliable detection of all single-nucleotide polymorphism classes and 1- or 3-base pair deletions. We examined the specificity, sensitivity and precision of this methodology. High-resolution melting analysis is an economical, sensitive and specific close-tube method and has a high utility for the detection of unknown mutations in cystic fibrosis DNA diagnostics.

Received October 7, 2008. Accepted May 6, 2009.

This study was supported by VZFNM00064203 EuroGentest (FP6-512145) and NR-9448-3/2007.

Corresponding author: Petra Křenková, Charles University in Prague, 2nd Faculty of Medicine and University Hospital Motol, Department of Biology and Medical Genetics, Cystic Fibrosis Centre, V Úvalu 84, 150 06 Prague 5, Czech Republic. Phone: (+420) 224 433 548; Fax: (+420) 224 433 520; e-mail: petra.krenkova@email.cz

Abbreviations: CF – cystic fibrosis, CFTR – cystic fibrosis transmembrane conductance regulator, DGGE – denaturing gradient gel electrophoresis, dHPLC – denaturing high-performance liquid chromatography, HA – heteroduplex analysis, HRM – high-resolution melting, PCR – polymerase chain reaction, SSCP – single-strand conformation polymorphism analysis, SNP – single nucleotide polymorphism, TGCE – temperature gradient capillary electrophoresis, T_m – melting temperature.

Introduction

To date, more than 1,600 mutations have been identified in the *c*ystic fibrosis transmembrane conductance regulator (*CFTR)* gene (http://www.genet.sickkids.on.ca/cftr). The *CFTR* gene consists of 27 exons (Riordan et al., 1989), thus making the detection of non-common mutations by sequencing laborious, expensive and time-consuming. To simplify the analysis of such a broad mutation spectrum, a rapid and reliable method is required. There are many available scanning methods, such as single-strand conformation polymorphism analysis (SSCP) (Orita et al., 1989), denaturing gradient gel electrophoresis (DGGE) (Lerman and Silverstein, 1987), temperature gradient capillary electrophoresis (TGCE) (Li et al., 2002), denaturing high-performance liquid chromatography (dHPLC) (Xiao and Oefner, 2001) or heteroduplex analysis (HA) (Highsmith et al., 1999) which are time-consuming and the sensitivity often depends on the experience of the operator. On the other hand, HRM presents a rapid, high-throughput, closed-tube method for mutation scanning and genotyping (Wittwer et al., 2003). The sample preparation consists of a standard PCR reaction with a dsDNA intercalation fluorescent dye and does not require any post-PCR handling. Products can be analysed directly after PCR amplification using specially designed instruments for high-resolution melting (HRM) analysis. The homozygous, heterozygous and wild-type samples are differentiated according to their melting profile, which is represented by plotting fluorescence over the temperature range. The heterozygous genotype is distinguished from a wild-type sample by different melting temperatures (T_m) and the shape of the melting curve, whereas homozygous genotypes are distinguished only by a change in T_m.

Folia Biologica (Praha) 55, 238-242 (2009)

Many publications have documented the successful use of HRM for mutation scanning and genotyping (Wittwer et al., 2003; Liew et al., 2004; Reed and Wittwer, 2004; Zhou et al., 2004, 2005; Chou et al., 2005; Graham et al., 2005; Krypuy et al., 2006, 2007; Montgomery et al., 2007; Audrezet et al., 2008, Nguyem-Dumont et al., 2009). HRM is a mutation detection and scanning technique that has high reliability. It has been reported to have near 100% sensitivity and specificity when the analysed PCR products were up to 400 bp in length (White and Potts, 2006).

To evaluate the reliability of HRM in our laboratory, we tested the specificity, sensitivity and repeatability in detecting 16 representative mutations (19 genotypes) within six exons of the *CFTR* gene. In comparison to previously published studies we focused on the utilization of Rotor-Gene™ 6000. This instrument has a specially tuned high-intensity optical channel and extreme thermal resolution (± 0.02 °C). Due to the unique rotary design there is the highest thermal uniformity between samples (± 0.01 °C), hence there is no need for temperature shifting. Compared to most block-based systems the light in our case is highly focused, while in the block-based systems it is most intense in its centre and becomes dispersed on the edges. This leads to optical variability and non-uniformity within the analysed plate. We also used the IdahoTechnology dye, which provides highest sensitivity compared to all subsequent proprietary dyes from other companies. All these properties make this technical platform unique and highly suitable for the diagnostic setting.

Material and Methods

DNA samples

We selected clinical samples derived from CF patients of our Institute with known genotypes previously determined by sequencing analysis. Genomic DNA was extracted from leukocytes of peripheral blood using a commercial PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions or by the "salting out" procedure. All DNA samples were diluted in the same buffer PURGENE DNA Hydration Solution (Gentra Systems) at the concentration 15 ng/µl.

Polymerase chain reaction conditions

Human genomic DNA was amplified by polymerase chain reaction (PCR) using previously published primers (Macek et al., 1997) in the presence of the intercalating fluorescent dye LCGreen plus (Idaho Technology, Salt Lake City, UT). Amplification efficiency was monitored using real-time PCR. PCR reactions were performed in 10 µl reaction volume which consisted of 1× PCR buffer including 2 mM $MgCl_2$ (final concentration), 0.3 µM of each primer, 1 mM of dNTPs, 1× LCGreen plus, 1 U of FastStart Taq DNA Polymerase (Roche Diagnostics, Indianapolis, IN) and 30 ng of genomic DNA. To determine the precision, all PCR reactions were performed five times.

PCR cycling and HRM analysis were performed in Rotor-Gene™ 6000 (Corbett Life Science, Qiagen, San Francisco, CA). The amplicons were run according to the following conditions: one cycle of initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 20 s, annealing for 30 s, 72 °C for 40 s and one cycle of final extension at 72 °C for 2 min. The annealing temperature was 62 °C for amplicons 10, 11, 14b and 22, 67 °C for amplicon 4 and 58 °C for amplicon 7. Following amplification, PCR products were denatured at 95 °C for 1 min and rapidly cooled to 25 °C for 1 min to form heteroduplexes.

Melting conditions

Melting analysis was performed immediately after PCR in the same instrument. The fluorescence signal was acquired from 65 °C to 95 °C at a ramp rate 0.1 °C/s. Melting data were visualized and analysed using Rotor-Gene™ 6000 Series Software 1.7 (Corbett Life Science). Melting curves of examined samples were normalized and the difference temperature graphs were compared against wild-type control samples.

Results and Discussion

We scanned 19 different mutant genotypes (Table 1) located within six exons of the *CFTR* gene (4, 7, 10, 11, 14b and 22). Analysed mutations included all SNP classes (which divided SNPs into four groups according to the intensity of T_m change) and 1- or 3 base pair deletions and represent at least 76.5 % of all CF alleles detected in the Czech Republic.

Evaluated amplicons varied in size from 101 bp to 380 bp and had a GC content ranging from 33.7 % to 45.9 %. The tested samples were evaluated by initial visual inspection of melting curves and by software analysis. All examined samples with mutant heterozygous genotype were unambiguously distinguished from wild-type samples by a different shape of the melting curves (Figs 1A, 1B, 1C, 1D, 1E, 1F, 1G and 1H). Heterozygous profiles of p.G551D versus p.R553X and p.L1335F versus p.L1335P were not distinguishable (Figs. 1A and 1B).

A sample with homozygous genotype both for p.F508del and p.M470V mutations showed a similar melting pattern as the wild-type control samples and the T_m shift not easily distinguished (Fig. 1C). The presence of the homozygous mutations was confirmed by mixing (1 : 1) the PCR product with the wild-type PCR product, denaturing and reanalysing the melting. In that way, heterozygosity of both mutations was established and the detection of the homozygous mutations by melting analysis was obvious – the melting pattern was similar as the original sample with heterozygous genotype p.M470V/p.F508del (Fig. 1D).

We scanned nine samples for exon 4 (three heterozygous, six wild-type controls), eight samples for exon 7 (four heterozygous, four wild-type controls), nine samples for exon 10 (four heterozygous, one homozygous

Table 1. CFTR mutations analysed in the study

Exon	Genotype	Nucleotide Change	SNP class*
4	c.621+1G>T	G>T	2
	p.Y122X	T>A	4
	c.574delA	del	--
7	p.R347P	G>C	3
	p.I336K	T>A	4
10	p.F508del/p.M470V/p.M470V	del/A>G/A>G	1
	p.F508del/p.F508del/p.M470V/p.M470V	del/del/A>G/A>G	1
	p.F508del/p.M470V	del/A>G	1
	p.M470V	A>G	1
	p.I507del	del	--
11	c.1717-1G>A	G>A	1
	c.1811+1G>C	G>C	3
	c.1717-1G>A / c.1811+1G>C	G>A / G>C	1/3
	p.G551D	G>A	1
	p.R553X	C>T	1
14b	c.2789+5G>A	G>A	1
22	p.L1335F	C>T	1
	p.L1335P	T>C	1
	p.L1324P	T>C	1

* (Venter et al., 2001)
SNP class 1 represents C/T and G/A base changes with typical T_m melting curve shift larger than 0.5 °C. SNP class 2 represents C/A and G/T base changes, class 3 C/G base change with T_m melting curve shift between 0.5–0.2 °C. SNP class 4 comprises A/T base change, which caused T_m melting curve shift smaller than 0.2 °C.

and four wild-type controls), twelve samples for exon 11 (seven heterozygous, five wild-type controls), seven samples for exon 14b (two heterozygous, five wild-type controls) and seven samples for exon 22 (three heterozygous, four wild-type controls). To determine the precision, each sample was prepared five times. In total, we analysed 120 wild-type melting curves and 140 mutant melting curves with 100% sensitivity and 96% specificity.

Currently, there is discussion whether mutation scanning techniques still have a role in DNA diagnostics, since many argue that lowering costs for sequencing render these unnecessary. However, even in the case of sequencing, false positivity/negativity could occur if one does not sequence both strands. Usually, in a routine diagnostic setting, laboratories only sequence one strand – an approach associated with higher risk of error. We have confirmed this contentious issue (currently in press) within our participation in the Eurogentest (www.eurogentest.org) consortium. Moreover, next-generation sequencing techniques still do not have the desirable sensitivity and specificity, since enrichment strategies are not yet optimized for DNA diagnostics (Hert et al., 2008; Voelkerding et al., 2009).

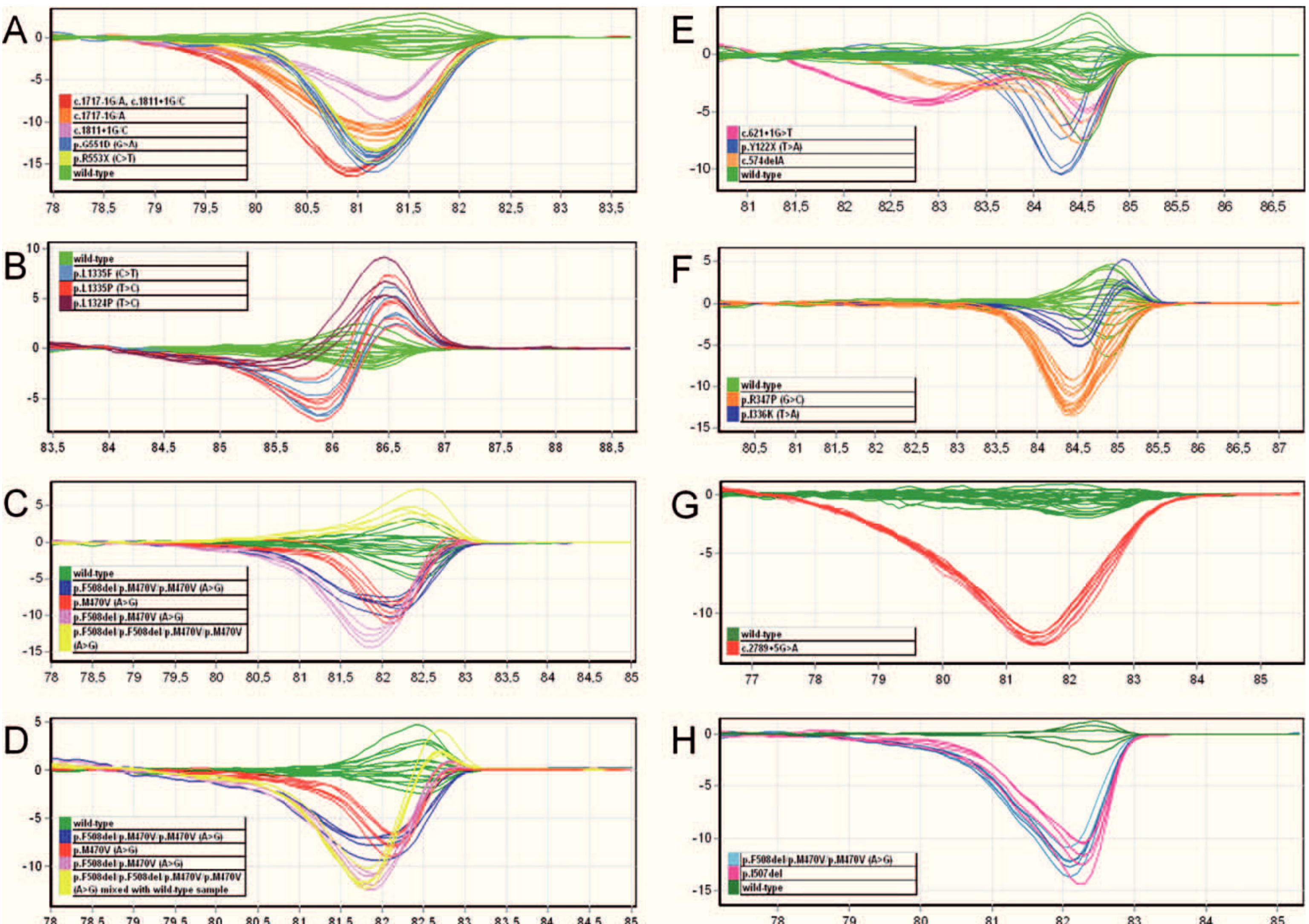
Therefore, there is still space for mutation pre-scanning prior to sequencing the "positives", as we have proved in the case of HRM. In this respect, we have studied a particular technical variant of HRM, based on the RotorGene™ 6000 device and the original HRM dye developed by IdahoTechnology. In this way we accounted for previously described disadvantages of alternative HRM platforms.

In our hands HRM allowed easy detection of all SNP classes as well as 1- and 3-base pair deletions. Heterozygous mutations belonging to the first SNP class cause the biggest change of T_m and should thus be the easiest mutations to detect. We detected this group very clearly, and we were also able to simply identify the most difficult fourth SNP class (Figs. 1E and 1F). The detection of homozygous mutations is complicated since generally the T_m difference is not high enough to allow proper discrimination. By generating "artificial" heterozygous samples from the 1 : 1 mixture, we were able to accurately detect homozygous sample.

All analysed samples with heterozygous mutant genotypes were unambiguously distinguished from wild-type samples. This method exhibits very high specificity and sensitivity, making it suitable for its use as a pre-screening method in diagnostics.

In some cases false positives appeared, hence we reached an almost 96 % of specificity. This could have been caused by analysing various old DNA samples, which were isolated by different techniques. Various DNA storage solution buffers can affect the melting behaviour in HRM, leading to broader dispersal of melting profiles, and thus could have contributed to false-positive "calls" by the software. It is also possible that the observed false-positive rates could be lowered following further optimization. Nevertheless, in diagnostic setting there is usually no time to perform such optimization, since it is expected that the technique should provide uniform results.

Figs. 1A–1H demonstrate the repeatability of the method; the melting curves of each sample were prepared in one day, by one analyst in the same instrument. Excellent repeatability is demonstrated by the overlapping curves of multiple samples for both the wild-type and the mutant samples.



Figs 1A-1H. Normalized difference graphs. Each genotype was melted and displayed in five replicates. A – exon 11. B – exon 22. C – exon 10. D – exon 10; homozygous sample (yellow colour) was mixed 1 : 1 with wild-type PCR product, denatured and reanalysed. E – exon 4. F – exon 7. G – exon 14b. H – exon 10.

In conclusion, HRM analysis is an economical, sensitive and specific close-tube method that can dramatically reduce the need for sequencing. Consequently, it has a high utility for the detection of unknown mutations in CF DNA diagnostics. The only caveat, which pertains to all other PCR-based techniques, is the quality of template DNA to which one needs to pay extra attention. We hope that our experience could be applicable to other HRM diagnostic applications.

Acknowledgements

We thank Andrea Tesoriero, Thomas Kaiser and Pavel Votýpka for their assistance with the manuscript.

References

Audrezet, M. P., Dabricot, A., Le Marechal, C., Ferec, C. (2008) Validation of high-resolution DNA melting analysis for mutation scanning of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. *J. Mol. Diag.* **10**, 424-434.

Cystic Fibrosis Mutation DataBase (CFMDB) (http://www.genet.sickkids.on.ca/cftr)

Eurogentest (http://www.eurogentest.org)

Graham, R., Liew, M., Meadows, C., Lyon, E., Wittwer, C. T. (2005) Distinguishing different DNA heterozygotes by high-resolution melting. *Clin. Chem.* **51**, 1295-1298.

Hert, D. G., Fredlake, C. P., Barron, A. E. (2008) Advantages and limitations of next-generation sequencing technologies: a comparison of electrophoresis and non-electrophoresis methods. *Electrophoresis* **29**, 4618-4626.

Highsmith, W., Jr., Jin, Q., Nataraj, A., O'Connor, J., Burland, V., Baubonis, W., Curtis, F., Kusukawa, N., Garner, M. M. (1999) Use of a DNA toolbox for the characterization of mutation scanning methods. I: construction of the toolbox and evaluation of heteroduplex analysis. *Electrophoresis* **20**, 1186–1194.

Chou, L. S., Lyon, E., Witter, C. T. (2005) A comparison of high-resolution melting analysis with denaturing high-performance liquid chromatography for mutation scanning: cystic fibrosis transmembrane conductance regulator gene as a model. *Am. J. Clin. Pathol*. **124**, 330-338.

Krypuy, M., Newnham, G. M., Thomas, D. M., Conron, M. and Dobrovic, A. (2006) High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer* **6**, 295.

Krypuy, M., Ahmed, A. A., Etemadmoghadam, D., Hyland, S. J. (2007) Australian Ovarian Cancer Study Group, DeFazio, A., Fox, S. B., Brenton, J. D., Bowtell, D. D., Dobrovic, A. High resolution melting for mutation scanning of TP53 exons 5-8. *BMC Cancer* **7**, 168.

Lerman, L. S., Silverstein, K. (1987) Computational simulation of DNA meeting and its application to denaturing gradient gel electrophoresis. *Methods Enzymol.* **155**, 482-501.

Li, Q., Liu, Z., Monroe, H., Culiat, C. T. (2002) Integrated platform for detection of DNA sequence variants using capillary array electrophoresis. *Electrophoresis* **23**, 1499-1511.

Liew, M., Pryor, R., Palais, R., Meadows, C., Erali, M., Lyon, E., Wittwer, C. (2004) Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin. Chem.* **50**, 1156-1164.

Macek, M. jr., Mercier, B., Macková, A., Miller, P. W., Hamosh, A., Férec, C., Cutting, G. R. (1997) Sensitivity of the denaturing gradient gel electrophoresis technique in detection of known mutations and novel Asian mutations in the *CFTR* gene. *Hum. Mut.* **9**, 136-147.

Montgomery, J., Wittwer, C. T., Kent, J. O., Zhou, L. (2007) Scanning the cystic fibrosis transmembrane conductance regulator gene using high-resolution DNA melting analysis. *Clin. Chem.* **53**, 1960-1962.

Nguyen-Dumont, T, Calvez-Kelm F. L, Forey, N., McKay-Chopin, S., Garritano, S., Gioia-Patricola, L., De Silva, D., Weigel, R., Sangrajrang, S., Lesueur, F., Tavtigian, S. V.; Breast Cancer Family Registries (BCFR) and Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab). (2009) Description and validation of high-throughput simultaneous genotyping and mutation scanning by high-resolution melting curve analysis. *Hum. Mutat.* **30**, 1-7.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., Sekiya, T. (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA* **86**, 2766–2770.

Reed, G. H., Wittwer, C. T. (2004) Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin. Chem.* **50**, 1748-1754.

Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066-1073.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L. (2001) The sequence of the human genome. *Science* **291**, 1304-1351.

Voelkerding, K. V., Dames, S. A., Durtschi, J. D. (2009) Next-generation sequencing: from basic research to diagnostics. *Clin. Chem*. **55**, 641-658.

White, H. and Potts, G. (2006) Mutation scanning by high resolution melt analysis. Evaluation of Rotor-Gene 6000 (Corbett Life Science), HR-1 and 384 well LightScanner (Idaho Technology). National Genetic Reference Laboratory. Wessex (http://www.ngrl.org.uk/Wessex/downloads.html).

Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G., Pryor, R. J. (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin. Chem.* **49**, 853-860.

Xiao, W., Oefner, P. J. (2001) Denaturing high-performance liquid chromatography: a review. *Hum. Mut.* **17**, 439-474.

Zhou, L., Myers, A. N., Vandersteen, J. G., Wang, L., Wittwer, C. T. (2004) Closed-tube genotyping with unlabeled oligonucleotide probes and a saturating DNA dye. *Clin. Chem.* **50**, 1328-1335.

Zhou, L., Wang, L., Palais, R., Pryor, R. and Wittwer, C. T. (2005) High-resolution DNA melting analysis for simultaneous mutation scannig and genotyping in solution. *Clin. Chem.* **51**, 1770-1777.

3.4. Makukh et al. (2010) A high frequency of the Cystic Fibrosis 2184insA mutation in Western Ukraine: genotype-phenotype correlations, relevance for newborn screening and genetic testing. J Cyst Fibros. (IF: 3.190)

Tato studie vznikla za podpory projektu Eurogentest, v době, kdy naše pracoviště působilo jako školící centrum pro metodiku detekce mutací v genu *CFTR* pro ostatní laboratoře zemí střední a východní Evropy. S ohledem na nedostatečnou kvalitu poskytovaných genetických služeb na Ukrajině, způsobenou podfinancováním veřejného zdravotnictví, bylo hlavním cílem zvýšit záchytnost populačně specifických CF alel. Současně jsme chtěli napomoci zavedení novorozeneckého skríningu CF na Ukrajině, neboť včasná diagnóza a léčba příznivě ovlivňuje průběh choroby a zároveň sníží náklady na léčbu tohoto onemocnění.

Incidence onemocnění na Ukrajině byla stanovena na základě náhodného DNA testování 720 jedinců na časté mutace v genu *CFTR* na 1:3300 novorozenců, což každoročně znamená narození až 150 dětí postižených CF. V západní části země, odkud pochází většina probandů z této studie, byla během let 1998-2008 stanovena diagnóza klasické CF u 132 probandů, u nichž se testováním panelu 10 nejčastějších evropských mutací podařilo identifikovat 76% CF alel (201/264).

V této studii bylo analyzováno 57 CF pacientů, u nichž nebyly identifikovány obě CF alely. Pomocí komerčního kitu Elucigene^{Tm} CF-EU1 byl simultánně testován panel 32 CF mutací, následovaný analýzou genových přeskupení genu *CFTR* metodou MLPA a sekvenační analýzou exonů 7 a 13 tohoto genu.

Sekvenace exonu 7 detekovala u jednoho pacienta novou dosud nepopsanou variantu Y362X způsobující zařazení předčasného stop kodonu. Sekvenace exonu 13 odhalila neobvykle vysokou frekvenci mutace 2184insA. Ta byla detekována u 17 nepříbuzných CF pacientů, z nichž dva byli homozygoti, a navýšila populační záchytnost o 7.2% CF alel. Vzhledem ke sporadickému výskytu této mutace v okolních zemích se zdá, že má svůj původ ve slovanské populaci západní Ukrajiny a odtud se šířila dále.

Mutace 2184insA představuje druhou nejčastější mutaci na západní Ukrajině a vzhledem k velkému množství jedinců ukrajinské národnosti žijící v zahraničí (přes 4 milióny v Rusku, 2 milióny v USA a 1 milión v Kanadě) by měla být součástí testovacího panelu CF pacientů ukrajinského původu. Donedávna nebylo možné testovat ji v rámci žádného komerčně diagnostického kitu, nyní je součástí nově vyvinutých souprav Devyser CFTR Core (Devyser), CF StripAssay (ViennaLab) a v brzké době uveřejněného kitu xTAG Cystic Fibrosis 71 kit v2+16 (Luminex).

Tato studie jako první poskytla přehled spektra mutací na západní Ukrajině a přinesla detailní zhodnocení korelace mezi genotypem a fenotypem u pacientů s mutací 2184insA. Mutační záchytnost byla navýšena na téměř 84%, čímž byly splněny požadavky na zavedení dvoustupňového (IRT/DNA) novorozeneckého skríningu.

ELSEVIER

Journal of Cystic Fibrosis 9 (2010) 371–375

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Short Communication

A high frequency of the Cystic Fibrosis 2184insA mutation in Western Ukraine: Genotype–phenotype correlations, relevance for newborn screening and genetic testing☆

Halyna Makukh [a], Petra Křenková [b], Marta Tyrkus [a], Lyudmyla Bober [a], Miroslava Hančárová [b], Oleh Hnateyko [a], Milan Macek Jr. [b,*]

[a] *Institute of Hereditary Pathology of the Ukrainian Academy of Medical Sciences, Lviv, Ukraine*
[b] *Department of Biology and Medical Genetics, 2nd Faculty of Medicine of Charles University Prague and University Hospital Motol, V Úvalu 84; Prague 5, CZ 15006, Czech Republic*

Received 18 April 2010; received in revised form 2 June 2010; accepted 7 June 2010
Available online 24 July 2010

Abstract

We present the first comprehensive report on the distribution and genotype–phenotype correlations of CF-causing mutations in Western Ukraine (former Galicia). The 2184insA mutation was identified in 17 unrelated CF patients, 2 of whom are homozygotes for this allele. This mutation is associated with the classical form of CF. The high frequency of 2184insA mutation (7.20% of all mutated CF chromosomes) suggests that it is likely of Galician origin, from where it has spread throughout Europe and beyond. The achieved 83.71% mutation detection rate fulfills the minimal pre-requisite for introduction of the "two-tier" (IRT/DNA) newborn screening program.
© 2010 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.

Keywords: Cystic fibrosis (OMIM 219700); *CFTR* gene; Genotype phenotype correlations; Western Ukraine; 2184insA; DNA diagnostics; Newborn screening; Sequencing

1. Introduction

The incidence of CF in Ukraine has not been determined at nation-wide or regional levels, thus far. However, our previous results based on testing of 720 random individuals for common *CFTR* alleles suggest a carrier frequency of 1 in 29 (data not shown). Therefore, the extrapolated frequency of CF homozygotes is approximately 1 in 3300 live births. Based on these estimates and the annual national birth rate of 509,000 newborns, 143 children with CF are expected to be born every year in the country [1]. Given the average annual birth rate within the last decade (1998–2008) of 119,000 newborns in Western Ukraine (WU), 47 of them with CF should be born in this region.

The core of historic Galicia comprises current regions of Lviv, Ternopil and Ivano-Frankivsk, from where most of the CF patients included in this report originate (Fig. 1). The region's dominant Ukrainian population (approximately 90%) is complemented by Russian (2.7%), Polish (2.1%), Romani (1.9%), Hungarian and Ashkenazi Jewish (1.5% each), Slovak (1.0%), German, Armenian and Czech (together 0.3%) minorities [extrapolated WU 2001 census; 2].

Within the last decade (1998 to 2008), molecular genetic screening of common European CF-causing mutations was

Abbreviations: BMI, body mass index; CF, Cystic Fibrosis; CFF, Cystic Fibrosis Foundation; *CFTR*, gene for the Cystic Fibrosis transmembrane conductance regulator protein; CFLD, Cystic Fibrosis liver disease; DNA, deoxyribonucleic acid; IRT, immunoreactive trypsinogen; FVC, forced vital capacity; FEV_1, forced expiratory volume at one second; M, million; MLPA, multiplex ligation-dependent probe amplification; PS, pancreatic sufficiency; PCR, polymerase chain reaction; WU, Western Ukraine.

☆ Presented in a poster form at the 32nd European Cystic Fibrosis Conference, Brest, France, June 2009. Křenková P, Makukh H, Hančárová M, Dvořáková L, Štambergová A, Macek M Jr. *CFTR* gene analysis in the Western Ukrainian population: an unusually high frequency of the 2184insA mutation. J Cyst Fibros 2009; 8(S2):S6.

* Corresponding author. Tel.: +420 224433501; fax: +420 224433520.
E-mail address: milan.macek.jr@lfmotol.cuni.cz (M. Macek).

1569-1993/$ - see front matter © 2010 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jcf.2010.06.001

performed in a total of 420 individuals of WU origin where CF was clinically suspected, including the examination of 630 first degree relatives from these families. From the total number of cases studied the diagnosis of the classical form of CF was unambiguously established in 132 patients (132/420; 31.43%) using consensual diagnostic criteria [3]. Since this cohort is representative of the WU population (Fig. 1), we could characterize the distribution of the most common CF-causing alleles by utilizing a panel of 10 mutations comprised within set a "home brew" methods (F508del, CFTRdele2,3(21 kb), I507del, 1677delTA, G542X, N1303K, W1282X, G551D, R553X, 1717-1G>A) [4,5]. Moreover, we continuously improved CF DNA diagnostics by successfully participating in the CF Thematic Network external quality assessment scheme since 2005 (cf. eqascheme.org).

In this study, in 57 patients where one or both *CFTR* mutations remained unidentified, we initially examined 23 additional common European CF alleles [6] (E60X, P67L, G85E, R117H, 621+1G>T, 711+1G>T, 1078delT, R334W, R347P, A455E, S549R T>R, R560T, 1898+1G>A, 2184delA, 2789+5G>A, 3120+1G>A, M1101K, D1152H, R1162X, 3659delC, 3849+10kbC>T, S1251N, 3905insT) comprised within a commercial assay, followed by DNA sequencing of selected *CFTR* exons 7 and 13, since these contain majority of the mutations found in Central and Eastern European CF populations [6,7]. The scope of this project was limited by the short duration of the Eurogentest project (www.eurogentest.org) incoming fellowship and its major aim was to provide sufficient evidence for public health purposes aimed at the introduction of newborn screening (IRT/DNA) in Ukraine.

To our knowledge, this is the first comprehensive report on the distribution of *CFTR* mutations in WU and their genotype–phenotype correlations, which has general implications for genetic testing and newborn screening in patients of this origin.

2. Methods

Leukocyte DNA was extracted either by the Proteinase K/phenol-based procedure or by our modification of the "salting out" technique [8]. Extended *CFTR* mutation/rearrangement screening was performed in sequential order by a/commercial assay comprising 32 most common European CF-causing mutations — Elucigene^{Tm} CF-EU1 (Gen-Probe Life Sciences Ltd.; United Kingdom), b/multiplex ligation-dependent probe amplification (MLPA)-SALSA MLPA KIT P091-B1 *CFTR* (MRC-Holland; The Netherlands) and c/sequencing on the 3130xl Genetic Analyzer (Applied Biosystems; USA).

3. Results

The "starting" distribution of CF mutations in the WU cohort based on our previous analyses [4,5] is listed in Table 1, whereby 76.14% (201/264 of all CF alleles) were identified (Table 1).

Fig. 1. Origin of patients with the 2184insA mutation included in this study.

Table 1
Distribution of *CFTR* mutations in Western Ukrainian CF patients.

CFTR mutations	N. of alleles	%
F508del (c.1520_1522delTCT)	143	54.17
2184insA (c.2052_2053insA)	19	7.20
N1303K (c.3909C>G)	13	4.92
CFTRdele2,3(21 kb) (c.54_273)	11	4.17
G542X (c.1624G>T)	7	2.65
W1282X (c.3846G>A)	6	2.27
1898+1G>A (c.1766+1G>A)#	3	1.14
2143delT (c.2012delT)#	3	1.14
621+1G>T (c.489+1G>T)#	2	0.76
R334W (c.1000C>T)#	2	0.76
3272-11 A>G (c.3140-11A>G) #	2	0.76
3849+10kbC>T (c.3717+12191C>T)#	2	0.76
185+1G>T (c.53+1G>T)#	1	0.38
E92K (c.274G>A)#	1	0.38
R347H (c.1040G>A)#	1	0.38
Y362X (c.1086 T>A)*	1	0.38
1717-1G>A (c.1585-1G>A)	1	0.38
R553X (c.1657C>T)	1	0.38
2183AA>G (c.2051_2052delAA)#	1	0.38
2721del11 (c.2589_2599delAATTTGGTGCT)#	1	0.38
Identified total	221	83.71
Unidentified	43	16.29

CFTR nomenclature is listed where applicable as by its more frequently used "legacy name" followed by the cDNA name in parenthesis [8]; mutations in italics were identified in this study, while the one in bold is novel; # these mutations were detected through previous collaborations (see Acknowledgment); * this mutation is novel.

We did not detect any intragenic *CFTR* rearrangements using MLPA in 63 *CFTR* genes (23.86%) with unknown CF mutations [9]. Sequencing of exon 7 revealed a novel allele Y362X [using the "legacy nomenclature"; 7]. The patient with this mutation bears the F508del allele in *trans* and suffers from the classical form CF associated with high sweat chloride concentrations. In exon 13 we found a high prevalence of the 2184insA mutation since this allele was detected in a total of 17 unrelated cases. Two patients are homozygotes for this mutation, while the analysis of parental origin revealed that 13 had F508del, 1 had the "Slavic" deletion CFTRdele2,3(21 kb) and one case had the R334W mutation in *trans*. Sixteen CF patients with 2184insA mutation originated were of WU origin, while only 1 case came from the eastern (Lugansk) part of the country. This mutation was not identified in 25 CF patients from the collaborating southeastern Zaporizhzhya CF centre (Fig. 1; data not shown). Identification of additional 20 CF mutations in the WU cohort increased the mutation detection rate to 83.71% (221/264; Table 1).

Genotype/phenotype correlations in 17 (8 females/9 males) bearing the 2184insA mutation are presented in Table 2. These data strongly suggest its "CF-causing" nature.

4. Discussion

Since the 2184insA mutation is not included within mutation panels of commonly used commercial assays [10], this allele is not identified by routine DNA diagnostics [11], thus far. As a consequence, there were only sporadic reports of this mutation in patients of Slavic origin [6,7,12–17] and of its associated phenotype, e.g. in a Polish patient [12]. The 2184insA mutation is located within the poly A tract in which deletions of one or two nucleotides are commonly found [9]. Consequently, the "mirror deletion" at this site, i.e. 2184delA, is included in the most frequently used commercial assays [10,11]. Similarly, the 2183AA>G belongs to the most common CF-causing mutations [6,17].

Of all populations studied to date, the prevalence of the 2184insA mutation is highest in WU (7.20% of all mutated CF chromosomes). Based on the observed population gradient, it is likely that the 2184insA mutation has its origin in Slavic populations of WU-Galicia from where it has disseminated throughout Europe and beyond.

More than half of the cases with this mutation were diagnosed within their first year of life, which attests the presence of typical CF symptoms in these children, and 1 patient presented with meconium ileus at birth (Table 2).

Fecal elastase-1, measured in 15 of the 17 cases with 2184insA, was low (Table 2), thereby substantiating the clinically ascertained pancreatic insufficiency [18]. Fecal elastase-1 levels in all patients treated at the Lviv CF centre are within the range of 1.6–566.0 µg/g of stool (average 6.5 µg/g; data not shown). Approximately 10% of them are pancreatic sufficient (PS, fecal elastase-1 > 200 µg/g). The percentage of PS cases and the high proportion of patients diagnosed within the first year of life are comparable to e.g. the CFF Registry [19]. This general comparison confirms that the clinical ascertainment of CF in WU for this study is not biased.

When comparing our CF patients with and without the 2184insA mutation, we found no significant differences in their age distribution within specific age brackets (0–1, 1–3, 3–7, 7–15, 15–18 and > 18 years of age), thereby demonstrating similar survival (data not shown). Although there were no differences in the presence of impaired glucose tolerance in patients older than 15 years of age (Table 2; data not shown for non-2184insA cases), the occurrence of CF liver disease (CFLD) was higher in patients with the 2184insA mutation (64.70% vs. 51.51%). We have also observed trends in the distribution of CF-specific infections: patients with the 2184insA mutation had a higher prevalence of *P. aeruginosa* (76.47% vs. 64.53%) and *S. aureus* (70.59% vs. 44.92%), but a lower rate of *H. influenzae* (11.76% vs. 44.94%) (Table 2). Although statistical calculations could not be performed due to the small sample size, based on the evidence presented this mutation can be regarded as "CF-causing".

In conclusion, the 2184insA is the second most common mutation in WU. As large Ukrainian populations are also found outside of the country with approximate estimates of 4.4 million (M) in Russia, 2 M in USA, 1 M in Canada, 0.25 M in Argentina and 0.3 M in Romania, this mutation should be included in the CF testing panels in patients of Ukrainian origin. Finally, the achieved mutation detection rate of 83.71% fulfills the minimal pre-requisite [20] for the introduction of the "two-tier" (IRT/DNA) newborn screening programs which could alleviate current imbalances in the level of CF clinical diagnosis in other regions of Ukraine with substantial WU populace [1,2].

Table 2
Phenotype parameters of Western Ukrainian CF patients with the 2184insA mutation.

No of patients	*2184insA*/allele in *trans*	Current age, full years	Age at diagnosis, month	Sweat test (Cl, mmol/l)	Meconium ileusI	CFLD	Weight for height z-score	Fecal elastase-1 ug/g of stool	Imapaired glucose tolerance	Infection				FVC		FEV_1		Bronchiectases	Blood oxygenation (%)	Finger clubbing	Compliance with therapy
										S. aureus	*P. aeruninosa*	*A. fumigatus*	*H. influenzae*	Liter	% predicted	Liter	% predicted				
1.	2184insA	9	39	87	No	No	−2.21	3.3	No	1	1	0	1	1.8	109	1.6	115	Yes	98	Yes	Good
2.	2184insA	*	42	105	No	Fibrosis	−4.63	NA*		0	1	0	0	NA		NA		Yes	NA	Yes	Unsatisfactory*
3.	F508del	9	3	98	No	No	−1.38	23.1	No	1	1	0	0	NA		NA		Yes	97	Yes	Good
4.	F508del	12	11	104	No	Fibrosis	−2.16	11.3	Yes	1	1	0	0	1.3	100	1.2	106	Yes	99	Yes	Good
5.	F508del	13	60	94	No	fibrosis	−1.11	15.6	Yes	1	1	0	0	1.5	60	0.9	43	Yes	99	Yes	Good
6.	F508del	12	48	105	No	Fibrosis, hypoplasia of biliary ducts	−1.76	2.7	No	0	1	0	0	NA	40	NA	43	Yes	NA	Yes	Good
7.	F508del	6	8	100	No	Fibrosis, hypoplasia of biliary ducts	−1.83	1. 9	No	1	1	0	0	y.a		y.a		x	94	Yes	Satisfactory
8.	CFTRdele 2,3 (21kb)	5	4	86	No	Fibrosis	−0.56	NA	NA	1	0	0	0	y.a		y.a		No	NA	No	Unsatisfactory
9.	F508del	7	5	106	No	Fibrosis	0.95	3.2	No	0	1	0	0	1.2	103	1.1	109	No	99	No	Satisfactory
10.	F508del	7	44	105	No	No	−1.48	17.4	No	1	1	0	0	1.0	100	1.0	114	Yes	95	Yes	Good
11.	F508del	14	21	103	No	Fibrosis		44.3	Yes	1	1	1	0	2.1	87	1.7	82	Yes	98	Yes	Unsatisfactory
12.	F508del	3	1	105	Yes	Hypoplasia of biliary ducts	−2.22	8.7	No	0	0	0	1	y.a		y.a		No	NA	No	Unknown
13.	F508del	2	6	105	No	No	−0.70	NA	No	1	1	0	0	y.a		y.a		No	92	No	Good
14.	F508del	6	66	97	No	Fibrosis	NA	NA	NA	1	1	0	0	y.a		NA		Yes	94	Yes	Satisfactory
15.	F508del	5	2	73	No	No	NA	NA		0	0	0	0	y.a.		NA		No	NA	NA	Unknown **
16.	F508del	7	60	100	No	Cirrhosis	−1.96	432.1	No	1	0	0	0	1.0	90	1.0	103	Yes	99	Yes	Satisfactory
17.	R334W	27	5	95	No	No	−0.52	514.2	No	1	1	0	0	3.8	84	1.2	54	Yes	97	Yes	Good

Legend: * — died at age of 7 years; ** — lost from Lviv CF centre care; NA—not available or not performed due to young age (y.a); 1 — presence; 0 — absence; CFLD — Cystic Fibrosis liver disease; FVC — forced vital capacity, FEV_1 — forced expiratory volume at one second, including glucose tolerance test reflect 2009 measurements, bronchiectases were diagnosed by computed tomography.

Acknowledgment

Supported by the Eurogentest Fellowship (FP6-512148) to HM and by MZOFNM2005 from the Czech Ministry of Health to MM. We also acknowledge proofreading of the manuscript by Dr. A. Melnyk (Chicago, USA), graphical design by Dr. M. Turnovec (Prague, Czech Republic) and help of Dr. S. Koceva (Skopje, Macedonia) and Dr. J. Feigelson (Paris, France) with sequencing of selected WU patients.

References

[1] http://www.moz.gov.ua/.
[2] http://www.ukrcensus.gov.ua/eng/results/general/nationality.
[3] Rosenstein BJ, Cutting GR. The diagnosis of cystic fibrosis: a consensus statement. Cystic Fibrosis Foundation Consensus Panel. J Pediatr 1998;132(4):589–95.
[4] Makukh H, Koceva S, Zastavna D, Kornienko Y, Hnateyko O. The mutation analysis of CFTR gene by DGGE in CF patients from West Ukraine. Biopolymers and Cells 2001;17(4):319–24 Article in Ukrainian.
[5] Livshits LA. The molecular-genetic analysis of some exons of CFTR gene among CF parients from Ukraine. Cytol Genet 2000;34(4):6–9 Article in Ukrainian.
[6] Bobadilla JL, Macek Jr M, Fine JP, Farrell PM. Cystic fibrosis: a worldwide analysis of CFTR mutations — correlation with incidence data and application to screening. Hum Mutat 2002;19(6):575–606.
[7] Cystic Fibrosis Mutation DataBase: http://www.genet.sickkids.on.ca/cftr
[8] Makukh H, Zastavna D, Tyrkus M, Tretjak B, Chorna L. The manner of DNA extaction from blood leucocytes. Patent of serviceable model. Ukraine, 32044, UA. МПК (2006) G01N33/49. State institution «Institute of Hereditary Pathology». U200801896, 14.02.2008, 25.04.2008, bulletin №8.
[9] Férec C, Casals T, Chuzhanova N, et al. Gross genomic rearrangements involving deletions in the CFTR gene: characterization of six new events from a large cohort of hitherto unidentified cystic fibrosis chromosomes and meta-analysis of the underlying mechanisms. Eur J Hum Genet 2006;14(5):567–76.
[10] Čamajová J, Berwouts S, Matthijs G, Macek Jr M, Dequeker E. Variability in the use of CE-marked assays for in vitro diagnostics of CFTR gene mutations in European genetic testing laboratories. Eur J Hum Genet 2009;17(4):537–40.
[11] Berwouts S, Gordon JT, Rundell CA, Barton DE, Dequeker E. Evaluation and use of a synthetic quality control material, included in the European external quality assessment scheme for cystic fibrosis. Hum Mutat 2008;29 (8):1063–70.
[12] Minarowska A, Kowalczuk O, Kaczmarski M, Kowalczuk D, Minarowski L, Chyczewski L. Rare genotype del2, 3/2184insA in a cystic fibrosis patient. Folia Histochem Cytobiol 2007;45(1):47–9.
[13] Petrova NV, Kapranov NI, Ginter EK. Detection of frequent mutations of the CFTR gene in cystic fibrosis patients from Central Russia. Genetika 1997;33(1):106–9.
[14] Witt M, Pogorzelski A, Bal J, Rutkiewicz E, Majka L, Sobczynska A. Frequency of mutatons and genotypes of the CFTR gene in cystic fibrosis adults in Poland. Pneumonol Alergol Pol 1999;67(3–4):137–41.
[15] Aznarez I, Bal J, Casals T, et al. Analysis of mutations in the CFTR gene in patients diagnosed with cystic fibrosis in Poland. Med Wieku Rozwoj 2000;4(2):149–59.
[16] Balaščaková M, Piskáčková T, Holubová A, et al. Current diagnostic strategies and overview of preimplantation, prenatal and postnatal DNA diagnostics of cystic fibrosis in the Czech Republic. Czech Pediatrics 2008;63(2):62–75.
[17] World Health Organisation 2004; The molecular genetic epidemiology of cystic fibrosis—http://www.who.int/genomics/publications/en/
[18] Walkowiak J, Lisowska A, Przyslawski J, Grzymislawski M, Krawczynski M, Herzig KH. Faecal elastase-1 test is superior to faecal lipase test in the assessment of exocrine pancreatic function in cystic fibrosis. Acta Paediatr 2004;93(8):1042–5.
[19] Cystic Fibrosis Foundation Patient Registry. Annual data report to the center directors. Maryland: Bethesda; 2008. www.cff.org.
[20] Castellani C, Southern KW, Brownlee K, et al. European best practice guidelines for cystic fibrosis neonatal screening. J Cyst Fibros 2009;8(3): 153–73.

3.5. Křenková et al. (2012) Distribution of CFTR mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection of novel/de novo alleles and relevance for related/derived populations. J Cyst Fibros. (IF: 3.190)

Identifikace obou kauzálních mutací u CF pacientů je důležitá z hlediska vztahu mezi genotypem a fenotypem, kdy znalost přesné molekulární podstaty onemocnění umožňuje předpovědět průběh onemocnění a individuálně stanovit léčbu, včetně případné mutačně specifické terapie. Znalost patogenních mutací genu *CFTR* zároveň podpoří klinickou diagnózu, umožní budoucí prenatální diagnostiku a možnost stanovení přenosu těchto alel a rizik u dalších rodinných příslušníků. Vzhledem k velkému množství dosud identifikovaných mutací v genu *CFTR* a k jejich populačně specifickému zastoupení je při molekulárně genetickém vyšetření klíčové znát etnický původ probanda a distribuci i četnost mutací v příslušné populaci.

To bylo hlavním cílem této publikace, která sumarizuje výsledky dvou dekád výzkumné a diagnostické činnosti v oblasti molekulárně genetické diagnostiky cystické fibrózy v České republice. Práce přináší přehled spektra mutací nalezených u české populace, která populační skladbou reprezentuje obyvatelstvo střední Evropy, a výsledky tak mohou být užitečné i pro okolní státy s nízkou detekční záchytností, pro velké množství krajanů žijících v Severní Americe i pro vývoj populačně-specifických diagnostických panelů.

Z téměř 3000 vyšetřovaných rodin byla jednoznačná klinická diagnóza CF stanovena u 600 nepříbuzných probandů. Vhledem k autozomálně recesivnímu charakteru onemocnění spočívá strategie tzv. kaskádového molekulárně genetického vyšetření genu *CFTR* v postupném vylučování nejčastějších mutací v dané populaci. V současné době začínáme vyšetřením komerčního panelu 50 mutací (Elucigene CF-EU2v1^{TM}), následovaného analýzou genových přeskupení genu *CFTR* metodou MLPA a analýzou celé kódující sekvence genu *CFTR* pomocí mutačního skenování metodou HRM a sekvenační analýzy.

Tímto přístupem se podařilo detekovat 99.50% všech CF alel (1194/1200) v české populaci. Celkem bylo nalezeno 91 různých mutací, z nichž se pouze 7 vyskytovalo ve frekvenci nad 1% (F508del - 67.42%, CFTRdele2,3(21kb) - 5.75%, G551D - 2.92%, N1303K - 2.42%, G542X - 2.0%, 3849+10kbC>T - 1.67% a 1898+1G>A - 1.42%) a celých 52 mutací se vyskytovalo privátně pouze v jedné rodině.

Podařilo se detekovat 20 nových mutací a 1 polymorfismus, které byly nahlášeny do mezinárodní databáze Cystic Fibrosis Genetic Analysis Consortium Dabatase (CFGAC, http://www.genet.sick-kids.on.ca/cftr/). Poskytnutí jejich klinického dopadu na své nosiče by mohlo pomoci při predikci fenotypu v případě nálezu dané mutace u dalšího CF pacienta, neboť u vzácných mutací chybí funkční studie na proteinové úrovni demonstrující jejich patogenetický potenciál. Sdílení těchto údajů je velmi přínosné pro zhodnocení závažnosti mutací, potažmo formy onemocnění, a to především v případě prenatální diagnostiky. Jedna z těchto nových mutací vznikla na paternální alele mechanismem *de novo*, tj. ve frekvenci 1/1200 CF alel, což je ve shodě s publikovanými daty (Girodon et al. 2008). Dále byl na základě korelace mezi fenotypem a genotypem predikován nepatogenetický potenciál některých variant (V754M, S1456N).

Na základě těchto dat byla vypočtena detekční záchytnost komerčního kitu Elucigene CF-EU2v1™ , který je v českých diagnostických laboratořích hojně rozšířen, na téměř 91%. Metoda MLPA detekující delece/duplikace na úrovni celých exonů přispěla k dosažené populační záchytnosti 1% a mutační skenování se sekvenační analýzou detekovaly dalších 94 alel (7.8%). Ačkoliv komerční diagnostická souprava Elucigene CF-EU2v1™ dosahuje dostatečnou záchytnost pro využití v rutinní diagnostice i novorozeneckém skríningu CF, molekulárně genetická diagnostika u pacientů s CF by měla v každém případě zahrnovat všechny mutace, které se v příslušné populaci vyskytují s četností vyšší než 0.5 %, proto by mutace I336K a S945L měly být přidány do rutinně používaného testovacího panelu.

Konečně průběžné výsledky této studie byly použity i v rámci optimalizace alternativních protokolů novorozeneckého skríningu CF zahrnujících DNA diagnostiku mutací v genu *CFTR* v české populaci na našem pracovišti (Krulisova et al. 2012), který však byl pouze mým vedlejším projektem a nebude proto blíže diskutován.

ELSEVIER

Journal of Cystic Fibrosis xx (2012) xxx – xxx

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Short Communication

Distribution of *CFTR* mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection of novel/*de novo* alleles and relevance for related/derived populations☆

Petra Křenková [a], Tereza Piskáčková [a], Andrea Holubová [a], Miroslava Balaščaková [a], Veronika Krulišová [a], Jana Čamajová [a,b], Marek Turnovec [a], Malgorzata Libik [a], Patricia Norambuena [a], Alexandra Štambergová [a], Lenka Dvořáková [a], Veronika Skalická [c], Jana Bartošová [c], Tereza Kučerová [c], Libor Fila [d], Dana Zemková [c], Věra Vávrová [c], Monika Koudová [a,e], Milan Macek, Sr. [a], Alice Krebsová [a,f], Milan Macek Jr. [a,*]

[a] *Department of Biology and Medical Genetics, 2nd Faculty of Medicine of Charles University Prague and University Hospital Motol, Prague, Czech Republic*
[b] *Regional Authority of Public Health, Bánská Bystrica, Slovak Republic*
[c] *Department of Pediatrics, 2nd Faculty of Medicine of Charles University Prague and University Hospital Motol, Prague, Czech Republic*
[d] *Department of Pneumology, 2nd Faculty of Medicine of Charles University Prague and University Hospital Motol, Prague, Czech Republic*
[e] *GHC Genetics Ltd., Prague, Czech Republic*
[f] *Department of Cardiology, Humboldt University, Virchow Klinikum Charité, Berlin, Germany*

Received 15 October 2012; received in revised form 30 November 2012; accepted 2 December 2012

Abstract

Background: This two decade long study presents a comprehensive overview of the *CFTR* mutation distribution in a representative cohort of 600 Czech CF patients derived from all regions of the Czech Republic.
Methods: We examined the most common CF-causing mutations using the Elucigene CF-EU2v1™ assay, followed by MLPA, mutation scanning and/or sequencing of the entire *CFTR* coding region and splice site junctions.
Results: We identified 99.5% of all mutations (1194/1200 *CFTR* alleles) in the Czech CF population. Altogether 91 different *CFTR* mutations, of which 20 were novel, were detected. One case of *de novo* mutation and a novel polymorphism was revealed.
Conclusion: The commercial assay achieved 90.7%, the MLPA added 1.0% and sequencing increased the detection rate by 7.8%. These comprehensive data provide a basis for the improvement of CF DNA diagnostics and/or newborn screening in our country. In addition, they are relevant to related Central European populations with lower mutation detection rates, as well as to the sizeable North American "Bohemian diaspora".
© 2012 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.

Keywords: Cystic fibrosis; Czech Republic; *De novo CFTR* mutation; Central Europe; Newborn screening

Abbreviations: AT, Austrian/Austria; CE, Central Europe; CF, Cystic Fibrosis; CZ, Czech Republic/Czech; *CFTR*, cystic fibrosis transmembrane conductance regulator gene; DE, German/Germany; HU, Hungarian/Hungary; MLPA, multiplex ligation-dependent probe amplification; NBS, newborn screening; PL, Polish/Poland; SK, Slovak/Slovakia; UK, United Kingdom; USA, United States of America.

☆ Presented as a poster at the 33rd European Cystic Fibrosis Conference, Valencia, Spain, June 2010. Petra Křenková, Tereza Piskáčková, Miroslava Balaščaková, Andrea Holubová, Jana Čamajová, Miroslava Hančárová, Lenka Dvořáková, Veronika Skalická, Alexandra Štambergová, Věra Vávrová, Milan Macek Jr. Spectrum of *CFTR* Mutations in the Czech Republic. Journal of Cystic Fibrosis 2010; 9(S1):S2.

* Corresponding author at: Department of Biology and Medical Genetics, 2nd Faculty of Medicine of Charles University Prague and University Hospital Motol, V Úvalu 84; Prague 5, CZ 15006, Czech Republic. Tel.: +420 224433501; fax: +420 224433520.
E-mail address: milan.macek.jr@lfmotol.cuni.cz (M. Macek).

1569-1993/$ -see front matter © 2012 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jcf.2012.12.002

1. Introduction

The incidence of cystic fibrosis (CF) in the Czech Republic (CZ) was estimated using epidemiological approaches more than half a century ago (1 in 2700 live births), while recent outcomes of a nationwide CF newborn screening (NBS) revealed a two-fold lower incidence (1 in 4023 live births) [1]. Given the current annual birth rate of 108,673 [2], approximately 30 CF patients would be expected to be born each year.

The CZ population has German (Bavarian; DE), Northern Austrian (AT), Polish (PL), Slovak (SK) or Hungarian (HU) influences (arranged by the degree of their historical impact) and is a representative of the population composition of Central Europe (CE) [3,4]. Romani intermarriage and non-European immigration have remained marginal [2]. Therefore, generally speaking, the CZ population is stable and homogeneous [5].

Over the past two decades, the Prague CF Centre has performed DNA diagnostic testing for the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene in a group of almost 3000 CZ families. This facility serves as the national reference center, both for clinical and laboratory diagnosis of CF [6], within an official network of regional CF centers [7]. The Prague center also coordinates CF newborn screening for the western part of the country [1].

The aim of this study is to present results from a substantially larger and representative group of CZ CF patients, compared to the previous report published within a collaborative study [8]. In addition, data on intra-*CFTR* rearrangements, copy number and/or novel mutations are included. Significantly updated data provide a basis for improvement of CF DNA diagnosis and/or CF NBS in the CZ and related populations. Finally, the information is important for the selection of patients for mutation-specific therapies [9].

2. Methods

Diagnosis of CF was established in 600 unrelated CZ CF patients by using a combination of clinical and laboratory diagnostic criteria [10]. Their geographic origin is presented in Fig. 1. During this long-term population study, DNA diagnostic techniques have gradually evolved (data available upon request). Initially, we examined the most common CF causing mutations using “in house” methods, later followed by commercial assays (e.g. Elucigene CF-EU2v1™, Gen-Probe Life Sciences, UK). Patients with one or both unidentified mutations were then examined for intragenic rearrangements using multiplex ligation-dependent probe amplification (MLPA, MRC-Holland, Netherlands). Subsequently, negative cases were subjected to mutation scanning [11] and/or sequencing of the entire *CFTR* coding region and splice site junctions (Applied Biosystems, USA). Any detected *CFTR* mutations were verified in the index case's parents.

Fig. 1. Origin of 600 Czech CF patients. Legend: Origin of patients is based on the postal codes of their domicile. Number of patients in the regions: Capital Prague (Praha) Region — 99; Central Bohemia Region (i.e. regiona surrounding Prague) — 88; Ústí nad Labem Region — 52; Pilsen (Plzeň) Region — 48; Liberec Region — 41; South-Bohemian (České Budějovice) Region — 40; Hradec Králové Region — 37; Zlín Region — 32; Pardubice Region — 31; Moravian-Silesian (Ostrava) Region — 29; Vysočina (Jihlava) Region — 29; Karlovy Vary Region — 26; Olomouc Region — 25; South Moravian (Brno) Region — 23.

Please cite this article as: Křenková P, et al, Distribution of *CFTR* mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection..., J Cyst Fibros (2012), http://dx.doi.org/10.1016/j.jcf.2012.12.002

Table 1
Spectrum of *CFTR* mutations detected in Czech CF patients.

	Mutations/HGVS nomenclature/	Mutations/traditional nomenclature, legacy name/	Legacy exon/intron	No. of alleles	%
1.	c.1521_1523delCTT	F508del*#	Ex10	809	67.42
2.	c.54-5940_273+10250del21kb	CFTRdele2,3/21kb/*#	In1–In3	69	5.75
3.	c.1652G>A	G551D*#	Ex11	35	2.92
4.	c.3909C>G	N1303K*#	Ex21	29	2.42
5.	c.1624G>T	G542X*#	Ex11	24	2.00
6.	c.3718-2477C>T	3849+10kbC>T*#	In19	20	1.67
7.	c.1766+1G>A	1898+1G>A*#	In12	17	1.42
8.	c.1040G>C	R347P*#	Ex7	11	0.92
9.	c.2012delT	2143delT*#	Ex13	11	0.92
10.	c.3140-26A>G	3272-26A>G*#	In17a	8	0.67
11.	c.1007T>A	I336K#	Ex7	7	0.58
12.	c.3846G>A	W1282X*#	Ex20	7	0.58
13.	c.1657C>T	R553X*#	Ex11	6	0.50
14.	c.2657+5G>A	2789+5G>A*#	In14b	6	0.50
15.	c.2834C>T	S945L#	Ex15	6	0.50
16.	c.442delA	574delA#	Ex4	5	0.42
17.	c.489+1G>T	621+1G>T*#	In4	5	0.42
18.	c.2052_2053insA	2184insA#	Ex13	5	0.42
19.	**c.3009_3017delAGCTATAGC**	**3141del9&**	**Ex17a**	**5**	**0.42**
20.	c.366T>A	Y122X*#	Ex4	4	0.33
21.	c.[874G>A]+[2126G>A]	E292K/R709Q	Ex7/Ex13	4	0.33
22.	c.1585-1G>A	1717-1G>A*#	In10	4	0.33
23.	c.3454 G>C	D1152H*#	Ex18	4	0.33
24.	c.3484C>T	R1162X*#	Ex19	4	0.33
25.	c.4242+1G>T	4374+1G>T	In23	4	0.33
26.	c.1000C>T	R334W*#	Ex7	3	0.25
27.	**c.1767-?_2619+?del**	**CFTRdele13,14a**	**Ex13–Ex14a**	**3**	**0.25**
28.	c.3468+2_3468+3insT	3600+2insT	In18	3	0.25
29.	c.3469-?_3717+?dup	CFTRdup19	Ex19	3	0.25
30.	c.3964-78_4242+577del	CFTRdele22,23#	Ex22–Ex23	3	0.25
31.	**c.53+1G>T**	**185+1G>T**	**In1**	**2**	**0.17**
32.	c.54-1161_164+1603del2875	CFTRdele2	Ex2	2	0.17
33.	c.169T>G	W57G	Ex3	2	0.17
34.	c.254G>A	G85E*#	Ex3	2	0.17
35.	c.274G>T	E92X#	Ex4	2	0.17
36.	c.328G>C	D110H#	Ex4	2	0.17
37.	c.579+3A>G	711+3A>G#	In5	2	0.17
38.	c.3528delC	3659delC*#	Ex19	2	0.17
39.	**c.4127_4131delTGGAT**	**4259del5**	**Ex22**	**2**	**0.17**
40.	**c.1-?_1584+?del**	**CFTRdele1,10**	**Ex1–Ex10**	**1**	**0.08**
41.	c.115C>T	Q39X#	Ex1	1	0.08
42.	**c.79G>C**	**G27R**	**Ex2**	**1**	**0.08**
43.	c.[125C>T]+[223C>T]	S42F/R75X#	Ex2/Ex3	1	0.08
44.	c.164+1G>A	296+1G>A	In2	1	0.08
45.	c.274G>A	E92K#	Ex4	1	0.08
46.	c.349C>T	R117C*#	Ex4	1	0.08
47.	c.509G>A	R170H	Ex5	1	0.08
48.	**c.533G>A**	**G178E**	**Ex5**	**1**	**0.08**
49.	c.579+1G>T	711+1G>T*#	In5	1	0.08
50.	c.902A>G	Y301C	Ex7	1	0.08
51.	c.1040G>A	R347H*#	Ex7	1	0.08
52.	**c.1114C>T**	**Q372X**	**Ex7**	**1**	**0.08**
53.	c.1117-1G>A	1249-1G>A	In7	1	0.08
54.	c.1209+1G>A	1341+1G>A#	In8	1	0.08
55.	c.1519_1521delATC	I507del*#	Ex10	1	0.08
56.	c.1654C>T	Q552X#	Ex11	1	0.08
57.	c.1673T>C	L558S#	Ex11	1	0.08
58.	c.1679+1G>C	1811+1G>C	In11	1	0.08
59.	**c.1687T>C**	**Y563H**	**Ex12**	**1**	**0.08**
60.	c.1753G>T	E585X#	Ex12	1	0.08
61.	c.1766+1G>C	1898+1G>C	In12	1	0.08
62.	**c.2044delA**	**2176delA**	**Ex13**	**1**	**0.08**
63.	c.2051_2052delAAinsG	2183delAA>G#	Ex13	1	0.08

(continued on next page)

Please cite this article as: Křenková P, et al, Distribution of *CFTR* mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection..., J Cyst Fibros (2012), http://dx.doi.org/10.1016/j.jcf.2012.12.002

Table 1 (*continued*)

	Mutations/HGVS nomenclature/	Mutations/traditional nomenclature, legacy name/	Legacy exon/intron	No. of alleles	%
64.	c.2052delA	2184delA*#	Ex13	1	0.08
65.	c.2290C>T	R764X#	Ex13	1	0.08
66.	c.2490+1G>A	2622+1G>A#	In13	1	0.08
67.	c.2538G>A	W846X*#	Ex14a	1	0.08
68.	c.2551C>T	R851X#	Ex14a	1	0.08
69.	c.2589_2599delAATTTGGTGCT	2721del11	Ex14a	1	0.08
70.	**c.2705delG**	**2837delG**	**Ex15**	**1**	**0.08**
71.	**c.2789delG**	**2921delG**	**Ex15**	**1**	**0.08**
72.	**c.2803_2813delCTACCACTGGT**	**2935del11**	**Ex15**	**1**	**0.08**
73.	c.2856G>C	M952I	Ex15	1	0.08
74.	c.2991G>C	L997F#	Ex17a	1	0.08
75.	**c.3106delA**	**3238delA**	**Ex17a**	**1**	**0.08**
76.	**c.3136G>T**	**E1046X**	**Ex17a**	**1**	**0.08**
77.	c.3139G>C	G1047R	Ex17a	1	0.08
78.	c.3196C>T	R1066C*#	Ex17b	1	0.08
79.	**c.3196C>G**	**R1066G**	**Ex17b**	**1**	**0.08**
80.	c.3302T>G	M1101R	Ex17b	1	0.08
81.	**c.3310G>A**	**E1104K**	**Ex17b**	**1**	**0.08**
82.	**c.3353C>T**	**S1118F**	**Ex17b**	**1**	**0.08**
83.	c.3472C>T	R1158X*#	Ex19	1	0.08
84.	c.3587C>G	S1196X#	Ex19	1	0.08
85.	**c.3708delT**	**3840delT**	**Ex19**	**1**	**0.08**
86.	c.3937C>T	Q1313X#	Ex21	1	0.08
87.	c.3971T>C	L1324P	Ex22	1	0.08
88.	c.4003C>T	L1335F	Ex22	1	0.08
89.	c.4004T>C	L1335P	Ex22	1	0.08
90.	**c.4097T>A**	**I1366N**	**Ex22**	**1**	**0.08**
91.	c.4426C>T	Q1476X	Ex24	1	0.08
92.	Unknown			6	0.50
	Total			1200	100.00

Legend: Within the traditional nomenclature column: "*" mutations included in the Elucigene CF-EU2v1™ assay; "#" genotype–phenotype correlations of detected mutations are described in the CFTR2 database [21] with e.g. D1152H, L997F having "varying consequences"¨; "?" — Genomic position of breakpoints has not been identified, thus far. Novel mutations are formatted in bold and "&" the novel mutation 3141del9 was independently detected in five unrelated CZ families.

3. Results

Altogether we found 91 different *CFTR* mutations (Table 1), with only seven being present at a frequency >1%: F508del (67.42%), CFTRdele2,3(21kb) (5.75%), G551D (2.92%), N1303K (2.42%), G542X (2.0%), 3849+10kbC>T (1.67%) and 1898+1G>A (1.42%) (using the legacy/traditional nomenclature). More than half of all mutations (n=52) occurred within a single family. In addition, 20 novel mutations (Table 1) and a novel variant (S1456N) were discovered.

A *de novo* novel mutation 3840delT was detected on "fingerprinting-proven" paternal *CFTR* allele in an adolescent male patient who bears a second novel mutation in compound heterozygosity — 3840delT/2921delG. He was diagnosed clinically at 3 years of age (sweat test: 80 mmol/l), and suffers from chronic *Pseudomonas aeruginosa* colonization and pancreatic insufficiency.

In addition, we observed the previously described V754M mutation in *trans* to N1303K in an unaffected mother (sweat test: 40 mmol/l). Her first CF child bears a R709Q-E292K/N1303K in *trans*, while sequencing of exon 7, 13 and 21 during prenatal diagnosis of her second child, who is unaffected, revealed the R709Q-E292K/V754M genotype.

The S1455X mutation was observed in compound heterozygosity with the F508del in a male patient who was clinically diagnosed at age 7 years, in which "repeated bronchitis" led to sweat testing (mean concentration 70 mmol/l). Interestingly, the patient's asymptomatic father bears mutation S1455X in *trans* to a novel variant S1456N and the patient's apparently healthy brother has the maternal-F508del/S1456N genotype.

In aggregate, we identified 99.50% of all mutations (1194/1200 *CFTR* alleles) in the CZ CF population (Table 1).

4. Discussion

This study presents a comprehensive overview of the *CFTR* mutation distribution in a representative cohort of CZ CF patients originating from all CZ regions (Fig. 1). Integration of CF clinical expertise with DNA diagnostics at the Prague National Reference Center, together with consistent application of consensus CF clinical and laboratory diagnostic criteria, led to the identification of more than 99% of all *CFTR* mutations (Table 1).

There are over 10 million inhabitants in the country, which according to population genetic analyses, is a representative of the CE ethnic composition [3], with significant overlaps with

Please cite this article as: Křenková P, et al, Distribution of *CFTR* mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection..., J Cyst Fibros (2012), http://dx.doi.org/10.1016/j.jcf.2012.12.002

Table 2
Distribution of selected *CFTR* mutations in Central European populations.

Mutations/HGVS nomenclature/	Mutations/traditional nomenclature, legacy name/	Czech Republic 2012 (this study) (N=1200)	Slovakia 2010 (N=856)	Eastern Hungary 2011 (N=80)	Germany Bavaria 2002 (N=250)	Austria Tyrol 1997 (N=126)	Austria North– East, North– North 2002 (N=118)	Poland (N=1726)
c.1521_1523delCTT	F508del	67.42	66.80	70.00	74.00	74,60	70.30	57.0
c.54-5940_273+10250del21 kb	CFTRdele2,3/21kb	5.75	2.26	5.00	1.2*	2.6[#]	NA	1.80
c.1652G>A	G551D	2.91	<0.50	0.00	6.40	1.60	2.50	0.50
c.3909C>G	N1303K	2.42	2.03	5.00	2.40	0.00	NA	1.80
c.1624G>T	G542X	2.00	4.06	3.75	3.20	2.40	5.10	2.60
c.3718-2477C>T	3849+10kbC>T	1.67	4.28	0.00	NA	0.00	3.40	2.70
c.1766+1G>A	1898+1G>A	1.42	<0.50	0.00	NA	0.00	NA	NA
c.1040G>C	R347P	0.92	1.10	1.25	0.80	1.60	2.50	NA
c.2012delT	2143delT	0.92	1.10	0.00	NA	0.00	NA	NA
c.3140-26A>G	3272-26A>G	0.67	<0.50	0.00	NA	0.00	NA	NA
c.3846G>A	W1282X	0.58	<0.50	0.00	NA	0.00	NA	0.70
c.1007T>A	I336K	0.58	0.00	0.00	NA	0.00	NA	NA
c.1657C>T	R553X	0.50	0.90	0.00	1.20	0.00	NA	1.90
c.2657+5G>A	2789+5G>A	0.50	0.00	0.00	NA	2.40	NA	NA
c.2834C>T	S945L	0.50	0.00	0.00	NA	0.00	NA	NA
c.2052_2053insA	2184insA	0.42	1.58	5.00	NA	0.00	NA	NA

Legend: data for Slovakia [12], Eastern Hungary [14], Germany–Bavaria [13], Austria–Tyrol [18], Austria North East and North West [13], Poland and *[8], and [#] [16]. NA: not analyzed/data not available. N: number of analyzed CF alleles.

the SK, HU, AT and DE populations [3,4], and to a lesser degree, with its PL and DE Saxony neighbors [5]. Therefore, our data are pertinent to the related populations with lower mutation detection rates (Table 2) [8,12,13], with the exception of Eastern HU [14]. The CZ mutation distribution is also relevant to the North American "Bohemian diaspora" (residing mainly in Canada and the U.S. Midwest) consisting of over 1.2 million immigrants with descendants from historical CZ territories [15].

In the CE population, the frequency of the F508del mutation does not "rigorously" follow the expected "North-to-South" gradient, which could be due to the higher population heterogeneity in PL (Table 2). The "Slavic" mutation CFTRdele2,3/21kb/ [16] is relatively common in CE (Table 2). However, the G551D mutation is more common within the "core Hallstatt culture territory" [17] that comprises the current CZ, DE, AT and AT–Tyrol [18, Table 2]. Therefore, a "Celtic" origin of G551D is likely and is supported by its common origin and extrapolated "age" [19]. Similarly, we presume that the "Mediterranean" mutation G542X [8] is, according to historical patterns of CE colonization [17], the most prevalent in the Danube basin, i.e. in SK, HU, AT and DE.

In the CZ population the commercial assay Elucigene CF-EU2v1™ achieved mutation detection rates of 90.7%, MLPA added 1.0%, with the CFTRdele2,3/21 kb/mutation (5.75%) being independently confirmed by this assay. Mutation scanning and/or sequencing detected an extra 94 CF alleles, adding an additional 7.8%.

We have not included the male with the S1455X/F508del in our cohort, since he only suffers from "isolated elevated sweat chloride concentrations" [20] and does not meet the diagnostic criteria for CF [10]. Similarly, all cases with the R117H-IVS8 T(7) in *cis*, which did not meet the diagnostic criteria were also excluded from this study. Although the V754M mutation "is still under evaluation" in the CFTR2 database [21], we concluded that its pathogenic potential is limited. All "equivocal" cases have been enrolled into a long-term clinical monitoring program at our center [6,22].

The previously reported pathological L1-mediated retro-transpositional event [23] was detected in one patient who was compound heterozygous for the E92K/M952I mutations. It is likely that the pathogenetic impact of the "*CFTR*-related disorder associated" mutation M952I [22] was augmented by the Alu1-related molecular alternation of the *CFTR*, leading to a "classical" presentation of CF in this case.

The observed *de novo* novel mutation occurred on the paternal *CFTR* allele (father's age at conception was 25 years) at a rate of 1 in 1200 *CFTR* alleles examined, i.e. as previously reported [24].

In addition, previously unknown complex allele [25] comprising R709Q-E292K in *cis* in four unrelated families was discovered, with the R709Q being reported in AT [26].

In summary, we present a thorough overview of the *CFTR* mutation distribution in the CZ population, which demonstrates that integrated clinical and laboratory expertise [22] can yield very high mutation detection rates.

The Elucigene CF-EU2v1™ assay was shown to achieve sufficient mutation detection rates for multi-tier CF NBS (i.e. more than 85%), although the I336K and S945L, with frequency over 0.5% (Table 1), should also be included in the Czech national screening panel [1]. The CZ mutation distribution is relevant to related populations which have reported rather limited mutation spectra (e.g. Northern AT) and to the sizeable North American immigrant population derived from this part of CE.

Conflict of interest statement

The authors have no conflict of interest that could influence the content or processing of this manuscript.

Please cite this article as: Křenková P, et al, Distribution of *CFTR* mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection..., J Cyst Fibros (2012), http://dx.doi.org/10.1016/j.jcf.2012.12.002

Acknowledgment

Supported by CZ.2.16/3.1.00/24022OPPK, "Conceptual development project of research organization #00064203" (University Hospital Motol, Prague) from the Czech Ministry of Health, PRVOUK UK 2. LF and the European Commission projects (Eurogentest; FP7-HEALTH-F4-2010-261469) to MM Jr. We would like to acknowledge all collaborating clinicians whom we could not list individually and current and previous colleagues Nikola Bílá, Miroslava Hančárová, Magdalena Hérová, Milada Matějíčková, Jana Paděrová and Inna Soldátová from our department. We would like to thank our partners and long-time foreign collaborators Drs. Marie-Pierre Audrézet, Jian-Min Chen, Claude Férec and Bernard Mercier (Laboratoire de Génétique Moléculaire et d'Histocompatibilité, Centre Hospitalier Universitaire, Hôpital Morvan, Brest, France) and Timothy W. Hefferon (National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA) for their invaluable assistance. We would also like to acknowledge the support of Dr. Garry R. Cutting (MsKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, U.S.A.) who supported this study in the early to mid nineties. Finally, we would like to thank the Czech CF Association (www.cfklub.cz) for their continuous support.

References

[1] Balaščaková M, Holubová A, Skalická V, et al. Pilot newborn screening project for cystic fibrosis in the Czech Republic: defining role of the delay in its symptomatic diagnosis and influence of ultrasound-based prenatal diagnosis on the incidence of the disease. J Cyst Fibrosis 2009;8(3):224–7.
[2] Czech Statistical Office. http://www.czso.cz/eng/redakce.nsf/i/home.
[3] Lao O, Lu TT, Nothnagel M, et al. Correlation between genetic and geographic structure in Europe. Curr Biology 2008;18(16):1241–8.
[4] Heath SC, Gut IG, Brennan, et al. Investigation of the fine structure of European populations with applications to disease association studies. Eur J Hum Genet Dec 2008;16(12):1413–29.
[5] Zástěra J, Roewer L, Willuweit S, Sekerka P, Benešová L, Minárik M. Assembly of a large Y-STR haplotype database for the Czech population and investigation of its substructure. Forensic Sci Int Genet Apr 2010;4(3):e75–8.
[6] Mayell SJ, Munck A, Craig JV, et al. European Cystic Fibrosis Society Neonatal Screening Working Group. A European consensus for the evaluation and management of infants with an equivocal diagnosis following newborn screening for cystic fibrosis. J Cyst Fibrosis 2009;8(1):71–8.
[7] Official bulletin of the Czech Ministry of Health no. 4. http://www.mzcr.cz/Legislativa/dokumenty/vestnik-c4/2012_6288_2510_11.html; May 28, 2012. [establishing national network of *de iure* CF centers].
[8] Bobadilla JL, Macek Jr M, Fine JP, Farrell PM. Cystic fibrosis: a worldwide analysis of CFTR mutations—correlation with incidence data and application to screening. Hum Mutat Jun 2002;19(6):575–606.
[9] Amaral MD. Targeting CFTR: how to treat cystic fibrosis by CFTR-repairing therapies. Curr Drug Targets 2011;12(5):683–93.
[10] De Boeck K, Derichs N, Fajac I, et al. New clinical diagnostic procedures for cystic fibrosis in Europe. J Cyst Fibrosis 2011;Suppl. 2:S53–66.
[11] Křenková P, Norambuena P, Štambergová A, Macek Jr M. Evaluation of high-resolution melting (HRM) for mutation scanning of selected exons of the CFTR gene. Folia Biol (Praha) 2009;55(6):238–42.
[12] Šoltýsová A, Ficek A, Baldovič M, et al. Molecular diagnosis of cystic fibrosis in Slovakia: current status and outlook into the future ("Molekulárna diagnostika cystickej fibrózy na Slovensku: jej stav a perspektívne smerovanie"). CF Plus Motýlik 2010;5:40–1 [ISSN 1337–7183].
[13] The molecular genetic epidemiology of cystic fibrosis. Report of a joint meeting of WHO/ECFTN/ICF(M)A/ECFSGenoa, Italy; 19 June 2002 [www.cfww.org/docs/who/2002/who_hgn_cf_wg_04.02.pdf].
[14] Ivady G, Madar L, Nagy B, et al. Distribution of CFTR mutations in Eastern Hungarians: relevance to genetic testing and to the introduction of newborn screening for cystic fibrosis. J Cyst Fibros May 2011;10(3):217–20.
[15] United States Census Bureau 2010 — factfinder2.census.gov.
[16] Dörk T, Macek Jr M, Mekus F, et al. Characterization of a novel 21-kb deletion, CFTRdele2,3(21 kb), in the CFTR gene: a cystic fibrosis mutation of Slavic origin common in Central and East Europe. Hum Genet Mar 2000;106(3):259–68.
[17] Cunliffe Barry. The ancient Celts. Penguin Books; 2001 [ISBN-13: 978–0140254228].
[18] Stuhrmann M, Dörk T, Frühwirth M, et al. Detection of 100% of the CFTR mutations in 63 CF families from Tyrol. Clin Genet Oct 1997;52(4):240–6.
[19] Cashman SM, Patino A, Martinez A, et al. Identical intragenic microsatellite haplotype found in cystic fibrosis chromosomes bearing mutation G551D in Irish, English, Scottish, Breton and Czech patients. Hum Hered Jan-Feb 1995;45(1):6–12.
[20] Salvatore D, Tomaiuolo R, Vanacore B, Elce A, Castaldo G, Salvatore F. Isolated elevated sweat chloride concentrations in the presence of the rare mutation S1455X: an extremely mild form of CFTR dysfunction. Am J Med Genet A Mar 1 2005;133A(2):207–8.
[21] Clinical and Functional Translation of CFTR database (CFTR2). www.cftr2.org.
[22] Castellani C, Cuppens H, Macek Jr M, et al. Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. J Cyst Fibros May 2008;7(3):179–96.
[23] Chen JM, Masson E, Macek Jr M, et al. Detection of two Alu insertions in the CFTR gene. J Cyst Fibros Jan 2008;7(1):37–43 [Epub 2007 May 24].
[24] Girodon E, des Georges M, Medina R, et al. Occurrence of CFTR de novo mutations is not so rare. J Cyst Fibros Jun 2008;7(Suppl. 2):S6.
[25] El-Seedy A, Girodon E, Norez C, et al. CFTR mutation combinations producing frequent complex alleles with different clinical and functional outcomes. Hum Mutat Jun 7 2012, http://dx.doi.org/10.1002/humu.22129 [Epub ahead of print].
[26] Cystic Fibrosis Mutation Database (CFTR1). www.genet.sickkids.on.ca.

Please cite this article as: Křenková P, et al, Distribution of *CFTR* mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection..., J Cyst Fibros (2012), http://dx.doi.org/10.1016/j.jcf.2012.12.002

3.6. Tüttelmann et al. (2010) A common haplotype of protamine 1 and 2 genes is associated with higher sperm counts. Int J Androl. (IF: 3.591)

V evropských zemích je během posledních pár desetiletí sledována pomalá setrvalá tendence ke snižování kvality mužského ejakulátu. Za jednu z příčin je považován zvýšený výskyt umělých estrogenů v prostředí a další negativní civilizační vlivy. Neobjasněných příčin defektní spermatogeneze a zhoršených funkčních parametrů spermatu je však stále mnoho, a proto jsme se v naší práci zaměřili na studium příčin genetických, konkrétně na zhodnocení výskytu a stanovení frekvence mutací a polymorfismů protaminových genů (*PRM1, PRM2*) a objasnění vlivu těchto variant na poruchy spermiogeneze.

Předchozí studie prokázaly jednoznačný vztah aberantní exprese protaminů k mužské infertilitě, kdy správné množství obou proteinů je nezbytné pro správnou diferenciaci spermií (Carrell et al. 2008). Dále bylo prokázáno, že myši haploinsuficientní pro jeden z protaminových genů vykazují změny v uspořádání chromatinu a jaderné integritě. Tyto myši stále produkovaly spermie, ty však vykazovaly abnormální morfologii a sníženou pohyblivost a nebyly schopné oplodnit oocyt (Cho et al. 2001). Vzhledem k rozhodující roli protaminů v diferenciaci spermatid se tedy lze domnívat, že mutace těchto genů mohou vést k idiopatické infertilitě u mužů s normálním počtem spermií. Dosud publikovaná data zabývající se rolí protaminových variant a jejich asociací se semennými parametry v lidské neplodnosti jsou však neprůkazná, až rozporuplná (Jodar et al. 2011). Proto byla ve spolupráci s Univerzitou v Münsteru (Německo) provedena tato retrospektivní studie na representativním souboru německých neplodných mužů a kontrol.

Sekvenační analýza (podle Sangera) obou exonů s příslušným intronem genů *PRM1* a *PRM2* byla provedena na souboru pacientů s idiopatickou infertilitou německého původu: 1/ u 88 mužů s teratozoospermií (≤7% morfologicky normálních forem) a normálním počtem spermií ($\geq 20.10^6$/ml) simulující fenotyp prm-haploinsuficientní myši, 2/ u 83 mužů s teratozoospermií (≤7% morfologicky normálních forem) a sníženým počtem spermií ($< 20.10^6$/ml) a 3/ u 77 normozoospermických mužů (≥19% morfologicky normálních forem) s normálním počtem spermií ($\geq 20.10^6$/ml).

V genu *PRM1* byly detekovány tři již publikované varianty, dvě vzácné: c.54G>A (rs35262993, exon 1, synonymní) (Aoki et al. 2006a, Ravel et al. 2007, Imken et al. 2009) a c.102G>T (rs355576928, exon 1, nesynonymní R34S) (Iguchi et al. 2006, Aoki et al. 2006a, Ravel et al. 2007, Kichine et al. 2008, Jodar et al. 2011) a jedna častá: c.139A>C/g.230A>C (rs737008, exon 2, synonymní) (Tanaka et al. 2003, Iguchi et al. 2006, Aoki et al. 2006a, Ravel et al. 2007, Imken et al. 2009, Jodar et al. 2011), a to ve shodných alelických i genotypových frekvencích u všech testovaných skupin. Frekvence variant detekovaných v *PRM1* byla srovnatelná s publikovanými studiemi (Tanaka et al. 2003, Aoki et al. 2006a). Výsledky této práce ohledně varianty c.102G>T, kde dochází ke změně argininu za serin, jsou ve shodě s rozsáhlou studií (Kichine et al. 2008) a popírají patogenní potenciál této varianty zjištěný v dřívějších pracích (Iguchi et al. 2006, Ravel et al. 2007).

V genu *PRM2* bylo detekováno osm variant, kde šest bylo vzácných nahodile se vyskytujících v jednotlivých testovaných skupinách, jenž lze považovat spíše za benigní polymorfismy: c.66T>C (exon 1, synonymní) (Aoki et al. 2006a, Jodar et al. 2011), c.201C>T (exon 1, synonymní) (Jodar et al. 2011, Imken et al. 2009, Aoki et al. 2006a), c.271+10C>T/g.281C>T (rs740007626, intron) (Aoki et al. 2006a, Imken et al. 2009, Jodar et al. 2011), c.271+19C>T/g.290C>T (rs740007625, intron) (Jodar et al. 2011, Imken et al. 2009, Aoki et al. 2006a), c.271+29A>G/g.300A>G (intron), c.271+106C>A/g.377C>T (intron), a dvě časté varianty nalezené ve shodných alelických i genotypových frekvencích u všech testovaných skupin: c.271+27G>C/g.298G>C (rs16460222; intron) (Aoki et al. 2006a, Tanaka et al. 2003, Imken et al. 2009, Jodar et al. 2011), c.271+102C>A/g.373C>A (rs2070923, intron) (Jodar et al. 2011, Tanaka et al. 2003, Aoki et al. 2006a, Imken et al. 2009).

Mezi třemi častými polymorfismy (*PRM1* g.230A>C a P*RM2* g.298G>C/g.373C>A) byla zjištěna silná vazba a byly sestaveny haplotypy, jejichž zastoupení se mezi testovanými skupinami nelišilo. Avšak při testování vlivu haplotypů na semenné parametry v skupině všech 248 mužů analyzovaných dohromady byla odhalena statisticky významná asociace. Homozygotní nosiči haplotypu ACC měli dvojnásobně vyšší počet spermií než muži bez tohoto haplotypu (45x10^6/ml x 24.2x10^6/ml). Je možné,

že spermie nosičů jiného než ACC haplotypu nejsou životaschopné či podléhají negativní selekci. To by signalizovala i zjištěná signifikantní odchylka od Hardy-Weinbergovi rovnováhy u polymorfismů tvořících haplotyp. Vliv haplotypu ACC na ostatní semenné parametry (morfologii, motilitu) nebyl prokázán.

Jelikož poměr protaminů ve spermiích nebyl měřen, nelze ověřit vliv haplotypů na jejich expresi a případný aberantní poměr PRM1/2, který byl jednoznačně asociován s mužskou neplodností (Balhorn et al. 1988).

Další výzkumy by se měly ubírat směrem k analýze variant genu *TNP2* či promotorovým variantám protaminových genů, které dosud nebyly hojně zkoumány. Dopad ACC haplotypu na spermatogenezi by měl být ověřen dalšími studiemi na jiných populacích, aby bylo umožněno jeho případné klinické a diagnostické využití. Pro snadnou a rychlou detekci polymorfismů tvořících tento kauzální haplotyp by bylo vhodné zavést metodu HRM (genotypizaci variant metodou malých amplikonů), která byla v této dizertační práci detailně zhodnocena a na rozličných studiích potvrzena ideálnost a vhodnost této metody pro účely detekce neznámých genových variant i cíleně analyzovaných polymorfismů/mutací.

Konečně v rámci studia mužské neplodnosti jsem se podílela na replikačních studiích frekvence mutací v genu *ART3* v české populaci neplodných mužů (Norambuena et al. 2012a) a následně u německé populace (Norambuena et al. 2012b), které však byly pouze mými vedlejšími projekty a nebudou proto blíže diskutovány.

International Journal of Andrology
international journal of andrology ISSN 0105-6263

ORIGINAL ARTICLE

A common haplotype of *protamine* 1 and 2 genes is associated with higher sperm counts

F. Tüttelmann,*† P. Křenková,‡ S. Römer,† A. R. Nestorovic,§ M. Ljujic,§ A. Štambergová,‡ M. Macek Jr,‡ M. Macek Sr,‡ E. Nieschlag,* J. Gromoll* and M. Simoni†¶

*Institute of Human Genetics and †Centre of Reproductive Medicine and Andrology, University of Münster, Münster, Germany, ‡Institute of Biology and Medical Genetics, Centre of Reproductive Genetics, 2nd Faculty of Medicine and University Hospital Motol, Prague, Czech Republic, §Institute of Molecular Genetics and Genetic Engineering, Belgrade, Serbia, and ¶Department of Medicine, Endocrinology, Metabolism and Geriatrics, University of Modena and Reggio Emilia, Modena, Italy

Keywords:
male infertility, PRM1, PRM2, protamine, semen parameters, SNP

Correspondence:
Frank Tüttelmann, Institute of Human Genetics, Vesaliusweg 12–14, D-48149 Münster, Germany.
E-mail: frank.tuettelmann@ukmuenster.de

F. Tüttelmann and P. Křenková contributed equally to this study.

Received 13 July 2009; revised 3 September 2009; accepted 10 September 2009

doi:10.1111/j.1365-2605.2009.01003.x

Summary

Sperm chromatin compaction in the sperm head is achieved when histones are replaced by protamines during spermatogenesis. Haploinsufficiency of the protamine 1 (*PRM1*) or *PRM2* gene causes infertility in mice. However, the published data remain inconclusive about a role of *PRM1/2* variants in male infertility and their association with semen parameters. By full sequence analysis, we assessed the frequency of sequence variations in *PRM1* and *PRM2* in three groups of Caucasian patients with idiopathic teratozoospermia and normal (n = 88) or reduced sperm concentration (n = 83) and in men with a high percentage of normal sperm morphology and normal concentrations (n = 77). Two rare (c.54G>A and c.102G>T) and one common SNP (c.230A>C) were identified in *PRM1*. In *PRM2*, some rare heterozygous mutations and the two common intronic SNPs 298G>C and 373C>A were detected. None of the *PRM1/2* variants was associated with teratozoospermia or individually with other semen parameters. However, significant linkage disequilibrium was detected between the common SNPs of *PRM1* and *PRM2* which formed haplotypes. Analysis of the pooled group (n = 248) revealed that homozygous carriers of the common haplotype ACC had a twofold higher sperm concentration and count than men lacking this haplotype, with sperm counts of heterozygotes for ACC being midway between the homozygotes. This markedly decreased sperm output might either be caused by spermatozoa lacking the ACC haplotype not being viable, or subject to negative selection. In addition, a significant deviation from Hardy–Weinberg-Equilibrium of these SNPs might indicate natural selection in favour of the ACC allele which leads to higher sperm output and therefore better fertility. In conclusion, for the first time we describe an association of a common haplotype formed by *PRM1* and *PRM2* with sperm output in a large group of men.

Introduction

During spermatogenesis and DNA condensation, histones are replaced by transition proteins in round spermatids. Subsequently, these are replaced by protamines in elongating spermatids (Steger *et al.*, 2000). Several studies demonstrated altered expression of protamines in infertile males, and an abnormal protamine 1 (PRM1)/protamine 2 (PRM2) protein ratio has been described in sperm of infertile patients. This implies that the relative amount of each protein is important for proper sperm differentiation [reviewed by Oliva (2006) and Carrell *et al.* (2008)].

Knockout (KO) of either protamine gene in mice results in male infertility, caused by a reduction of the total amount of protamine formation, DNA damage and reduced sperm function. Haploinsufficiency of *Prm*1 or *Prm*2 in heterozygous mice causes an alteration in sperm chromatin assembly and nuclear integrity. These mice still

© 2009 The Authors

produce sperm, but these sperm exhibit abnormal morphology, combined with reduced motility and are thus unable to fertilize an oocyte (Cho *et al.*, 2001).

PRM1 and *PRM2* are located adjacently on chromosome 16p13.2 in humans (Fig. 1a). As a result of the crucial role of protamines in the differentiation of spermatids, changes in the gene's sequences may result in male infertility. Recent studies investigated protamine gene sequence variations in humans with ambiguous results. The first study by Tanaka *et al.* (2003) described several single nucleotide polymorphisms (SNPs) in *PRM1* and *PRM2*, but only a rare mutation (*PRM2* p.Q50X) found once was suggested to cause the man's infertility as it induces translation termination. Two rare transversions in *PRM1* (−107G>C in the promoter region and 102G>T in exon 1 changing the amino acid, R34S) with a minor allele frequency (MAF) of around 1% were repeatedly reported to be associated with male infertility (Aoki *et al.*, 2006; Iguchi *et al.*, 2006; Ravel *et al.*, 2007). However, a larger study of 672 fertile and over 300 infertile men could not confirm this finding (Kichine *et al.*, 2008). These SNPs were inconsistently described in men with isolated teratozoospermia (phenotype similar to KO mice) and/or oligozoospermia. In addition, some rare mutations in *PRM1/2*, one common SNP in *PRM1* (230A>C), and two common intronic SNPs in *PRM2* (298G>C and 373C>A) have been found without an association with male infertility. All of these studies either investigated small numbers, did not characterize the men analysed clinically with respect to their semen parameters or studied only *PRM1*. Therefore, we sequenced *PRM1* and *PRM2* in groups of idiopathic infertile patients with distinct teratozoospermia and normal or reduced sperm concentration and in men with both normal sperm morphology and concentration.

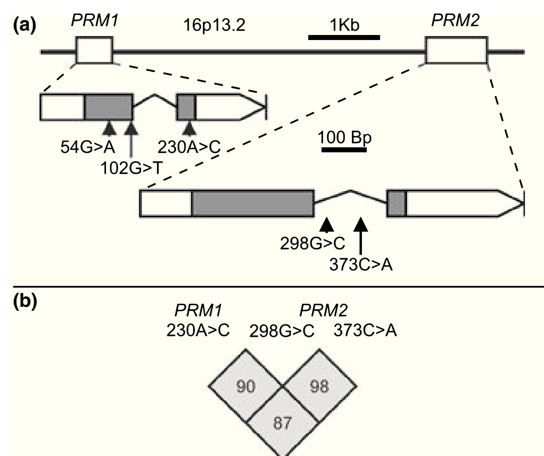


Figure 1 (a) Schematic representation of the *PRM1* and *PRM2* genes on chromosome 16p13.2 and respective transcriptional units (open bars) and open reading frame (shaded area) with the SNPs position marked. (b) Pairwise linkage disequilibrium (D′) of the three common SNPs.

Patients and methods

Study population

In a retrospective case–control study design, we selected Caucasian patients mostly of German origin (96%, according to self-report) with idiopathic infertility using the Androbase© database (Tüttelmann *et al.*, 2006). All men with azoospermia, known clinical (for example maldescended testes, varicocele and infections) and genetic (karyotype anomalies, Y-chromosomal deletions) causes of infertility were excluded. Of these men ($n = 944$), the 25th and 75th percentiles of percentage of morphologically normal sperm were calculated, resulting in 7 and 19% respectively. These were used as cut-off values to select similarly sized groups of men with normal sperm concentration [$\geq 20 \times 10^6$/mL according to WHO (World Health Organization, 1999)] and low percentage of normal morphology (≤7%, group 1, $n = 88$), resembling the phenotype of the KO mice with teratozoospermia and the group of patients first analysed by Iguchi *et al.* (2006); reduced sperm concentration ($< 20 \times 10^6$/mL) and ≤7% normal sperm morphology (group 2, $n = 83$) as SNPs were also reported to be associated with oligozoospermia (Ravel *et al.*, 2007) and men with normal sperm concentrations ($\geq 20 \times 10^6$/mL) and high percentage of normal sperm (≥19%, group 3, $n = 77$) as controls. Ancestry of patients was not significantly different between the three study groups with 94, 92 and 98% of German origin respectively.

All participants gave written informed consent for evaluation of their clinical data and genetic analysis of their donated DNA samples according to a protocol approved by the Ethics Committee of the Medical Faculty in Münster and State Medical Board.

Clinical parameters

All participants underwent a complete physical examination including ultrasonographic analysis of the scrotal content using a high-frequency 7.5-MHz convex scanner. Testicular volume was calculated using the ellipsoid method and summed as bi-testicular volume.

Analysis of sperm concentration was performed with Neubauer-improved chambers and motility was assessed on a heated (37 °C) microscope stage. Slides for morphology assessment were stained with modified Papanicolaou method and examined with phase contrast optics.

© 2009 The Authors

All values were determined in accordance with the current WHO criteria (World Health Organization, 1999). Progressive motility comprises WHO categories a and b. Total sperm count was calculated by multiplying semen volume by sperm concentration. In our laboratory, semen analysis is under constant internal and external quality control (Cooper *et al.*, 2002).

Serum concentrations of LH (normal range 2–10 U/L) and FSH (1–7 U/L) were determined using immunofluorometric assays (Autodelfia, Perkin Elmer, Freiburg, Germany) and serum testosterone (>12 nM) by a commercial direct solid-phase enzyme immunoassay (DRG AURICA ELISA Testosterone Kit; DRG Instruments, Marburg, Germany).

Genetic analyses

Genomic DNA was isolated from peripheral blood samples using FlexiGene DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Two 24-nucleotide PCR primer pairs were used to amplify the genetic region encompassing each of the protamine genes. *PRM1* was amplified using a 5′-end primer (5′-cccctggcatctataacaggccgc-3′) and the 3′-end primer (5′-tcaagaacaaggagagaagagtgg-3′), producing fragments of 557 nucleotides. For amplification of *PRM2*, 5′-end primer (5′-ctccagggcccactgcagcctcag-3′) and 3′-end primer (5′-gaattgctatggcctcacttggtg-3′) were used, producing fragments of 599 nucleotides.

PCR reactions were performed in 25 μL volumes which consisted of 1× PCR buffer including 1.5 mM $MgCl_2$, 200 μM of dNTPs, 0.4 μM of each primer, 0.1 U of Taq polymerase (Qiagen) and 100 ng of genomic DNA. PCR was performed using XP cycler (Bioer Technology, Hangzhou, China) with the following conditions: one cycle of initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 50 sec, 68 °C (*PRM1*) or 74 °C (*PRM2*) for 50 sec, 72 °C for 90 sec followed by one cycle of final extension at 72 °C for 10 min. The presence of products was checked by electrophoresis on a 1.5% agarose gel. The PCR-amplified fragments were subsequently purified using the QIA quick PCR Purification Kit (Qiagen), sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analysed by capillary electrophoresis with 3130xl Genetic Analyzer (Applied Biosystems).

Statistical analysis

Differences in clinical data of patients and controls were tested by either ANOVA when values were normally distributed, or the non-parametric Kruskal–Wallis test when the distribution normality test failed. *p*-values (two-sided) of <0.05 were considered statistically significant. All calculations were performed with GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA).

All genetic analyses were carried out with Stata/SE (StataCorp LP, version 9.1, College Station, TX, USA) using specific genetic subroutines (http://www.biostat-resources.com/stata/): HWSNP (Hardy–Weinberg equilibrium test for multiple SNPs) was used to test for departure from the Hardy–Weinberg equilibrium (HWE), GENCC (allele analysis for case–control genetic studies) to test differences in allele frequency comparing cases and controls, and QTLSNP (Quantitative trait loci SNP) to compare equality of means of clinical data across genotypes. This routine assumes a codominant genetic model to test for both additive and multiplicative effects, but dominant or recessive models can also be used. Haplotype analysis and linkage disequilibrium (LD) determination were performed with HaploView 4.1 (Barrett *et al.*, 2005). PHASE software, version 2.1 was used to estimate individual haplotypes (Stephens *et al.*, 2001; Stephens & Scheet, 2005).

Results

Clinical data

The anthropometric, hormone and semen parameters of the three study groups with low percentage of normal morphology and normal or reduced sperm concentration (group 1 and 2 respectively) and with high percentage of normal sperm and normal sperm concentration (group 3) are presented in Table 1. Testicular volume was lower and FSH higher in group 2 with reduced sperm count compared with both other groups with normal sperm concentration. All other anthropometric and hormone parameters were similar in all three groups. Duration of abstinence and semen volume were comparable between all groups. Sperm concentration and count as well as percentage of sperm with normal morphology were significantly different by selection. Percentage of progressively motile sperm followed the same direction as sperm morphology and was the lowest in group 2 with reduced sperm concentration and low normal morphology.

Genetic analyses

Allele frequencies and genotype distributions of the five SNPs in *PRM1* and *PRM2* are shown in Table 2. Apart from the three previously described SNPs 54G>A (rs35262993, exon 1, synonymous), 102G>T (rs35576928, exon 1, non-synonymous R34S), 230A>C (rs737008, exon 2, synonymous), no mutations were found in *PRM1*. The 54G>A and 102G>T SNPs were rare with an MAF of

© 2009 The Authors

Table 1 Comparison of anthropometric data, hormone and semen parameters between the three study groups presented as median and range (min–max)

	Group 1 (normal concentration, ≤7% normal forms) (*n* = 88)	Group 2 (reduced concentration, ≤7% normal forms) (*n* = 83)	Group 3 (normal concentration, ≥19% normal forms) (*n* = 77)	*p* (1 vs. 2, 1 vs. 3, 2 vs. 3)
Age [y]	35 (24–48)	33 (18–50)	34 (24–54)	NS
Height [cm]	182 (163–199)	182 (162–204)	180 (162–202)	NS
Weight [kg]	85 (62–127)	85 (60–160)	85 (60–138)	NS
BMI [kg/m^2]	25.8 (20.0–36.6)	25.8 (20.1–44.8)	25.6 (19.8–34.7)	NS
Testicular volume [ml]	54 (23–96)	45 (17–92)	51 (29–95)	<0.001, NS, <0.05
LH [U/L]	3.1 (1.0–7.7)	3.1 (0.9–9.6)	3.1 (0.8–8.6)	NS
FSH [U/L]	3.6 (1.3–15.2)	4.5 (1.2–23.0)	3.1 (0.4–10.8)	<0.05, NS, <0.001
Testosterone [nmol/L]	14.8 (7.2–29.4)	15.2 (6.4–31.3)	15.3 (7.0–30.3)	NS
Abstinence [d]	4 (2–7)	4 (2–7)	4 (2–7)	NS
Concentration [10^6/mL]	40.0 (20.0–177.0)	4.7 (0.2–19.5)	66.5 (20.0–228.0)	<0.001, <0.01, <0.001
Semen volume [mL]	3.5 (1.6–8.8)	3.6 (1.5–8.2)	3.8 (1.7–9.5)	NS
Total sperm count [10^6]	141 (18–677)	16 (1–111)	240 (46–1140)	<0.001, <0.01, <0.001
Normal morphology [%]	6 (1–7)	5 (1–7)	24 (19–44)	NS, <0.01, <0.001
a+b Motility [%]	48 (12–62)	36 (8–59)	52 (33–92)	<0.001, <0.05, <0.001

about 1% in all study groups. By contrast, the SNP 230A>C had an MAF of 30.1, 33.7 and 28.6% in the three study groups, respectively, which was not significantly different.

In *PRM2*, some rare heterozygous mutations were detected in one or two patients each. The two synonymous SNPs 66T>C (rs n/a) and 201C>T (rs n/a) are located in exon 1 of *PRM2*; 201C>T was found twice in group 1 and 66 T>C twice in group 2. Two intronic SNPs 281C>T (rs74007626) and 290C>T (rs74007625) were both found in one man with normal sperm concentration and morphology (controls, group 3). These four SNPs were also described by Aoki *et al.* (2006). In addition, two new mutations 300A>G (group 3) and 377C>T (group 2) in intron 1 of *PRM2* were each found once. The common SNPs 298G>C (rs1646022) and 373C>A (rs2070923) of *PRM2* were found with comparable MAFs of 46.4, 40.1, 50.7 and 30.7, 36.8, 30.1% respectively. [Correction added after online publication: 9 December 2009; amendment in the second SNP number from rs270923 to rs2070923].

All SNPs were in HWE in the respective groups except for the *PRM1* 230A>C and *PRM2* 373C>A in group 2. A similar deviation for *PRM2* 373C>A can be calculated from the data reported by Aoki *et al.* (2006) in their group of protamine deficient patients. The allele frequencies of the common SNPs of *PRM1/2* were similar to those reported in the public databases and in the literature. Neither comparison of allele frequencies, genotype distributions nor dominant or recessive models revealed significant differences between the three study groups.

Pairwise D' between the *PRM1* 230A>C and 298G>C/373C>A in *PRM2* was strong (Fig. 1b). Therefore, haplotypes could be constructed reliably for these three common SNPs spanning both protamine genes. The lowest probability for deriving the correct haplotype was 0.97 (PHASE software). The phased haplotypes are listed in Table 3 and their frequencies are compared among the three study groups without finding any significant differences.

Next, we tested the influence of the genotype on semen parameters in the pooled cohort of 248 men (Table 4). The distributions of *PRM1* 230A>C and *PRM2* 298G>C/373C>A deviated significantly from HWE when all men were considered together, while *PRM1* 54G>A and 102G>T did not (Table 2). In addition, the former common three SNPs showed a trend for an association with sperm concentration without reaching statistical significance. Median sperm concentration/count for men with genotypes *PRM1* 230AA, *PRM2* 298CC and 373CC were higher than the heterozygous group which exceeded the homozygous group respectively. Sperm morphology and motility were not associated with any SNP. These results indicated that the common haplotype ACC formed by the three SNPs is associated with higher sperm concentrations/counts. We therefore compared men homozygous for ACC (ACC/ACC, *n* = 57) with heterozygotes for ACC and any other haplotype (ACC/N, *n* = 105) and homozygotes for other haplotypes (N/N, *n* = 86). This analysis revealed significant differences ($p < 0.05$) for sperm concentration between carriers of ACC/ACC vs. ACC/N and N/N respectively, and for sperm counts between carriers of ACC/ACC and N/N (Fig. 2a,b). In addition, *p* for linear trend was calculated showing a highly significant association of the ACC-haplotype with sperm concentration ($p = 0.007$) and count ($p = 0.004$). These results were robust after permutation analysis: After randomly permuting the genotypes 10 000 times, *p*-values of 0.005 and 0.004

© 2009 The Authors

Table 2 Allele frequencies and genotype distributions of the three SNPs in *PRM1* and two SNPs in *PRM2* in the three study groups and in the pooled cohort of all men analysed

	Group 1 (normal conc., ≤7% normal forms) (*n* = 88)	Group 2 (reduced conc., ≤7% normal forms) (*n* = 83)	Group 3 (normal conc., ≥19% normal forms) (*n* = 77)	All men (*n* = 248)	*p*
PRM1 54G>A					
HWE test[a] (*p*)	NS	NS	NS	NS	
G frequency	98.9% (174)	98.8% (164)	98.7% (152)	98.8% (490)	
A frequency	1.1% (2)	1.2% (2)	1.3% (2)	1.2% (6)	NS
GG	97.7% (86)	97.6% (81)	97.4% (75)	97.6% (242)	NS
GA	2.3% (2)	2.4% (2)	2.6% (2)	2.4% (6)	NS
PRM1 102G>T					
HWE test[a] (*p*)	NS	NS	NS	NS	
G frequency	98.9% (174)	98.8% (164)	98.7% (152)	98.8% (490)	
T frequency	1.1% (2)	1.2% (2)	1.3% (2)	1.2% (6)	NS
GG	97.7% (86)	97.6% (81)	97.4% (75)	97.6% (242)	NS
GT	2.3% (2)	2.4% (2)	2.6% (2)	2.4% (6)	NS
PRM1 230A>C					
HWE test[a] (*p*)	NS	**0.028**	NS	**0.036**	
A frequency	69.9% (123)	66.3% (110)	71.4% (110)	69.2% (343)	
C frequency	30.1% (53)	33.7% (56)	28.6% (44)	30.8% (153)	NS
AA	50.0% (44)	49.4% (41)	53.2% (41)	50.8% (126)	NS
AC	39.8% (35)	33.7% (28)	36.4% (28)	36.7% (91)	NS
CC	10.2% (9)	16.9% (14)	10.4% (8)	12.5% (31)	NS
PRM2 298G>C					
HWE test[a] (*p*)	NS	NS	NS	**0.006**	
C frequency	53.6% (89)	59.9% (91)	49.3% (72)	54.3% (252)	
G frequency	46.4% (77)	40.1% (61)	50.7% (74)	45.7% (212)	NS
GG	30.7% (27)	39.5% (30)	30.1% (22)	34.1% (79)	NS
CG	39.8% (35)	40.8% (31)	38.4% (28)	40.5% (94)	NS
CC	23.9% (21)	19.7% (15)	31.5% (23)	25.4% (59)	NS
PRM2 373C>A					
HWE test[a] (*p*)	NS	**0.001**	NS	**0.003**	
C frequency	69.3% (115)	63.2% (96)	69.9% (102)	67.5% (313)	
A frequency	30.7% (51)	36.8% (56)	30.1% (44)	32.5% (151)	NS
CC	46.6% (41)	48.7% (37)	52.0% (38)	50.0% (116)	NS
AC	37.5% (33)	28.9% (22)	35.6% (26)	34.9% (81)	NS
AA	10.2% (9)	22.4% (17)	12.3% (9)	15.1% (35)	NS

No significant differences were found comparing allele and genotype frequencies between the study groups. *PRM1* 230A>C, *PRM2* 298G>C and 373C>A deviated significantly from HWE in the pooled cohort.
[a]Hardy–Weinberg-Equilibrium test.

Table 3 Frequency of haplotype formed by *PRM1* 230A>C, *PRM2* 298G>C and 373C>A and comparisons between the study groups

PRM1/2 haplotype	Group 1 (normal concentration, ≤7% normal forms) (*n* = 88)	Group 2 (reduced concentration, ≤7% normal forms) (*n* = 83)	Group 3 (normal concentration, ≥19% normal forms) (*n* = 77)	*P*
ACC	44.9% (79)	37.3% (62)	50.6% (78)	NS
CGA	28.4% (50)	30.1% (50)	27.3% (42)	NS
AGC	21.6% (38)	21.7% (36)	18.8% (29)	NS
AGA	3.4% (6)	6.6% (11)	1.9% (3)	NS
CGC	0.6% (1)	1.8% (3)	1.3% (2)	NS
CCC	1.1% (2)	1.8% (3)	0.0% ()	NS

No significant differences were found.

© 2009 The Authors
Journal compilation © 2010 European Academy of Andrology

Table 4 Semen parameters of all men (n = 248) according to the genotype of the three common SNPs in *PRM1* and *PRM2*

	n	Sperm concentration (10^6/mL)	Total sperm count (10^6)	Normal morphology (%)	a + b motility (%)
PRM1 230A>C					
AA	126	37.5	118	7	47
AC	91	26.5	100	6	47
CC	31	24.5	76	5	45
PRM2 298G>C					
GG	79	26.0	86	6	46
CG	94	27.7	109	7	47
CC	59	44.0	157	7	48
PRM2 373C>A					
CC	175	39.3	121	7	48
AC	81	26.4	100	6	48
AA	35	20.0	74	6	37

The differences are all statistically not significant, but note the trend for sperm concentration/count for all three SNPs with the genotypes.

were calculated. Median sperm concentrations for homozygotes of ACC, heterozygotes and homozygotes of *N* were 45.0, 28.0 and 24.2 × 10^6/mL respectively, and median total sperm counts were 159, 111 and 80 × 10^6. In addition, no influence of the haplotype on sperm morphology or motility was detected and testicular volume was not different between the groups (Fig. 2c,d,e). The distribution of the haplotype ACC also deviated significantly from HWE (p = 0.029).

Discussion

Proper remodelling leading to compaction of chromatin during the elongation of round spermatids requires correct interplay between the nuclear proteins where protamines replace histones. Recent studies investigated the role of *PRM1/2* sequence variants in male infertility after it was shown that male KO mice with *PRM* haploinsufficiency are infertile (Cho *et al.*, 2001). The clinical observation that infertile men may exhibit an abnormal PRM1/2 ratio additionally provokes an interest in the physiology of PRM synthesis (Steger *et al.*, 2008). Mutations and polymorphisms of the protamine genes might be involved in such abnormal production of the PRM proteins and thereby be related to male infertility, as well as in disorders of condensation and thus stability of sperm DNA.

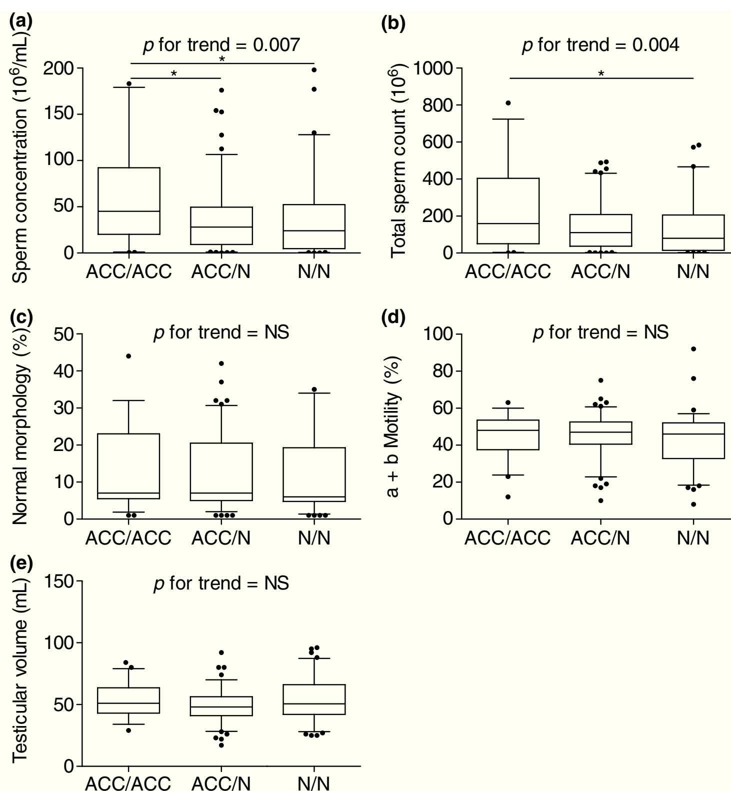


Figure 2 Boxplots (covering the 25–75th, whiskers the 5–95th percentile, median marked) comparing sperm concentration (a), total sperm count (b), percentage of normal morphology (c), percentage of a+b motility (d) and testicular volume (e) in study subjects grouped according to their combined *PRM1/PRM2* haplotype as ACC/ACC (n = 57), ACC/N(=other) (n = 105) and N/N (n = 86). Significant differences are marked with * (p < 0.05).

© 2009 The Authors

The limitations of the previous *PRM1/2* sequence studies were the limited number of patients, lack of detailed clinical characterization and analysis of only *PRM1*. We therefore analysed *PRM1* and *PRM2* in three groups of well-characterized men with either low percentage of normal sperm morphology and normal or reduced sperm concentration, or high normal morphology and normal sperm concentration. To avoid population stratification, all three study groups were selected from our patients in contrast to most other published studies where controls were selected e.g. from 'the general population', 'healthy men' or 'fathers'. Therefore, with respect to the endpoint of spermatogenesis evaluated in this study, population stratification is unlikely to interfere with the reported results.

In *PRM1*, we found the three previously described SNPs with frequencies comparable with the published reports (Tanaka *et al.*, 2003; Aoki *et al.*, 2006). None of these SNPs showed a significantly different distribution between our study groups. Concerning the rare 102G>T transversion changing the amino acid sequence, our findings agree with a recent large case–control study (Kichine *et al.*, 2008). Our observation renders an association of this SNP and male infertility or teratozoospermia, as proposed by the two earlier studies (Iguchi *et al.*, 2006; Ravel *et al.*, 2007), very unlikely. Nor was the other rare variant 54G>A of *PRM1* found to be associated with semen parameters, similar to earlier reports, and further supporting its role as an uncommon neutral variant. Both the previous and newly described rare variants in *PRM2* can most likely be considered neutral mutations, too, as none of these clustered in any study group. However, the 66T>C and 201C>T variants were only found in patients with either reduced normal sperm morphology and/or concentration as in the report by Aoki *et al.* (2006). Thus, the possibility that these are involved in male infertility cannot completely be excluded.

The three common SNPs of *PRM1* 230A>C and *PRM2* 298G>C/373C>A were neither distributed differently between the study groups nor associated with semen parameters individually. However, high LD was detected between these SNPs, which is not surprising as the two *PRM* genes are located in close proximity. The common haplotype ACC, which can be considered the wild type with an overall frequency of 44%, was significantly associated with sperm output. Neither an association of these individual SNPs nor their combined haplotype with semen parameters has been studied before. Homozygous carriers exhibited an about twofold higher sperm concentration and total sperm count than men not carrying this haplotype at all. Heterozygous carriers were found with 50% reduced sperm counts compared with the wild type. By general consent, higher sperm counts are an indicator of better fertility (Bonde *et al.*, 1998). No association of this haplotype with testicular volume was found, suggesting that the length of seminiferous tubules and therefore spermatogenic capacity were comparable between men with or without the ACC haplotype. Consequently, the ACC haplotype supposedly transfers an effect late during spermatogenesis or maturation of spermatozoa in the epididymis. Two explanations of the severely reduced sperm output in men without the ACC haplotype are possible: either sperm without the ACC haplotype are not viable or these are negatively selected against.

The other haplotypes of *PRM1/2* may lead to altered expression of protamines – probably involving an abnormal PRM1/2 ratio – which has clearly been shown to be associated with infertility (Oliva, 2006; Ravel *et al.*, 2007; Steger *et al.*, 2008). In addition, Aoki *et al.* (2005) have shown that sperm concentration, motility and morphology are significantly reduced in patients with an aberrant protamine ratio. As we did not measure the protamine contents of the ejaculate, we could not test this hypothesis.

As proposed by Carrell *et al.* (2007), protamines may act as a checkpoint for spermatogenesis, where abnormal PRM expression induces an apoptotic process which could explain the reduction of sperm output. The sequential process of histone replacement facilitates the remodelling of the male genome within the differentiating spermatid nucleus (Sassone-Corsi, 2002) and the regulation of protamine exchange might also be linked to a broader control of spermatogenesis. Further support for a role of the *PRM1/2* SNPs and their haplotype can be deduced from their frequency distribution. Natural selection is expected to enrich an allele in the general population and its frequency distribution will deviate from HWE. The common *PRM* SNPs and their haplotype ACC deviate from HWE, which might indicate natural selection involving this haplotype associated with higher sperm output, and therefore supposedly higher fertility.

The *PRM1/2*, and transition protein 2 (*TNP2*) genes are located in a compact gene cluster in mouse, rat and human (Oliva, 2006). According to the data from the HapMap project (http://www.hapmap.org), this cluster forms a large haplogroup for which the herein described SNPs in *PRM1/2* and their haplotype might be indicative. The transition proteins first replace histones and are then replaced by protamines during chromatin compaction. Therefore, variants of *TNP2* might well be involved in disturbed spermatogenesis, probably during an earlier step than protamines. Up-to-date, sequence variations of *TNP2* have not been as extensively studied as those of PRM1/2 and the results remain inconclusive (Miyagawa *et al.*, 2005; Tüttelmann *et al.*, 2007). Recently, an SNP in the promoter region of *PRM1* (−190C>A, rs2301365) was

© 2009 The Authors

found more frequently in infertile patients than in the controls with an MAF 33 and 18% respectively (Gazquez *et al.*, 2008). Unfortunately, sperm counts were not reported for patients and are not known for controls in that study. But as the patients were undergoing assisted reproduction, overall lower sperm counts than in the controls can be assumed. As the *PRM1* −190C>A SNP also lies within the haplogroup described above, the study by Gazquez *et al.* (2008) further supports the influence of this region on male fertility.

The protamine gene cluster contains a fourth gene, protamine 3 (*PRM3*), located between the *PRM2* and *TNP2* genes. The *PRM3* gene has been poorly studied, and its function remains unclear. Oliva (2006) notes it could well be a pseudogene because of its debated expression in humans and should not be called protamine at all because the predicted amino-acid sequence is not related to the other protamines. A recent study in mice demonstrated that *PRM3* is conserved in diverse mammals and that the PRM3 protein is located in the cytoplasm rather than in the nucleus. Prm3 KO mice are fertile but display reduced sperm motility (Grzmil *et al.*, 2008). As, according to HapMap, all these genes are inherited together, not only *PRM1/2* but also variations in *TNP2* or *PRM3* could be causative for the reduced sperm output found.

In conclusion, we describe for the first time an association of a common haplotype formed by *PRM1* and *PRM2* with sperm output in a large group of men. Although a link to chromatin packaging and the tightly regulated process of spermatogenesis is likely, the mechanisms underlying such quality control leading to reduced sperm numbers remain unclear.

Acknowledgements

The authors would like to thank the patients and physicians who took care of them at the Centre of Reproductive Medicine and Andrology and Susan Nieschlag, MA, for language editing of the manuscript. This study was supported by the Czech Ministry of Health IGA NR9448-3/2007.

Disclosure

The authors have nothing to disclose.

References

Aoki, V. W., Liu, L. & Carrell, D. T. (2005) Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. *Human Reproduction (Oxford, England)* 20, 1298–1306.

Aoki, V. W., Christensen, G. L., Atkins, J. F. & Carrell, D. T. (2006) Identification of novel polymorphisms in the nuclear protein genes and their relationship with human sperm protamine deficiency and severe male infertility. *Fertility and Sterility* 86, 1416–1422.

Barrett, J. C., Fry, B., Maller, J. & Daly, M. J. (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics (Oxford, England)* 21, 263–265.

Bonde, J. P., Ernst, E., Jensen, T. K., Hjollund, N. H., Kolstad, H., Henriksen, T. B. *et al.* (1998) Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. *Lancet* 352, 1172–1177.

Carrell, D. T., Emery, B. R. & Hammoud, S. (2007) Altered protamine expression and diminished spermatogenesis: what is the link? *Human Reproduction Update* 13, 313–327.

Carrell, D. T., Emery, B. R. & Hammoud, S. (2008) The aetiology of sperm protamine abnormalities and their potential impact on the sperm epigenome. *International Journal of Andrology* 31, 537–545.

Cho, C., Willis, W. D., Goulding, E. H., Jung-Ha, H., Choi, Y. C., Hecht, N. B. *et al.* (2001) Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nature Genetics* 28, 82–86.

Cooper, T. G., Bjorndahl, L., Vreeburg, J. & Nieschlag, E. (2002) Semen analysis and external quality control schemes for semen analysis need global standardization. *International Journal of Andrology* 25, 306–311.

Gazquez, C., Oriola, J., de Mateo, S., Vidal- Taboada, J. M., Ballesca, J. L. & Oliva, R. (2008) A common protamine 1 promoter polymorphism (-190 C->A) correlates with abnormal sperm morphology and increased protamine P1/P2 ratio in infertile patients. *Journal of Andrology* 29, 540–548.

Grzmil, P., Boinska, D., Kleene, K. C., Adham, I., Schluter, G., Kamper, M. *et al.* (2008) Prm3, the fourth gene in the mouse protamine gene cluster, encodes a conserved acidic protein that affects sperm motility. *Biology of Reproduction* 78, 958–967.

Iguchi, N., Yang, S., Lamb, D. J. & Hecht, N. B. (2006) An SNP in protamine 1: a possible genetic cause of male infertility? *Journal of Medical Genetics* 43, 382–384.

Kichine, E., Msaidie, S., Bokilo, A. D., Ducourneau, A., Navarro, A., Levy, N. *et al.* (2008) Low-frequency protamine 1 gene transversions c.102G->T and c.-107G->C do not correlate with male infertility. *Journal of Medical Genetics* 45, 255–256.

Miyagawa, Y., Nishimura, H., Tsujimura, A., Matsuoka, Y., Matsumiya, K., Okuyama, A. *et al.* (2005) Single-nucleotide polymorphisms and mutation analyses of the TNP1 and TNP2 genes of fertile and infertile human male populations. *Journal of Andrology* 26, 779–786.

Oliva, R. (2006) Protamines and male infertility. *Human Reproduction Update* 12, 417–435.

Ravel, C., Chantot-Bastaraud, S., El Houate, B., Berthaut, I., Verstraete, L., De Larouziere, V. *et al.* (2007) Mutations in the protamine 1 gene associated with male infertility. *Molecular Human Reproduction* 13, 461–464.

Sassone-Corsi, P. (2002) Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science* 296, 2176–2178.

Steger, K., Pauls, K., Klonisch, T., Franke, F. E. & Bergmann, M. (2000) Expression of protamine-1 and -2 mRNA during human spermiogenesis. *Molecular Human Reproduction* 6, 219–225.

Steger, K., Wilhelm, J., Konrad, L., Stalf, T., Greb, R., Diemer, T. *et al.* (2008) Both protamine-1 to protamine-2 mRNA ratio and Bcl2 mRNA content in testicular spermatids and ejaculated spermatozoa discriminate between fertile and infertile men. *Human Reproduction* 23, 11–16.

© 2009 The Authors
Journal compilation © 2010 European Academy of Andrology

Stephens, M. & Scheet, P. (2005) Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. *American Journal of Human Genetics* 76, 449–462.

Stephens, M., Smith, N. J. & Donnelly, P. (2001) A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics* 68, 978–989.

Tanaka, H., Miyagawa, Y., Tsujimura, A., Matsumiya, K., Okuyama, A. & Nishimune, Y. (2003) Single nucleotide polymorphisms in the protamine-1 and -2 genes of fertile and infertile human male populations. *Molecular Human Reproduction* 9, 69–73.

Tüttelmann, F., Luetjens, C. M. & Nieschlag, E. (2006) Optimising workflow in andrology: a new electronic patient record and database. *Asian Journal of Andrology* 8, 235–241.

Tüttelmann, F., Rajpert-De Meyts, E., Nieschlag, E. & Simoni, M. (2007) Gene polymorphisms and male infertility – a meta-analysis and literature review. *Reproductive Biomedicine Online* 15, 643–658.

World Health Organization. (1999) WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4th edn. Cambridge University Press, Cambridge.

© 2009 The Authors

4. ZÁVĚR

1/ Podrobným a rozsáhlým zhodnocením metody HRM a třech různých platforem umožňujících tuto metodu (LightScanner^{TM}, LightCycler 480 a RotorGene6000) byla jednoznačně potvrzena její optimální využitelnost v detekci mutací v rutinní DNA diagnostice. HRM představuje rychlou, citlivou a finančně nenáročnou metodu, jejíž všechny kroky mohou být provedeny v jedné zkumavce v jednom přístroji, což snižuje riziko kontaminace na minimum a výrazně šetří čas. Simultánní kombinace mutačního skenování a genotypizace častých polymorfismů ještě více snižuje potřebu sekvenační analýzy. I přes rychlé rozšiřování metody sekvenování nové generace (NGS), která je charakteristická svou náročností na vybavení, náklady na provoz a obtížným vyhodnocením získaných dat, si HRM zachovává v DNA diagnostice stále své významné místo. Poskytnutím metodických pokynů, návodů a validačních koncepcí jsme se pokusili usnadnit implementaci této technologie a umožnit její úspěšné využití i v dalších laboratořích.

2/ Díky integraci přesné klinické diagnostiky a komplexního molekulárně genetického přístupu (zahrnujícího i metodu HRM) v rámci Národního centra pro diagnostiku a léčbu cystické fibrózy ve Fakultní nemocnici v Praze Motole (Věstník Ministerstva zdravotnictví č. 4/2012 z 28. května 2012) se podařilo zmapovat téměř kompletní spektrum populačně specifických mutací u českých pacientů s tímto onemocněním (>99% populační záchytnost). To má význam pro a/ zkvalitnění poskytovaných genetických služeb u pacientů s CF, b/ další rozvoj novorozeneckého skríningu CF a c/ následnou aplikaci včasné a správné léčby/terapie, což příznivě ovlivní průběh a prognózu onemocnění a umožní snížit léčebné náklady. Současně jsme přispěli ke zvýšení kvality molekulárně genetické diagnostiky CF na západní Ukrajině, kde ekonomické a metodické limitace umožňovaly testovat pouze panel světově nejčastějších mutací, a to zásluhou odhalené mutace 2184insA, která se ukázala být po mutaci F508del druhou nejfrekventovanější mutací nalézanou u CF pacientů ukrajinské národnosti. Zvýšením populační záchytnosti CF mutací byly splněny podmínky pro zavedení novorozeneckého skríningu. Vzhledem k vysokému počtu Ukrajinců pracujících a žijících

na našem území jsou zjištěné výsledky přínosné i pro české laboratoře, kdy je při testování probandů ukrajinské národnosti nezbytné myslet na testování této mutace, která není součástí celorepublikově hojně rozšířeného diagnostického kitu firmy Elucigene.

3/ Mutační analýzou kandidátních genů *PRM1 a PRM2* pro mužskou infertilitu se potvrdilo, že mutace v těchto genech jsou vzácné a nejsou přímou příčinou poruch spermiogeneze. Podařilo se však nalézt statisticky signifikantní asociaci mezi haplotypem ACC, tvořeným třemi častými polymorfismy těchto genů, a koncentrací spermií a jejich celkovým počtem. Před praktickou implementací těchto výsledků do rutinní diagnostiky je nezbytné ověřit platnost nálezu na dalších souborech a populacích.

5. PŘEHLED PUBLIKACÍ, POSTERŮ A PŘEDNÁŠEK

5.1. Publikace

1. Křenková P, Piskáčková T, Holubová A, Balaščaková M, Krulišová V, Camajová J, Turnovec M, Libik M, Norambuena P, Stambergová A, Dvořáková L, Skalická V, Bartošová J, Kučerová T, Fila L, Zemková D, Vávrová V, Koudová M, Macek M, Krebsová A, Macek M Jr. Distribution of CFTR mutations in the Czech population: Positive impact of integrated clinical and laboratory expertise, detection of novel/de novo alleles and relevance for related/derived populations. J Cyst Fibros. 2012 Dec 28. doi:pii: S1569-1993(12)00235-4. 10.1016/j.jcf.2012.12.002. [Epub ahead of print]. (IF 3.190)

2. Krulišová V, Balaščaková M, Skalická V, Piskáčková T, Holubová A, Paděrová J, Křenková P, Dvořáková L, Zemková D, Kračmar P, Chovancová B, Vávrová V, Stambergová A, Votava F, Macek M Jr. Prospective and parallel assessments of cystic fibrosis newborn screening protocols in the Czech Republic: IRT/DNA/IRT versus IRT/PAP and IRT/PAP/DNA. Eur J Pediatr. 2012;171(8):1223-9. (IF 1.879)

3. Norambuena PA, Diblík J, Křenková P, Paulasová P, Macek M Sr, .Macek M Jr. ADP-ribosyltransferase 3 (ART3) variant is associated with reduced sperm counts in Czech males: case/control association study replicating results from a Japanese population. Neuro Endocrinol Lett. 2012;33(1):48-52. (IF 1.296)

4. Mattocks CJ, Morris MA, Matthijs G, Swinnen E, Corveleyn A, Dequeker E, Müller CR, Pratt V, Wallace A; EuroGentest Validation Group (Mike Zoccoli, Jana Camajova, Petra Krenkova, Patricia Norambuena, Alexandra Stambergova, Milan Macek, Isabelle Moix, Patrick M. Bossuyt, Els Voorhoeve, Bert Bakker, Sarah Berwouts, Tom Janssens, Ivo Salden, Trudi McDevitt, David Barton, Jean Amos-Wilson, Ian Mann , Hans Scheffer). Eur J Hum Genet. 2010;18(12):1276-88. (IF 4.400), citováno: 10x

5. Makukh H, Krenková P, Tyrkus M, Bober L, Hancárová M, Hnateyko O, Macek M Jr. A high frequency of the Cystic Fibrosis 2184insA mutation in Western Ukraine: genotype-phenotype correlations, relevance for newborn screening and genetic testing. J Cyst Fibros. 2010;9(5):371-5. (IF 3.190), citováno: 2x

6. Tüttelmann F, Křenková P, Römer S, Nestorovic AR, Ljujic M, Stambergová A, Macek Jr M, Macek Sr M, Nieschlag E, Gromoll J, Simoni M. A common haplotype of protamine 1 and 2 genes is associated with higher sperm counts. Int J Androl. 2010;33(1):e240-8. (IF 3.591), citováno: 4x

7. Norambuena PA, Copeland JA, Krenková P, Stambergová A, Macek M Jr. Diagnostic method validation: High resolution melting (HRM) of small amplicons genotypingfor the most common variants in the MTHFR gene. Clin Biochem. 2009 Aug;42(12):1308-16. (IF 2.076), citováno: 18x

8. Krenková P, Norambuena P, Stambergová A, Macek M Jr. Evaluation of high-resolution melting (HRM) for mutation scanning of selected exons of the CFTR gene. Folia Biol (Praha). 2009; 55(6):238-242. (IF 1.151), citováno: 3x

9. van der Stoep N, van Paridon CD, Janssens T, Krenkova P, Stambergova A, Macek M, Matthijs G, Bakker E. Diagnostic guidelines for high-resolution melting curve (HRM) analysis: an interlaboratory validation of BRCA1 mutation scanning using the 96-well LightScanner. Hum Mutat. 2009;30(6):899-909. (IF 5.686), citováno: 47x

10. Balaščaková M, Piskáčková T, Holubová A, Raušová E, Kazárová V, Krebsová A, Koudová M, Štambergová A, Čamajová J, Norambuena P, Křenková P, Votava F, Vávrová V, Macek M st., Macek M ml. Současné metodické postupy a přehled preimplantační, prenatální a postnatální DNA diagnostiky cystické fibrózy v České republice. Česko-slovenská Pediatrie. 2008; 63(2):62-75. (bez IF)

11. Macek M ml, Čamajová J, Křenková P, Norambuena P, Goetz P, Macek M st, Havlovicová M, Štambergová A. EuroGentest: evropský projekt zaměřený na harmonizaci a zvýšení úrovně genetických služeb. Česko-slovenská Pediatrie. 2007; 62(7-8):444-446. (bez IF)

5.2. Postery

1. Chrudimská Jana, Křenková Petra, Macek Milan jr., Macek Milan sr. "Distribution of the insLQ variant in luteinizing hormone receptor (LHCGR) in fertile Czech male and female controls". Ovarian Club II. Praha, listopad 2012.
2. J. Chrudimská, P. Křenková, M. Macek jr., M. Macek sr. "Distribuce polymorfismu insLQ v genu pro receptor luteinizačního hormonu (*LHCGR*) v české populaci". 11. česko-slovenská konference reprodukční gynekologie a 22. sympozium asistované reprodukce. Brno, listopad 2012.
3. P. Norambuena, P. Krenkova, F. Tuettelmann, S. Kliesch, P. Paulasova, A. Stambergova, M. Macek Jr., M. Macek Sr. "ADP-ribosyltransferase variant (ART3) in Czech and German males with reduced sperm counts". European Society of Human Reproduction and Embryology (ESHRE). Istanbul, Turecko, červenec, 2012.
4. L. Dvořáková, J. Paděrová, D. Chudoba, D. Novotná, H. Kuželová, P. Křenková, J. Drábová, Z. Zmítková, M. Macek Jr, M. Macek Sr. "Possibility of MLPA prenatal detection of the most clinically important chromosomal abnormalities". European Society of Human Genetics Conference. Norimberk, Německo, červen 2012
5. Krenkova P., Tuettelmann F., Kliesch S., Paulasova P., Diblik J., Macek M. jr, Macek M. sr. "*PRM1* and *PRM2* gene polymorphisms in Czech and German men with idiopathic oligozoospermia". European Society of Human Genetics Conference. Norimberk, Německo, červen 2012.
6. Norambuena P, Krenkova P, Stambergova A, Macek M Jr., Macek M Sr. "PARP-3 polymorphism of an ADP-ribosyltransferase 3 (*ART3*) is associated with reduced sperm count in Czech dysfertile men". The 12th International Congress of Human Genetics (ICHG)and the American Society of Human Genetics 61st Annual Meeting (ASHG). Montreal, Kanada. říjen, 2011.
7. Krulisova V, Balascakova M, Skalicka V, Piskackova T, Holubova A, Stambergova A, Dvorakova L, Krenkova P, Zemkova D, Kracmar P, Vavrova V,

Macek M Jr., and Votava F. "The Comparison of Parallel IRT/DNA and IRT/PAP/DNA+ST Cystic Fibrosis Newborn Screening Protocols in the Czech Republic". The 7th ISNS European Neonatal Screening Regional Meeting. Ženeva, Švýcarsko, srpen 2011.

8. Křenková P., Tuettelmann F., Paulasová P., Diblík J., Macek M. jr, Macek M. sr. "*PRM1* and *PRM2* gene polymorphisms in Czech men with idiopathic oligozoospermia, normozoospermic men and men with proven fertility". European Society of Human Genetics Conference. Amsterodam, Nizozemí, květen 2011.

9. Křenková P., Tuettelmann F., Paulasová P., Diblík J., Macek M. jr, Macek M. sr. "*PRM1* and *PRM2* gene polymorphisms in Czech men with idiopathic oligozoospermia, normozoospermic men and men with proven fertility". Studentská vědecká konference 2.LF Univezity Karlovy. Praha, duben, 2011.

10. Norambuena PA, Krenková P, Stambergová A, Macek M Sr and Macek M Jr. “An ADP-ribosyltransferase 3 (*ART3*) polymorphism is associated with reduced sperm count in Czech men". Studentská vědecká konference 2.LF Univezity Karlovy. Praha, duben, 2011.

11. Petra Krenkova, Tereza Piskackova, Miroslava Balascakova, Andrea Holubova, Jana Camajova, Miroslava Hancarova, Lenka Dvorakova, Veronika Skalicka,, Alexandra Stambergova, Vera Vavrova, Milan Macek Jr. "Spectrum of *CFTR* mutations in the Czech Republic". 33rd European Cystic Fibrosis Conference. Valencie, Španělsko, červen 2010.

12. P. Krenkova, H. Makukh, M. Hancarova, L. Dvorakova, A. Stambergova, M. Macek Jr. "*CFTR* gene analysis in the Western-Ukrainian population: an unusually high frequency of the 2184insA mutation". 32nd European Cystic Fibrosis Conference. Brest, Francie, červen 2009.

13. P. Krenkova, F. Tüttelmann, J. Gromoll, P. Norambuena, I. Eliasova, M. Simoni, M. Macek jr., M. Macek sr. "Comparison of *PRM1* and *PRM2* genes polymorphisms in fertile Czech and normozoospermic German men". European Society of Human Genetics Conference. Vídeň, Rakousko, květen 2009.

14. F. Tüttelmann, P. Křenková, A. Štambergová, M. Macek jr., M.. Macek Sr, E. Nieschlag, J. Gromoll, M. Simoni. "Protamine 1 and 2 sequence variants in teratozoospermic men with normal vs. reduced sperm concentration and normozoospermic men". The 11th World Congress on Controversies in Obstetrics, Gynecology & Infertility (COGI). Paříž, Francie, listopad 2008.

15. F. Tüttelmann, P. Křenková, A. Štambergová, M. Macek jr., M.. Macek Sr, E. Nieschlag, J. Gromoll, M. Simoni. "Protamine 1 and 2 sequence variants in teratozoospermic men with normal vs. reduced sperm concentration and normozoospermic men". 5th European Congress of Andrology. Řím, Itálie, listopad 2008

16. P Křenková, F Tüttelmann, A Stambergova, M Macek jr., M. Macek Sr, E Nieschlag, J Gromoll, M Simoni. "Molekulárně genetická studie vztahu genů protamin 1 a protamin 2 k poruchám spermiogeneze". 7. česko-slovenská konference reprodukční gynekologie a 18. sympozium asistované reprodukce. Brno, listopad 2008.

17. Macek M Jr, Křenková P, Norambuena P and Štambergová A. "Evaluation of high-resolution melting (HRM) for mutation scanning of the *CFTR* gene". North American CF Conference (NACFC). Orlando, Florida, USA, říjen 2008.

18. Křenková P, Štambergová A, Norambuena P and Macek M Jr. "Mutation scanning of the *CFTR* gene by high resolution melting analysis (HRM)". 31th European Cystic Fibrosis Conference. Praha, červen 2008.

19. Norambuena P, Copeland J, Křenková P, Macek M Jr and Štambergová A. "Genotyping of MTHFR 677C>T and 1298 A>C Variants by High Resolution Melting of Small Amplicons: an Example of Method Validation". European Society of Human Genetics Conference. Barcelona, Španělsko, květen-červen 2008.

20. Balaščaková M, Křenková P, Norambuena P, Fialová M, Štambergová A, Macek M Jr. "Selekční výhoda nosičství mutace F508del ve vztahu k laktózové

intolerancí". Studentská vědecká konference 2.LF Univezity Karlovy. Praha, duben 2008.

5.3. Přednášky

1. Norambuena P, Křenková P, Štambergová A, Macek M Jr., Macek M Sr. "ADP-ribosyltransferase 3 (*ART3*) variant is associated with idiopathic oligospermia". 10. Česko-slovenská konference reprodukční gynekologie a 21. Sympózium asistované reprodukce. Brno, listopad 2011.

2. P Křenková, F Tüttelmann, S Römer, AR Nestorovic, M Ljujic, A Štambergová, M Macek Jr., E. Nieschlag, J Gromoll, M Simoni, M Macek Sr. "ACC haplotyp protaminových genů *PRM1* a *PRM2* je asociován s vyšším počtem spermií". 8. česko-slovenská konference reprodukční gynekologie a 19. sympozium asistované reprodukce. Brno, 2009.

3. Norambuena P and Krenkova P. "Our experiences with HRM method evaluation for gene scanning and small amplicon genotyping". Seminar - "Roche Your Partner in Discovery". Praha, červen 2008.

4. Norambuena P, Copeland J, Krenkova P, Nestorovic A, Stambergova A and Macek M Jr. "Genotyping of MTHFR 677 C>T and 1298 A>C polymorphisms by High Resolution Melting of Small Amplicons". 11. celostátní konference DNA diagnostiky. Praha, prosinec 2007.

5. Křenková P, Štambergová A, Norambuena P, Macek M jr. "Využití analýzy křivek tání s vysokým rozlišením (HRM) pro detekci mutací/SNP v *CFTR* a *BRCA 1* genu". Celostátní sjezd Společnosti lékařské genetiky ČLS a 40. výroční cytogenetické konference. Praha, září 2007.

6. Štambergová A, Křenková P, Norambuena P and Macek M Jr. "Zkušeností s analyzátorem křivek tání s vysokým rozlišením (HRMCA) pro detekci mutací/SNP genu CFTR a BRCA1". "HRM – High Resolution Melting" Workshop. Genetica. Praha, květen 2007.

7. Štambergová A, Křenková P, Norambuena P. "První zkušenosti s analyzátorem křivek tání s vysokým rozlišením (HRMCA) pro detekci mutací/SNP CFTR a BRCA 1 genu". Workshop „Detekce mutací“ pořádaný firmou KRD. Praha, únor 2007.

5.4. Ostatní

1. 1. místo za nejlepší abstrakt 19. sympozia asistované reprodukce, Brno, listopad 2009: P Křenková, F Tüttelmann, S Römer, AR Nestorovic, M Ljujic, A Štambergová, M Macek Jr., E. Nieschlag, J Gromoll, M Simoni, M Macek Sr. "ACC haplotyp protaminových genů *PRM1* a *PRM2* je asociován s vyšším počtem spermií".
2. Úspěšná účast v mezilaboratorní externí kontrole kvality 2012 – vyšetření nejčastějších aneuploidií chromozomů 13, 18, 21 X a Y metodou QF-PCR

6. SEZNAM ZKRATEK

ADP	adenosindifosfát
AMH	anti-müllerický hormon
AR	androgenní receptror
ATP	adenosintrifosfát
AZF	azoospermický faktor
bp	base pair, pár bazí
BRCA1	breast cancer 1, gen pro karcinom prsu a ovárií
CBAVD	kongenitální bilaterální absence (ageneze) vas deferens
CF	cystická fibróza
CFGAC	Cystic Fibrosis Genetic Analysis Consortium Database, databáze CFTR1
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator, transmembránový regulátor vodivosti
CFTR2	databáze Clinical and functional translation of CFTR
ČR	Česká republika
DGGE	Denaturing Gradient Gel Elektrophoresis, denaturační gradientová gelová elektroforéza
dHPLC	denaturing High Performance Liquid Chromatography, denaturační vysoce účinná kapalinová chromatografie
DNA	deoxyribonukleová kyselina
dsDNA	double-stranded DNA, dvouřetězcová DNA
ENaC	Epithelial Sodium Channel, epitelový sodíkový kanál
EU	Evropská Unie
FN	false negativity, falešná negativita
FP	false positivity, falešná pozitivita
FSH	folikulistimulační hormon
FSHR	receptor pro folikulistimulační hormon
GnRH	gonadotropin-releasing hormon, gonadotropiny uvolňující hormon
HA	Heteroduplex Analysis, analýza heteroduplexů
HRM	High Resolution Meltig, vysokorozlišovací analýza křivek tání
IRT	imunoreaktivní trypsinogen
kb	kilobáze

kDa	kilodalton
KO	knockout, cílené vyřazení z funkce
LH	luteinizační hormon
LHR	receptor pro luteinizační hormon
MLPA	Multiplex Ligation-dependent Probe Amplification, multiplexní amplifikace prob závislá na ligaci
mRNA	mediátorová ribonukleová kyselina
MSD	Membrane Spanning Domains, transmembránová doména
MTHFR	methylentetrahydrofolátreduktáza
NBD	Nucleotide Binding Domain, nukleotid vazebná doména
ORCC	Outwardly Rectified Chloride Channel, chloridový kanál
PCR	Polymerase Chain Reaction, polymerázová řetězová reakce
PKA	proteinkináza A
PKC	proteinkináza C
PRM	protamin
R	regulační doména
RT-PCR	Real-Time Polymerase Chain Reaction, kvantitativní PCR v reálném čase
SNPs	Single Nukleotide Polymorphisms, jednonukleotidové polymorfismy
SOX9	SRY- box 9
SRY	sex determining region on chromosome Y, oblasti určující pohlaví na chromozomu Y
SSCP	Single-Strand Conformation Polymorphism, metoda jednořetězcového konformačního polymorfismu
ssDNA	single-stranded DNA, jednořetězcová DNA
TGCE	Temperature Gradient Capillary Electrophoresis, kapilární elektroforéza s teplotním gradientem
Tm	melting temperature, teplota tání
TN	true negativity, správná negativita
TNP	tranziční protein
TP	true positivity, správná pozitivita
TTGE	Temporal Temperature Gradient Elektrophoresis, elektroforéza s teplotním gradientem
USA	United States of America, Spojené státy americké
v-LHβ	variantní β podjednotka luteinizačního hormonu
WHO	World Health Organisation, Světová zdravotnická organizace

7. SEZNAM CITOVANÉ LITERATURY

Achard, C., C. Courtillot, O. Lahuna, G. Meduri, J. C. Soufir, P. Liere, A. Bachelot, H. Benyounes, M. Schumacher, F. Kuttenn, P. Touraine & M. Misrahi (2009) Brief Report: Normal Spermatogenesis in a Man with Mutant Luteinizing Hormone. *New England Journal of Medicine,* 361**,** 1856-1863.

Ahda, Y., J. Gromoll, A. Wunsch, K. Asatiani, M. Zitzmann, E. Nieschlag & M. Simoni (2005) Follicle-stimulating hormone receptor gene haplotype distribution in normozoospermic and azoospermic men. *Journal of Andrology,* 26**,** 494-499.

Ammer, H., A. Henschen & C. H. Lee (1986) ISOLATION AND AMINO-ACID-SEQUENCE ANALYSIS OF HUMAN-SPERM PROTAMINE-P1 AND PROTAMINE-P2 - OCCURRENCE OF 2 FORMS OF PROTAMINE-P2. *Biological Chemistry Hoppe-Seyler,* 367**,** 515-522.

Aoki, V. W. & D. T. Carrell (2003) Human protamines and the developing spermatid: their structure, function, expression and relationship with male infertility. *Asian Journal of Andrology,* 5**,** 315-324.

Aoki, V. W., G. L. Christensen, J. F. Atkins & D. T. Carrell (2006a) Identification of novel polymorphisms in the nuclear protein genes and their relationship with human sperm protamine deficiency and severe male infertility. *Fertility and Sterility,* 86**,** 1416-1422.

Aoki, V. W., L. H. Liu & D. T. Carrell (2005) Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. *Human Reproduction,* 20**,** 1298-1306.

Aoki, V. W., L. H. Liu, K. P. Jones, H. H. Hatasaka, M. Gibson, C. M. Peterson & D. T. Carrell (2006b) Sperm protamine 1/protamine 2 ratios are related to in vitro fertilization pregnancy rates and predictive of fertilization ability. *Fertility and Sterility,* 86**,** 1408-1415.

Audrezet, M. P., A. Dabricot, C. Le Marechal & C. Ferec (2008) Validation of high-resolution DNA melting analysis for mutation scanning of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Journal of Molecular Diagnostics,* 10**,** 424-434.

Balascakova, M., A. Holubova, V. Skalicka, D. Zemkova, P. Kracmar, L. Gonsorcikova, J. Camajova, T. Piskackova, J. Lebl, P. Drevinek, V. Gregor, V. Vavrova, F. Votava & M. Macek (2009) Pilot newborn screening project for cystic fibrosis in the Czech Republic: Defining role of the delay in its symptomatic diagnosis and influence of ultrasound-based prenatal diagnosis on the incidence of the disease. *Journal of Cystic Fibrosis,* 8**,** 224-227.

Balaščaková, M., T. Piskáčková, A. Holubová, E. Raušová, V. Kazárová, A. Krebsová, M. Koudová, A. Štambergová, J. Čamajová, P. Norambuena, P. Křenková, F. Votava,

V. Skalická, V. Vávrová, M. Macek st. & M. Macek ml. (2008) Současné metodické postupy a přehled preimplantační, prenatální a postnatální DNA diagnostiky cystické fibrózy v České republice. *Česko-slovenská Pediatrie*, 63, 62-75.

Balhorn, R., S. Reed & N. Tanphaichitr (1988) ABERRANT PROTAMINE-1 PROTAMINE-2 RATIOS IN SPERM OF INFERTILE HUMAN MALES. *Experientia,* 44**,** 52-55.

Belokopytova, I. A., E. I. Kostyleva, A. N. Tomilin & V. I. Vorobev (1993) HUMAN MALE-INFERTILITY MAY BE DUE TO A DECREASE OF THE PROTAMINE-P2 CONTENT IN SPERM CHROMATIN. *Molecular Reproduction and Development,* 34**,** 53-57.

Bobadilla, J. L., M. Macek, J. P. Fine & P. M. Farrell (2002) Cystic fibrosis: A worldwide analysis of CFTR mutations - Correlation with incidence data and application to screening. *Human Mutation,* 19**,** 575-606.

Braun, R. E. (2001) Packaging paternal chromosomes with protamine. *Nature Genetics,* 28**,** 10-12.

Carrell, D. T., B. R. Emery & S. Hammoud (2007) Altered protamine expression and diminished spermatogenesis: what is the link? *Human Reproduction Update,* 13**,** 313-327.

Carrell, D. T., B. R. Emery & S. Hammoud (2008) The aetiology of sperm protamine abnormalities and their potential impact on the sperm epigenome. *International Journal of Andrology,* 31**,** 537-545.

Carrell, D. T. & L. H. Liu (2001) Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. *Journal of Andrology,* 22**,** 604-610.

Carson, M. R., S. M. Travis & M. J. Welsh (1995) THE 2 NUCLEOTIDE-BINDING DOMAINS OF CYSTIC-FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) HAVE DISTINCT FUNCTIONS IN CONTROLLING CHANNEL ACTIVITY. *Journal of Biological Chemistry,* 270**,** 1711-1717.

Chevaillier, P., N. Mauro, D. Feneux, P. Jouannet & G. David (1987) ANOMALOUS PROTEIN COMPLEMENT OF SPERM NUCLEI IN SOME INFERTILE MEN. *Lancet,* 2**,** 806-807.

Cho, C., H. Jung-Ha, W. D. Willis, E. H. Goulding, P. Stein, Z. Xu, R. M. Schultz, N. B. Hecht & E. M. Eddy (2003) Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. *Biology of Reproduction,* 69**,** 211-217.

Cho, C., W. D. Willis, E. H. Goulding, J. H. Haesook, Y. C. Choi, N. B. Hecht & E. M. Eddy (2001) Haploinsufficiency of protamine-1 or-2 causes infertility in mice. *Nature Genetics,* 28**,** 82-86.

Chou, L. S., E. Lyon & C. T. Wittwer (2005) A comparison of high-resolution melting analysis with denaturing high-performance liquid chromatography for mutation scanning - Cystic fibrosis transmembrane conductance regulator gene as a model. *American Journal of Clinical Pathology,* 124**,** 330-338.

Ciccone, N. A. & U. B. Kaiser (2009) The biology of gonadotroph regulation. *Current Opinion in Endocrinology Diabetes and Obesity,* 16**,** 321-327.

Dadoune, J. P. (1995) The nuclear status of human sperm cells. *Micron,* 26**,** 323-345.

De Kretser, D. M. & H. W. G. Baker (1999) Infertility in men: Recent advances and continuing controversies. *Journal of Clinical Endocrinology & Metabolism,* 84**,** 3443-3450.

de Yebra, L., J. L. Ballesca, J. A. Vanrell, L. Bassas & R. Oliva (1993) COMPLETE SELECTIVE ABSENCE OF PROTAMINE-P2 IN HUMANS. *Journal of Biological Chemistry,* 268**,** 10553-10557.

de Yebra, L., J. L. Ballesca, J. A. Vanrell, M. Corzett, R. Balhorn & R. Oliva (1998) Detection of P2 precursors in the sperm cells of infertile patients who have reduced protamine P2 levels. *Fertility and Sterility,* 69**,** 755-759.

Denning, C. R., S. C. Sommers & H. J. Quigley (1968) INFERTILITY IN MALE PATIENTS WITH CYSTIC FIBROSIS. *Pediatrics,* 41**,** 7-&.

Domenjoud, L., G. Nussbaum, I. M. Adham, G. Greeske & W. Engel (1990) GENOMIC SEQUENCES OF HUMAN PROTAMINES WHOSE GENES, PRM1 AND PRM2, ARE CLUSTERED. *Genomics,* 8**,** 127-133.

Du, J. W., K. Y. Xu, L. Y. Fang & X. L. Qi (2012) Association between mutations of the luteinizing hormone beta subunit and female infertility. *Molecular Medicine Reports,* 5**,** 473-476.

Dufresne, S. D., D. R. Belloni, W. A. Wells & G. J. Tsongalis (2006) BRCA1 and BRCA2 mutation screening using SmartCycler II high-resolution melt curve analysis. *Archives of Pathology & Laboratory Medicine,* 130**,** 185-187.

Emery, B. R. & D. T. Carrell (2006) The effect of epigenetic sperm abnormalities on early embryo-genesis. *Asian Journal of Andrology,* 8**,** 131-142.

Engel, W., S. Keime, H. Kremling, H. Hameister & G. Schluter (1992) THE GENES FOR PROTAMINE-1 AND PROTAMINE-2 (PRM1 AND PRM2) AND TRANSITION PROTEIN 2 (TNP2) ARE CLOSELY LINKED IN THE MAMMALIAN GENOME. *Cytogenetics and Cell Genetics,* 61**,** 158-159.

Er, T. K. & J. G. Chang (2012) High-resolution melting: Applications in genetic disorders. *Clinica Chimica Acta,* 414**,** 197-201.

Erali, M., K. V. Voelkerding & C. T. Wittwer (2008) High resolution melting applications for clinical laboratory medicine. *Experimental and Molecular Pathology,* 85**,** 50-58.

Erali, M. & C. T. Wittwer (2010) High resolution melting analysis for gene scanning. *Methods,* 50**,** 250-261.

Estivill, X., C. Bancells, C. Ramos, A. Piazza, A. Carbonara, G. Mastella, A. Bonizzato, G. Castaldi, E. Dalcamo, M. Ferrari, P. Gasparini, G. Guanti, G. B. Leoni, P. F. Pignatti, P. Ronchetto, M. Seia, F. Torricelli, M. Goossens, F. ChevalierPorst, D. Bozon, B. SimonBouy, D. Feldmann, J. Elion, J. C. Kaplan, C. Ferec, M. Claustres, C. Clavel, E. Puchelle, J. Lunardi, M. Mathieu, H. Scheffer, D. J. J. Halley, A. M. W. vandenOuweland, A. Tijmensen, T. Casals, F. J. Gimenez, L. Ramos, M. Beneyto, J. Benitez, A. Palacio, B. Tummler, I. Bauer, T. Meitinger, A. Claass, M. Lindner, E. Schroder, M. Stuhrmann, J. Cassiman, H. Cuppens, P. Cochaux, J. Poncin, L. Messian, V. S. Baranov, T. E. Ivaschenko, M. Bakay, J. Bal, M. Witt, M. Kanavakis, M. Tzetis, T. Antoniadi, J. Lavinha, P. Pacheco, A. Duarte, P. Loureiro, L. Kalaydjieva, D. Angelicheva, A. Jordanova, A. Savov, K. Eiklid, L. Holmberg, C. Schaedel, M. Ozguc, A. Gocmen, H. Erdern, S. LiechtiGallati, M. Nemeti, G. Fekete, T. Klaassen, M. Schwarz, M. Schwartz, M. Macek, A. Krebsova, V. Vavrova, B. Kerem, D. Aveliovich, V. Ferak, L. Kadasi, H. Kayserova, D. Glavac, M. RavnikGlavac, G. D. Efremov, N. CankiKlein & J. Kere (1997) Geographic distribution and regional origin of 272 cystic fibrosis in European populations. *Human Mutation,* 10**,** 135-154.

Ferlin, A., F. Raicu, V. Gatta, D. Zuccarello, G. Palka & C. Foresta (2007) Male infertility: role of genetic background. *Reproductive Biomedicine Online,* 14**,** 734-745.

Gadsby, D. C., P. Vergani & L. Csanady (2006) The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature,* 440**,** 477-483.

Gatewood, J. M., G. R. Cook, R. Balhorn, C. W. Schmid & E. M. Bradbury (1990) ISOLATION OF 4 CORE HISTONES FROM HUMAN SPERM CHROMATIN REPRESENTING A MINOR SUBSET OF SOMATIC HISTONES. *Journal of Biological Chemistry,* 265**,** 20662-20666.

Gazquez, C., J. Oriola, S. de Mateo, J. M. Vidal-Taboada, J. L. Ballesca & R. Oliva (2008) A common protamine 1 promoter polymorphism (-190 c -> A) correlates with abnormal sperm morphology and increased protamine P1/P2 ratio in infertile patients. *Journal of Andrology,* 29**,** 540-548.

Graham, R., M. Liew, C. Meadows, E. Lyon & C. T. Wittwer (2005) Distinguishing different DNA heterozygotes by high-resolution melting. *Clinical Chemistry,* 51**,** 1295-1298.

Girodon, E., M. des Georges & R. Medina (2008) Occurrence of CFTR de novo mutations is not so rare. *Journal of Cystic Fibrosis*, 7, Suppl 2, S6.

Grievink, H. & K. M. Stowell (2008) Identification of ryanodine receptor 1 single-nucleotide polymorphisms by high-resolution melting using the LightCycler 480 System. *Analytical Biochemistry,* 374, 396-404.

Gundry, C. N., S. F. Dobrowolski, Y. R. Martin, T. C. Robbins, L. M. Nay, N. Boyd, T. Coyne, M. D. Wall, C. T. Wittwer & D. H. F. Teng (2008) Base-pair neutral homozygotes can be discriminated by calibrated high-resolution melting of small amplicons. *Nucleic Acids Research,* 36, 3401-3408.

Gundry, C. N., J. G. Vandersteen, G. H. Reed, R. J. Pryor, J. Chen & C. T. Wittwer (2003) Amplicon melting analysis with labeled primers: A closed-tube method for differentiating homozygotes and heterozygotes. *Clinical Chemistry,* 49**,** 396-406.

Hammoud, S., B. R. Emery, V. W. Aoki & D. T. Carrell (2007) Identification of genetic variation in the 5 ' and 3 ' non-coding regions of the protamine genes in patients with protamine deregulation. *Archives of Andrology,* 53**,** 267-274.

Hantash, F. M., J. B. Redman, K. Starn, B. Anderson, A. Buller, M. J. McGinniss, F. Quan, M. Peng, W. M. Sun & C. M. Strom (2006) Novel and recurrent rearrangements in the CFTR gene: clinical and laboratory implications for cystic fibrosis screening (vol 95, pg 361, 2000). *Human Genetics,* 119**,** 352-352.

Harris, A. (1992) CYSTIC-FIBROSIS GENE. *British Medical Bulletin,* 48**,** 738-753.

Hemann, M. T., K. L. Rudolph, M. A. Strong, R. A. DePinho, L. Chin & C. W. Greider (2001) Telomere dysfunction triggers developmentally regulated germ cell apoptosis. *Molecular Biology of the Cell,* 12**,** 2023-2030.

Herrmann, M. G., J. D. Durtschi, L. K. Bromley, C. T. Wittwer & K. V. Voelkerding (2006) Amplicon DNA melting analysis for mutation scanning and genotyping: Cross-platform comparison of instruments and dyes. *Clinical Chemistry,* 52**,** 494-503.

Highsmith, W. E., Q. Jin, A. J. Nataraj, J. M. O'Connor, V. D. Burland, W. R. Baubonis, F. P. Curtis, N. Kusukawa & M. M. Garner (1999) Use of a DNA toolbox for the characterization of mutation scanning methods. I: Construction of the toolbox and evaluation of heteroduplex analysis. *Electrophoresis,* 20**,** 1186-1194.

Houštěk, J. & V. Vávrová (1962) K výskytu cystické fibrosy pankreatu v ČSSR. *Česko-slovenská Pediatrie*, 17, 445-451.

Hwang, T. C. & D. N. Sheppard (2009) Gating of the CFTR Cl- channel by ATP-driven nucleotide-binding domain dimerisation. *Journal of Physiology-London,* 587, 2151-2161.

Iguchi, N., S. Yang, D. J. Lamb & N. B. Hecht (2006) An SNP in protamine 1: a possible genetic cause of male infertility? *Journal of Medical Genetics,* 43, 382-384.

Imken, L., H. Rouba, B. El Houate, N. Louanjli, A. Barakat, A. Chafik & K. McElreavey (2009) Mutations in the protamine locus: association with spermatogenic failure? *Molecular Human Reproduction,* 15, 733-738.

Jager, S. (1990) SPERM NUCLEAR-STABILITY AND MALE-INFERTILITY. *Archives of Andrology,* 25, 253-259.

Jodar, M., J. Oriola, G. Mestre, J. Castillo, A. Giwercman, J. M. Vidal-Taboada, J. L. Ballesca & R. Oliva (2011) Polymorphisms, haplotypes and mutations in the protamine 1 and 2 genes. *International Journal of Andrology,* 34, 470-485.

Kerem, B. S., J. M. Rommens, J. A. Buchanan, D. Markiewicz, T. K. Cox, A. Chakravarti, M. Buchwald & L. C. Tsui (1989) IDENTIFICATION OF THE CYSTIC-FIBROSIS GENE - GENETIC-ANALYSIS. *Science,* 245, 1073-1080.

Khara, K. K., M. Vlad, M. Griffiths & C. R. Kennedy (1997) Human protamines and male infertility. *Journal of Assisted Reproduction and Genetics,* 14, 282-290.

Kichine, E., S. Msaidie, A. D. Bokilo, A. Ducourneau, A. Navarro, N. Levy, P. Terriou, P. Collignon, G. Boetsch, J. Chiaroni & M. J. Mitchell (2008) Low-frequency protamine 1 gene transversions c.102G -> T and c.-107G -> C do not correlate with male infertility. *Journal of Medical Genetics,* 45, 255-256.

Knauff, E. A. H., L. Franke, M. A. van Es, L. H. van den Berg, Y. T. van der Schouw, J. S. E. Laven, C. B. Lambalk, A. Hoek, A. J. Goverde, S. Christin-Maitre, A. J. Hsueh, C. Wijmenga, B. Fauser & P. O. F. C. Dutch (2009) Genome-wide association study in premature ovarian failure patients suggests ADAMTS19 as a possible candidate gene. *Human Reproduction,* 24, 2372-2378.

Kobayashi, H., A. Sato, E. Otsu, H. Hiura, C. Tomatsu, T. Utsunomiya, H. Sasaki, N. Yaegashi & T. Arima (2007) Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. *Human Molecular Genetics,* 16, 2542-2551.

Krulisova, V., M. Balascakova, V. Skalicka, T. Piskackova, A. Holubova, J. Paderova, P. Krenkova, L. Dvorakova, D. Zemkova, P. Kracmar, B. Chovancova, V. Vavrova, A. Stambergova, F. Votava & M. Macek (2012) Prospective and parallel assessments of cystic fibrosis newborn screening protocols in the Czech Republic: IRT/DNA/IRT versus IRT/PAP and IRT/PAP/DNA. *European Journal of Pediatrics,* 171, 1223-1229.

Kuhnert, B. & E. Nieschlag (2004) Reproductive functions of the ageing male. *Human Reproduction Update,* 10, 327-339.

Kurtz, K., F. Martinez-Soler, J. Ausio & M. Chiva (2007) Acetylation of histone H4 in complex structural transitions of spermiogenic chromatin. *Journal of Cellular Biochemistry,* 102, 1432-1441.

Latronico, A. C., J. Anasti, I. J. P. Arnhold, R. Rapaport, B. B. Mendonca, W. Bloise, M. Castro, C. Tsigos & G. P. Chrousos (1996) Testicular and ovarian resistance to

luteinizing hormone caused by inactivating mutations of the luteinizing hormone-receptor gene. *New England Journal of Medicine,* 334**,** 507-512.

Lerman, L. S. & K. Silverstein (1987) COMPUTATIONAL SIMULATION OF DNA MELTING AND ITS APPLICATION TO DENATURING GRADIENT GEL-ELECTROPHORESIS. *Methods in Enzymology,* 155**,** 482-501.

Li, Q. B., Z. W. Liu, H. Monroe & C. T. Culiat (2002a) Integrated platform for detection of DNA sequence variants using capillary array electrophoresis. *Electrophoresis,* 23**,** 1499-1511.

Li, T. C., M. Makris, M. Tomsu, E. Tuckerman & S. Laird (2002b) Recurrent miscarriage: aetiology, management and prognosis. *Human Reproduction Update,* 8**,** 463-481.

Liew, M., R. Pryor, R. Palais, C. Meadows, M. Erali, E. Lyon & C. Wittwer (2004) Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clinical Chemistry,* 50**,** 1156-1164.

Lofrano-Porto, A., G. B. Barra, L. A. Giacomini, P. P. Nascimento, A. C. Latronico, L. A. Casulari & F. D. D. Neves (2007) Luteinizing hormone beta mutation and hypogonadism in men and women. *New England Journal of Medicine,* 357**,** 897-904.

Lubamba, B., B. Dhooghe, S. Noel & T. Leal (2012) Cystic fibrosis: Insight into CFTR pathophysiology and pharmacotherapy. *Clinical Biochemistry,* 45**,** 1132-1144.

Lyczak, J. B., C. L. Cannon & G. B. Pier (2002) Lung infections associated with cystic fibrosis. *Clinical Microbiology Reviews,* 15**,** 194-+.

Macek, M. ml., J. Čamajová, P. Křenková, P. Norambuena,, P. Goetz, M. Macek st., M. Havlovicová & A. Štambergová (2007) EuroGentest: evropský projekt zaměřený na harmonizaci a zvýšení úrovně genetických služeb. *Česko-slovenská Pediatrie*, 62, 444-446.

Mafra, F. A., B. Bianco, D. M. Christofolini, A. M. B. Souza, K. Zulli & C. P. Barbosa (2010) Luteinizing hormone beta-subunit gene (LH beta) polymorphism in infertility and endometriosis-associated infertility. *European Journal of Obstetrics & Gynecology and Reproductive Biology,* 151**,** 66-69.

Margraf, R. L., R. Mao, W. E. Highsmith, L. M. Holtegaard & C. T. Wittwer (2006) Mutation scanning of the RET protooncogene using high-resolution melting analysis. *Clinical Chemistry,* 52**,** 138-141.

Martins, R. P., G. C. Ostermeier & S. A. Krawetz (2004) Nuclear matrix interactions at the human protamine domain - A working model of potentiation. *Journal of Biological Chemistry,* 279**,** 51862-51868.

Mattocks, C. J., M. A. Morris, G. Matthijs, E. Swinnen, A. Corveleyn, E. Dequeker, C. R. Muller, V. Pratt, A. Wallace & G. EuroGentest Validation (2010) A standardized framework for the validation and verification of clinical molecular genetic tests. *European Journal of Human Genetics,* 18**,** 1276-1288.

Matzuk, M. M. & D. J. Lamb (2008) The biology of infertility: research advances and clinical challenges. *Nature Medicine,* 14**,** 1197-1213.

Miescher, F. (1874) Das Protamin - Eine neue organische Basis aus den Samenf - den des Rheinlachses. *Berichte der Deutschen Chemischen Gesellschaft*, 7, 376.

Montgomery, J., C. T. Wittwer, J. O. Kent & L. M. Zhou (2007) Scanning the cystic fibrosis transmembrane conductance regulator gene using high-resolution DNA melting analysis. *Clinical Chemistry*, 53, 1891-1898.

Morral, N., J. Bertranpetit, X. Estivill, V. Nunes, T. Casals, J. Gimenez, A. Reis, R. Varonmateeva, M. Macek, L. Kalaydjieva, D. Angelicheva, R. Dancheva, G. Romeo, M. P. Russo, S. Garnerone, G. Restagno, M. Ferrari, C. Magnani, M. Claustres, M. Desgeorges, M. Schwartz, M. Schwarz, B. Dallapiccola, G. Novelli, C. Ferec, M. Dearce, M. Nemeti, T. Kere, M. Anvret, N. Dahl & L. Kadasi (1994) THE ORIGIN OF THE MAJOR CYSTIC-FIBROSIS MUTATION (DELTA-F508) IN EUROPEAN POPULATIONS. *Nature Genetics,* 7, 169-175.

Nagasaki, K., N. Katsumata, Y. Ogawa, T. Kikuchi & M. Uchiyama (2010) Novel C617Y mutation in the 7th transmembrane segment of luteinizing hormone/choriogonadotropin receptor in a Japanese boy with peripheral precocious puberty. *Endocrine Journal,* 57, 1055-1060.

Niel, F., J. Martin, F. Dastot-Le Moal, B. Costes, B. Boissier, V. Delattre, M. Goossens & E. Girodon (2004) Rapid detection of CFTR gene rearrangements impacts on genetic counselling in cystic fibrosis. *Journal of Medical Genetics,* 41.

Norambuena, P. A., J. A. Copeland, P. Krenkova, A. Stambergova & M. Macek (2009) Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene. *Clinical Biochemistry,* 42, 1308-1316.

Norambuena, P. A., J. Diblík, P. Křenková, P. Paulasová, M. Macek Sr & M..Macek M Jr (2012a) ADP-ribosyltransferase 3 (ART3) variant is associated with reduced sperm counts in Czech males: case/control association study replicating results from a Japanese population. *Neuroendocrinology Letters*, 33, 48-52.

Norambuena, P. A., P. Křenková, F. Tuettelmann, S. Kliesch, P. Paulasová, A. Štambergová, M. Macek Sr & M. Macek Jr (2012b) ADP-ribosyltransferase variant

(ART3) in Czech and German males with reduced sperm counts. *Human Reproduction*, 27, Suppl. 2, P018.

Nuti, F. & C. Krausz (2008) Gene polymorphisms/mutations relevant to abnormal spermatogenesis. *Reproductive Biomedicine Online,* 16, 504-513.

Oliva, R. (2006) Protamines and male infertility. *Human Reproduction Update,* 12, 417-435.

Oliva, R. & G. H. Dixon (1991) VERTEBRATE PROTAMINE GENES AND THE HISTONE-TO-PROTAMINE REPLACEMENT REACTION. *Progress in Nucleic Acid Research and Molecular Biology,* 40, 25-94.

Oppenheimer, E.H. & J. R. Esterly (1970) Observations on cystic fibrosis of the pancreas. VI. The uterine cervix. *The Journal of Pediatrics*, 77, 991-995.

Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi & T. Sekiya (1989) DETECTION OF POLYMORPHISMS OF HUMAN DNA BY GEL-ELECTROPHORESIS AS SINGLE-STRAND CONFORMATION POLYMORPHISMS. *Proceedings of the National Academy of Sciences of the United States of America,* 86, 2766-2770.

Piasecka, M. & J. Kawiak (2003) Sperm mitochondria of patients with normal sperm motility and with asthenozoospermia: morphological and functional study. *Folia Histochemica Et Cytobiologica,* 41, 125-139.

Pohunek, P. & J. Lebl (2008) Doc. Věra Vávrová a historie cystické fibrózy v České republice. *Česko-slovenská Pediatrie*, 63, 59-61.

Poongothai, J., T. S. Gopenath & S. Manonayaki (2009) Genetics of human male infertility. *Singapore Medical Journal,* 50, 336-347.

Qiao, J., B. Han, B. L. Liu, X. Chen, Y. Ru, K. X. Cheng, F. G. Chen, S. X. Zhao, J. Liang, Y. L. Lu, J. F. Tang, Y. X. Wu, W. L. Wu, J. L. Chen, M. D. Chen & H. D. Song (2009) A Splice Site Mutation Combined with a Novel Missense Mutation of LHCGR Cause Male Pseudohermaphroditism. *Human Mutation,* 30, E855-E865.

Raivio, T., I. Huhtaniemi, R. Anttila, M. A. Siimes, L. Hagenas, C. Nilsson, K. Pettersson & L. Dunkel (1996) The role of luteinizing hormone-beta gene polymorphism in the onset and progression of puberty in healthy boys. *Journal of Clinical Endocrinology & Metabolism,* 81, 3278-3282.

Ramanujam, L. N., W. X. Liao, A. C. Roy, A. Loganath, H. H. Goh & S. C. Ng (1999) Association of molecular variants of luteinizing hormone with menstrual disorders. *Clinical Endocrinology,* 51, 243-246.

Ravel, C., S. Chantot-Bastaraud, B. El Houate, I. Berthaut, L. Verstraete, V. De Larouziere, D. Lourenco, A. Dumaine, J. M. Antoine, J. Mandelbaum, J. P. Siffroi & K. McElreavey (2007) Mutations in the protamine 1 gene associated with male infertility. *Molecular Human Reproduction,* 13, 461-464.

Reed, G. H. & C. T. Wittwer (2004) Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clinical Chemistry,* 50, 1748-1754.

Riordan, J. R., J. M. Rommens, B. S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. L. Chou, M. L. Drumm, M. C. Iannuzzi, F. S. Collins & L. C. Tsui (1989) IDENTIFICATION OF THE CYSTIC-FIBROSIS GENE - CLONING AND CHARACTERIZATION OF COMPLEMENTARY-DNA. *Science,* 245, 1066-1072.

Rowe, S. M., S. Miller & E. J. Sorscher (2005) Mechanisms of disease: Cystic fibrosis. *New England Journal of Medicine,* 352**,** 1992-2001.

Sheppard, D. & J. Welsh (1999) Structure and Function of the CFTR Chloride Channel. *Physiological Reviews,* 79, 23-45.

Schlicker, M., V. Schnulle, L. Schneppel, V. I. Vorobev & W. Engel (1994) DISTURBANCES OF NUCLEAR CONDENSATION IN HUMAN SPERMATOZOA - SEARCH FOR MUTATIONS IN THE GENES FOR PROTAMINE-1, PROTAMINE-2 AND TRANSITION PROTEIN-1. *Human Reproduction*, 9, 2313-2317.

Simoni, M., J. Gromoll, W. Hoppner, A. Kamischke, T. Krafft, D. Stahle & E. Nieschlag (1999) Mutational analysis of the follicle-stimulating hormone (FSH) receptor in normal and infertile men: Identification and characterization of two discrete FSH receptor isoforms. *Journal of Clinical Endocrinology & Metabolism,* 84, 751-755.

Simoni, M., F. Tuttelmann, C. Michel, Y. Bockenfeld, E. Nieschlag & J. Gromoll (2008) Polymorphisms of the luteinizing hormone/chorionic gonadotropin receptor gene: association with maldescended testes and male infertility. *Pharmacogenetics and Genomics,* 18**,** 193-200.

Simpson, J.L. (2005) Hledání významu screeningu cystické fibrózy. *Gynekologie po promoci*, 1, 26-31.

Sims, E. J., A. Clark, J. McCormick, G. Mehta, G. Connett, A. Mehta & U. K. C. F. Database (2007) Cystic fibrosis diagnosed after 2 months of age leads to worse outcomes and requires more therapy. *Pediatrics,* 119, 19-28.

Steger, K., L. Fink, K. Failing, R. M. Bohle, S. Kliesch, W. Weidner & M. Bergmann (2003) Decreased protamine-1 transcript levels in testes from infertile men. *Molecular Human Reproduction,* 9, 331-336.

Takahashi, K., K. Karino, H. Kanasakia & K. Miyazakia (2004) Altered kinetics of pituitary response to gonadotropin-releasing hormone in women with variant luteinizing hormone: correlation with ovulatory disorders. *Hormone Research,* 61, 27-32.

Takahashi, K., T. Ozaki, M. Okada, H. Kurioka, H. Kanasaki & K. Miyazaki (1999) Increased prevalence of luteinizing hormone beta-subunit variant in patients with premature ovarian failure. *Fertility and Sterility,* 71, 96-101.

Tanaka, H., Y. Miyagawa, A. Tsujimura, K. Matsumiya, A. Okuyama & Y. Nishimune (2003) Single nucleotide polymorphisms in the protamine-1 and-2 genes of fertile and infertile human male populations. *Molecular Human Reproduction,* 9, 69-73.

Taulan, M., A. Girardet, C. Guittard, J. P. Altieri, C. Templin, C. Beroud, M. des Georges & M. Claustres (2007) Large genomic rearrangements in the CFTR gene contribute to CBAVD. *Bmc Medical Genetics,* 8.

Ulcova-Gallova, Z., V. Bouse, L. Svabek, J. Turek & Z. Rokyta (2002) Endometriosis in reproductive immunology. *American Journal of Reproductive Immunology,* 47**,** 269-274.

Valdes-Socin, H., R. Salvi, A. F. Daly, R. C. Gaillard, P. Quatresooz, P. Tebeu, F. P. Pralong & A. Beckers (2004) Brief report: Hypogonadism in a patient with a mutation in the luteinizing hormone beta-subunit gene. *New England Journal of Medicine,* 351**,** 2619-2625.

Valdes-Socin, H., R. Salvi, A. Thiry, A. F. Daly, F. P. Pralong, R. Gaillard & A. Beckers (2009) Testicular Effects of Isolated Luteinizing Hormone Deficiency and Reversal by Long-Term Human Chorionic Gonadotropin Treatment. *Journal of Clinical Endocrinology & Metabolism,* 94**,** 3-4.

Vávrová, V. (2006) Problémy v diagnostice cystické fibrózy – potřeba novorozeneckého screeningu. *Česko-slovenská Pediatrie*, 12,703-709.

Vávrová, V., D. Zemková, H. Slavíková (2001) Sweat chloride concentrations in Czech CF patiens, CF carriers and apparently healthy controls. *Journal of Cystic Fibrosis*. Abstracts of the 24th European Cystic Fibrosis Conference, P51.

Vávrová, V., D. Zemková, M. Macek, M. jr., P. Pohunek (2000) Cystická fibróza u dětí- ale nejen u nich. *Forum Medicinae,* 3, 49-55.

Venkatesh, S., R. Kumar, D. Deka, M. Deecaraman & R. Dada (2011) Analysis of sperm nuclear protein gene polymorphisms and DNA integrity in infertile men. *Systems Biology in Reproductive Medicine,* 57, 124-132.

Venter, J. C., M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, G. G. Sutton, H. O. Smith, M. Yandell, C. A. Evans, R. A. Holt, J. D. Gocayne, P. Amanatides, R. M. Ballew, D. H. Huson, J. R. Wortman, Q. Zhang, C. D. Kodira, X. Q. H. Zheng, L. Chen, M. Skupski, G. Subramanian, P. D. Thomas, J. H. Zhang, G. L. G. Miklos, C. Nelson, S. Broder, A. G. Clark, C. Nadeau, V. A. McKusick, N. Zinder, A. J. Levine, R. J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. M. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A. E. Gabrielian, W. Gan, W. M. Ge, F. C. Gong, Z. P. Gu, P. Guan, T. J. Heiman, M. E. Higgins, R. R. Ji, Z. X. Ke, K. A. Ketchum, Z. W. Lai, Y. D. Lei, Z. Y. Li, J. Y. Li, Y. Liang, X. Y. Lin, F. Lu, G. V. Merkulov, N. Milshina, H. M. Moore, A. K. Naik, V. A. Narayan, B. Neelam, D. Nusskern, D. B. Rusch, S. Salzberg, W. Shao, B. X. Shue, J. T. Sun, Z. Y. Wang, A. H. Wang, X. Wang, J. Wang, M. H. Wei, R. Wides, C. L. Xiao, C. H. Yan, et al. (2001) The sequence of the human genome. *Science,* 291, 1304-+.

Viguie, F., L. Domenjoud, M. F. Rousseaumerck, J. P. Dadoune & P. Chevaillier (1990) CHROMOSOMAL LOCALIZATION OF THE HUMAN PROTAMINE GENES, PRM1 AND PRM2, TO 16P13.3 BY INSITU HYBRIDIZATION. *Human Genetics,* 85**,** 171-174.

Vilfan, I. D., C. C. Conwell & N. V. Hud (2004) Formation of native-like mammalian sperm cell chromatin with folded bull protamine. *Journal of Biological Chemistry,* 279**,** 20088-20095.

Walker, I. D. (2000) Thrombophilia in pregnancy. *Journal of Clinical Pathology,* 53**,** 573-580.

Weiss, J., L. Axelrod, R. W. Whitcomb, P. E. Harris, W. F. Crowley & J. L. Jameson (1992) HYPOGONADISM CAUSED BY A SINGLE AMINO-ACID SUBSTITUTION IN THE BETA SUBUNIT OF LUTEINIZING-HORMONE. *New England Journal of Medicine,* 326**,** 179-183.

Welsh, M.J., B. W. Ramsey, F. Accurso & G. R. Cutting (2001) Cystic fibrosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Basis of Inherited Disease*. 8th ed. New York: McGraw-Hill Inc., Vol III, Chapter 201.

White, H., G. L. Potts & N. C. P. Cross (2006) NGRL (Wessex) evaluation of high resolution melt curve analysis for mutation scanning. *Journal of Medical Genetics,* 43, S85-S85.

Willmore-Payne, C., J. A. Holden, S. Tripp & L. J. Layfield (2005) Human malignant melanoma: detection of BRAF- and c-kit-activating mutations by high-resolution amplicon melting analysis. *Human Pathology,* 36, 486-493.

Wischmann, T., H. Stammer, H. Scherg, I. Gerhard & R. Verres (2001) Psychosocial characteristics of infertile couples: a study by the 'Heidelberg Fertility Consultation Service". *Human Reproduction,* 16, 1753-1761.

Wittwer, C. T., G. H. Reed, C. N. Gundry, J. G. Vandersteen & R. J. Pryor (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical Chemistry,* 49, 853-860.

Wojdacz, T. K. & A. Dobrovic (2007) Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Research,* 35.

Xiao, W. Z. & P. J. Oefner (2001) Denaturing high-performance liquid chromatography: A review. *Human Mutation,* 17, 439-474.

Zalenskaya, I. A. & A. O. Zalensky (2002) Telomeres in mammalian male germline cells. *International Review of Cytology - a Survey of Cell Biology, Vol 218,* 218, 37-67.

Zegers-Hochschild, F., G. D. Adamson, J. de Mouzon, O. Ishihara, R. Mansour, K. Nygren, E. Sullivan, S. Vanderpoel, Icmart & Who (2009) International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009. *Fertility and Sterility,* 92, 1520-1524.

Zhang, X. Y., M. San Gabriel & A. Zini (2006) Sperm nuclear histone to protamine ratio in fertile and infertile men: Evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. *Journal of Andrology,* 27, 414-420.

Zhou, L. M., A. N. Myers, J. G. Vandersteen, L. Wang & C. T. Wittwer (2004) Closed-tube genotyping with unlabeled oligonucleotide probes and a saturating DNA dye. *Clinical Chemistry,* 50, 1328-1335.

Zhou, L. M., L. Wang, R. Palais, R. Pryor & C. T. Wittwer (2005) High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. *Clinical Chemistry,* 51, 1770-1777.

Zielenski, J. (2000) Genotype and phenotype in cystic fibrosis. *Respiration,* 67**,** 117-133.

Zielenski, J. & L. C. Tsui (1995) Cystic fibrosis: Genotypic and phenotypic variations. *Annual Review of Genetics,* 29**,** 777-807.

Manuál CorPotocol 6000, High Resolution Melt Assay Design and Analysis. Corbett, 2006.

World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th ed. Geneva: World Health Organization; 2010.

http://cikgurozaini.blogspot.cz/2011/06/spermatogenesis-and-oogenesis.html

www.cftrscience.com

www.cftr2.org

www.eurogentest.org

www.genet.sickkids.on.ca/cftr/

www.novorozenecky-screening.cz

www.repromeda.cz/komplexni-diagnostika-neplodnosti.html