UNIVERZITA KARLOVA V PRAZE

2. LÉKAŘSKÁ FAKULTA

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Vplyv variant v génoch asociovaných s kancerogenézou na predispozíciu a fenotyp hereditárnych a sporadických nádorových ochorení gastrointestinálneho traktu

Dizertačná práca

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Obsah

Zoznam skratiek

KRK - kolorektálny karcinóm

WHO - World Health Organization

HNPCC - Hereditary nonpolyposis colorectal cancer

MMR - mismatch repair

MSI - microsatellite instability

FAP - familiárna adenomatózna polypóza

AFAP - atenuovaná familiárna adenomatózna polypóza

GIT - gastrointestinálny trakt

MAP - *MUTYH* asociovaná polypóza

PJS - Peutz-Jeghersov syndróm

JP - juvenilná polypóza

CS - Cowdenovej syndróm

PHTS - *PTEN* hamartomatous tumour syndromes

ÚZIS ČR - Ústav zdravotnických informací a statistiky ČR

UICC - Union for International Cancer Control

LU - lymfatické uzliny

MCR - mutation cluster region

UMD - universal mutation database

CMMR-D - constitutional mismatch repair-deficiency

Úvod

Genetická podstata nádorových ochorení

Je všeobecne známe, že nádorové ochorenie je genetické ochorenie spôsobené mutáciami rôznych génov. Ktorákoľvek bunka mnohobunkového organizmu, ktorá unikne prísnym regulačným mechanizmom zabezpečujúcich jej správnu funkciu, môže byť základom pre rozvoj nádoru. Kľúčovými pre rozvoj nádorov sú tri skupiny génov: proto-onkogény, ktoré v normálnych bunkách podporujú rast, delenie a diferenciáciu, tumor supresorové gény, ktoré naopak potláčajú proliferáciu buniek, a reparačné gény, ktoré sa podieľajú na opravách poškodenia DNA. Mutácie niektorých génov v zárodočných bunkách sú zodpovedné za vzácne dedičné predispozície s rôznou mierou rizika vzniku nádoru v priebehu života daného jedinca. S rozvojom molekulárno-genetických metód dochádza k postupnému odhaľovaniu vzťahov medzi mutáciami jednotlivých génov a vznikom rôznych nádorov. Získané poznatky sa využívajú hlavne pre rýchlu diagnostiku a prevenciu ochorenia, ale taktiež pre výber optimálnej liečby. Okrem spontánneho vzniku mutácií nezanedbateľnú úlohu pri karcinogenéze zohrávajú aj rôzne environmentálne faktory.

Dedičné genetické faktory u nádorových ochorení

Relatívne malá skupina nádorových ochorení (5-10%) je podmienená mutáciami génov v zárodočných bunkách [1]. Zárodočné mutácie, pokiaľ nie sú geneticky letálne, sa môžu prenášať z generácie na generáciu a v závislosti od konkrétneho génu výrazne zvýšiť riziko vzniku rôznych typov nádorov. U pacientov so zárodočnými mutáciami génov predisponujúcich k nádorovému ochoreniu nemusí bezpodmienečne dôjsť k iniciácií karcinogenézy. K iniciácii a rozvoju nádorového ochorenia sú nevyhnutné somatické mutácie ďalších génov a ich vzájomné interakcie s faktormi životného prostredia, ktoré môžu celý proces ovplyvniť a značne urýchliť. Identifikácia predisponujúcich génov umožňuje detekciu nádorov v počiatočnom štádiu a zároveň určitú možnosť prevencie ich vzniku. V rodinách so známou dedičnou genetickou predispozíciou môže analýza príslušného génu odhaliť nositeľov zárodočnej mutácie ešte pred rozvinutím ochorenia a tým poskytnúť čas pre prípadnú prevenciu a odpovedajúcu formu starostlivosti o týchto členov rodiny. V súčasnosti je známych niekoľko dedičných predispozícií

spojených s rôznym rizikom vzniku nádorového ochorenia. Prehľad tých najčastejších je uvedený v Tabuľke 1.

Syndróm	**OMIM**	**Gén**	**Frekvencia**	**Penetrancia***
Hereditárny nepolypózny kolorektálny karcinóm (HNPCC)	120435 120436	*MLH1,MSH2,MSH6,PMS2*	1/400	90%
Hereditárny karcinóm prsníka a vaječníkov	113705 600185	*BRCA1, BRCA2*	1/500	85%
Neurofibromatóza typ 1 (NF1)	162200	*NF1*	1/3000	100%
Familiárna adenomatózna polypóza (FAP)	175100	*APC*	1/5000- 1/10000	100%
Retinoblastóm	180200	*RB*	1/13500- 1/25000	90%
Mnohopočetná endokrinná neoplázia (MEN2)	131100	*RET*	1/30000	70%
Tuberózna skleróza	191100 191092	*TSC1, TSC2*	1/30000	95-100%
Von Hippel-Lindau syndróm (VHL)	193300	*VHL*	1/36000	90-95%
Ataxia telangiektázia	208900	*ATM*	1/30000- 1/100000	100%
Neurofibromatóza typ 2 (NF2)	101000	*NF2*	1/30000	100%
Nevoidný bazocelulárny karcinóm	109400	*PTCH*	1/57000	90%
Juvenilný polypózny syndróm (JP)	174900	*SMAD4, BMPR1A*	1/100000	95%
Peutz-Jeghersov syndróm (PJS)	175200	*STK11*	1/200000	95-100%
Cowdenovej syndróm (CS)	158350	*PTEN*	1/200000	90-95%
Li-Fraumeni syndróm	151623	*TP53*	neznáma	90-95%
Dedičná forma melanómu 1 a 2	155600	*CDKN2A, CDK4*	neznáma	100%
Difúzny karcinóm žalúdka	137215	*CDH1*	neznáma	90%

Tabuľka 1. Najčastejšie nádorové predispozície s katalógovým číslom OMIM (Online Mendelian Inheritance in Man), kauzálnym génom a odhadovanou frekvenciou. *Penetrancia uvádza kumulatívne riziko klinickej manifestácie (benígne aj malígne prejavy) do 70. roku života. Spracované podľa http://www.ncbi.nlm.nih.gov/omim.

Kolorektálny karcinóm

Najčastejšie sa vyskytujúcim nádorom tráviaceho traktu je rakovina hrubého čreva a konečníka, kolorektálny karcinóm (KRK). Podľa medzinárodnej klasifikácie ochorení pre onkológiu vydávanej medzinárodnou zdravotníckou organizáciou (WHO) [2], má kolorektálny karcinóm označenie C18-21. V Českej republike je incidencia tohto ochorenia mimoriadne vysoká a vyznačuje sa vysokou mortalitou [3]. Prevažnú väčšinu KRK (~75%) predstavujú sporadické prípady, kde dominantnú úlohu v etiológii zohrávajú environmentálne faktory. Familiárne prípady, s výskytom ochorenia v rodine ale bez známej genetickej príčiny, tvoria asi ~20% všetkých pacientov s KRK. Najmenšiu skupinu (~3-5%) reprezentujú hereditárne prípady s geneticky známou príčinou vzniku ochorenia [4] .

Hereditárne syndrómy s predispozíciou k kolorektálnemu karcinómu

Prehľad týchto syndrómov je uvedený v Tabuľke 2. Najčastejším ochorením patriacim do tejto skupiny je hereditárny nepolypózny karcinóm (HNPCC), nazývaný tiež Lynchov syndróm, ktorý je zodpovedný za 2-4% všetkých prípadov KRK [5]. Lynchov syndróm je zapríčinený zárodočnými mutáciami v jednom zo štyroch mismatch reparačných (MMR) génov (*MLH1, MSH2, MSH6* a *PMS2*). Vyznačuje sa skorým vznikom KRK, u žien aj karcinómom maternice, vaječníkov resp. ďalšími malignitami, často už pred 50. rokom života [6]. Charakteristickým znakom pacientov s Lynchovým syndrómom je prítomnosť mikrosatelitovej nestability (MSI) v nádoroch v dôsledku chybnej funkcie MMR systému [7].

Familiálna adenomatózna polypóza (FAP) je ďalším dobre popísaným dedičným syndrómom, ktorý je zodpovedný za <1% všetkých prípadov KRK. Typickým znakom je masívna polypóza (>100-1000 polypov) v gastrointestinálnom trakte (GIT) a takmer 100% riziko vzniku KRK u nosičov zárodočnej mutácie *APC* génu [8]. Miernejšia forma FAP, s menej ako 100 polypmi, sa označuje ako atenuovaná FAP (AFAP). Fenotypové varianty tohto ochorenia s extraintestinálnymi symptómami (osteómy, epidermoidné cysty, dezmoidy, kongenitálna hypertrofia pigmentového epitelu sietnice, hepatoblastómy a nádory štítnej žľazy) sa označujú ako Gardnerov a Turcotov syndróm [8].

MUTYH asociovaná polypóza (MAP) je autozomálne recesívne ochorenie zapríčinené bialelickými mutáciami génu *MUTYH* [9]. Fenotyp je podobný pacientom s atenuovanou FAP - počet adenomatóznych polypov býva v rozmedzí 20-100 a priemerný vek v čase diagnózy je 45 rokov (rozsah 12-68 rokov) [10]. Strata funkcie génu *MUTYH* vedie k akumulácií somatických mutácií, hlavne G:C>A:T v genóme, vrátane napr. génu *APC*, čo má za následok vznik predovšetkým adenómov a KRK [9].

Osobitnú skupinu predstavujú vzácne autozomálne dominantné hamartomatózne polypózne syndrómy (Tabuľka 2). Tieto na rozdiel od ostatných polypóznych syndrómov, kde hlavným histologickým typom polypu sú adenómy, sa vyznačujú vznikom predovšetkým hamartomatóznych polypov [11]. Spoločne predstavujú menej ako 1% všetkých prípadov KRK. Do tejto skupiny patria Peutz-Jeghersov syndróm (PJS), juvenilná polypóza (JP) a *PTEN* hamartomatózny polypózny syndróm (PHTS). PHTS zahŕňa Cowdenovej syndróm, Bannayan-Riley-Ruvalcaba syndróm a všetky ostatné syndrómy so zárodočnou mutáciou génu *PTEN* [11].

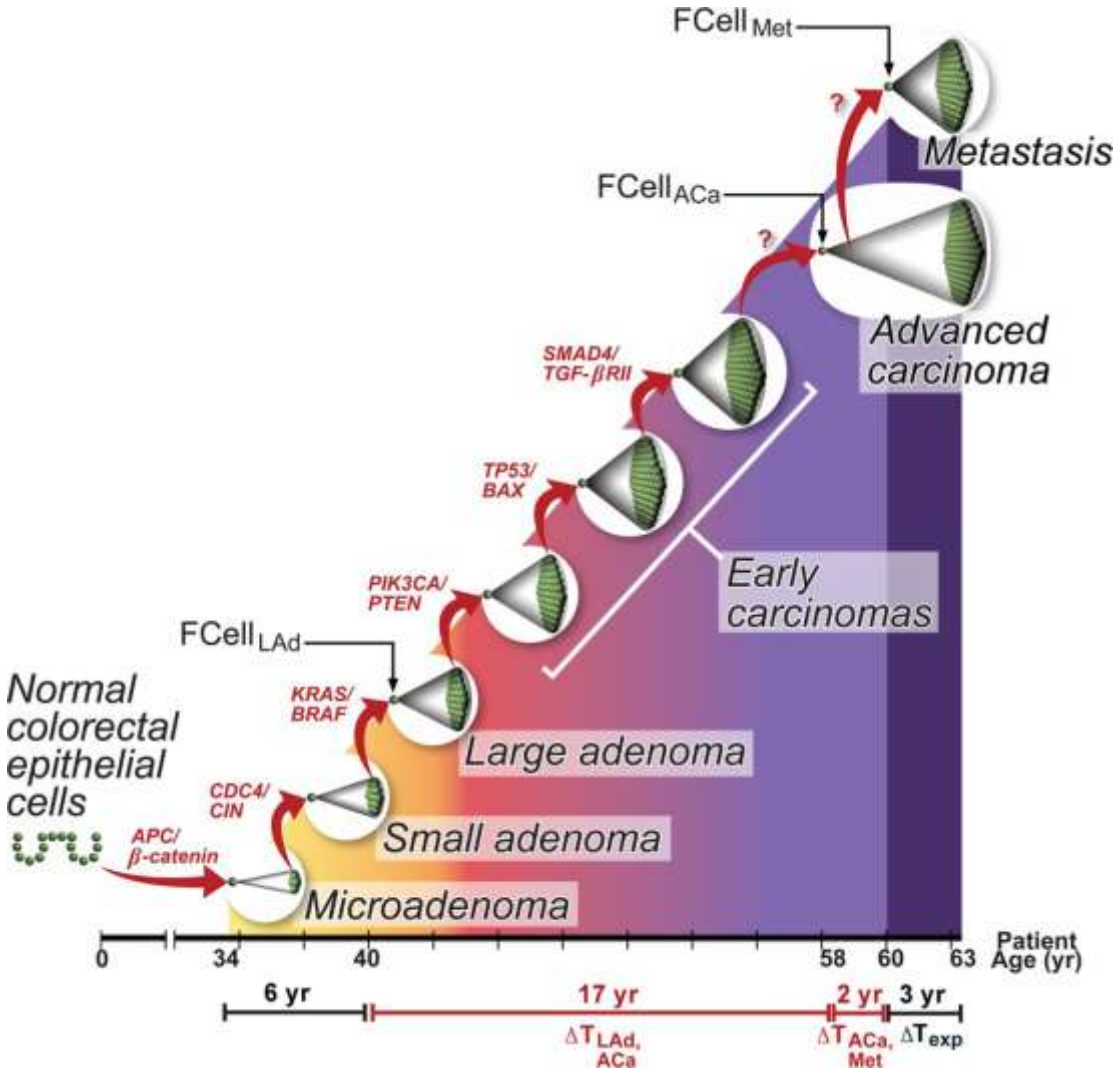
Syndróm	Gén	Inci-dencia	Dedi-čnosť	Počet polypov	Lokalizácia	Histológia	Pene-trancia (%)	Riziko KRK (%)	Iné symptómy
Adenomatózne syndrómy									
Hereditárny nepolypózny kolorektálny karcinóm (HNPCC)	*MLH1* *MSH2* *MSH6* *PMS2*	1:500	AD	0 až 30	hr. črevo	adenómy	~80	~80	nádory: maternice, vaječníkov, žalúdka, močových ciest a iné
Familiálna adenomatózna polypóza (FAP)	*APC*	1:10000	AD	>100	hr. črevo duodenum žalúdok	adenómy	~100	100	nádory: dezmoidné, osteómy kongenitálna hypertrofia pigmentového epitelu sietnice, hepatoblastómy, štítna žľaza
MUTYH asociovaná polypóza (MAP)	*MUTYH*	<1:10000	AR	<100	hr. črevo duodenum žalúdok	adenómy	~100	80-100	nádory: extraintestinálne
Hamartomatózne syndrómy									
Peutz-Jeghersov syndróm (PJS)	*STK11*	1:120000	AD	<20	t. črevo hr. črevo žalúdok	PJS polypy	vysoká	70	mukokutánna/periorálna pigmentácia nádory: GIT, prsníka, vaječníkov a Sertoliho buniek
Juvenilná polypóza (JP)	*SMAD4* *BMPR1A*	1:100000	AD	~5 až >100	hr. črevo konečník žalúdok t.črevo	juvenilné polypy	>90	~70	*SMAD* pozitívni: teleangiektázie
Cowdenovej syndróm (CS)	*PTEN*	1:200000	AD	?	t. črevo hr. črevo žalúdok	juvenilné, hyperplastické, zápalové polypy	~100	nízke	mukokutánne lézie nádory: prsníka, štítnej žľazy, maternice a iné

Tabuľka 2. Hereditárne syndrómy s predispozíciou k KRK
Spracované podľa http://www.ncbi.nlm.nih.gov/omim

Patogenéza kolorektálneho karcinómu

Kolorektálny karcinóm slúži ako ideálny model somatickej evolúcie epitelových nádorov hlavne pre svoju dobrú dostupnosť, možnosť sledovania a dobrú znalosť rôznych štádií neoplastického procesu. Prvý model postupnej akumulácie rôznych genetických zmien vedúcich k vzniku KRK načrtol Kinzler a Vogelstein [12]. K vzniku malignity pri tomto type nádorov (a aj u väčšiny ostatných solídnych nádorov) predpokladali minimálne sedem genetických zmien, ktoré postupnou akumuláciou vedú k transformácií normálnej epitelovej bunky tráviaceho traktu až na bunku schopnú invadovať vzdialené tkanivá resp. orgány (Obr. 1). I keď vznik mutácií je náhodný proces, ich selekcia a poradie, v akom k danej selekcii dochádza, sú javy nenáhodné a podliehajú rôznym evolučným tlakom [12]. Proces karcinogenézy KRK začína inaktiváciou signálnej dráhy WNT [13], často v dôsledku mutácie *APC* resp. *CTNNB1* génu, pri ktorej dôjde k transformácií normálneho epitelu na dysplastický/malý adenóm [14,15]. Mutácia v géne *KRAS*, delécia dlhého ramienka chromozómu 18 (18q) a inaktivácia génu *TP53* sa podieľajú na premene malého dysplastického adenómu až na veľký pokročilý karcinóm, pričom táto fáza u sporadických KRK trvá približne 17 rokov [16,17]. Prechod pokročilého karcinómu do štádia schopného metastázovať je veľmi rýchly, zvyčajne trvá menej ako 2 roky (Obr. 1) [17]. Dôvodom prečo je prechod zo štádia veľkého adenómu k pokročilému karcinómu pomalý, v porovnaní či už s prechodom normálneho epitelu na dysplastický/malý adenóm alebo s prechodom pokročilého karcinómu k metastázam, je pravdepodobne zapríčinený nevyhnutnou akumuláciou oveľa väčšieho počtu mutácií a klonálnych selekcií ako sa doposiaľ predpokladalo. Bolo zistené, že jedna bunka KRK môže získať až 76 bodových mutácií a 9 veľkých génových prestavieb, ktoré sa rôznou mierou podieľajú na karcinogenéze [18,19].

Patogenéza hereditárnych KRK sa od sporadických KRK líši prítomnosťou mutácie v niektorom z dôležitých génov uplatňujúcich sa pri vzniku nielen KRK už v zárodočnej línií, čím sa mimoriadne zvyšuje pravdepodobnosť a skracuje čas potrebný k zahájeniu neoplastického procesu. Riziko ale aj doba nevyhnutná k iniciácií karcinogenézy odpovedá dôležitosti konkrétneho mutovaného génu v určitej signálnej dráhe, narušením ktorej sa celý proces zahájí. Príkladom je FAP, kde zárodočné mutácie génu *APC*, ktorý zohráva dôležitú úlohu aj pri sporadických KRK, sú spájané s takmer 100% penetranciou a priemerným vekom nástupu malígneho ochorenia (pokiaľ pacient nie je sledovaný) 40 rokov (Tab.1 a 2) [8].



Obrázok 1. Somatická evolúcia sporadického KRK (Prevzaté od Jones et al. 2007 [17]).

Aktualizovaný model neoplastickej transformácie normálnej epitelovej bunky s mutáciami príslušných génov a predpokladaným časom nevyhnutným k jednotlivým prechodom rôznymi štádiami. Posledné dve štádia označené otáznikom nie sú spájané so žiadnymi známymi genetickými aberáciami.

$\Delta T_{LAd,ACa}$ - čas nevyhnutný pre prechod od pokročilého adenómu k pokročilému karcinómu

$\Delta T_{ACa,Met}$ - čas nevyhnutný pre prechod od pokročilému karcinómu k vzniku metastatickej bunky

ΔT_{exp} - čas nevyhnutný pre expanziu metastatickej bunky

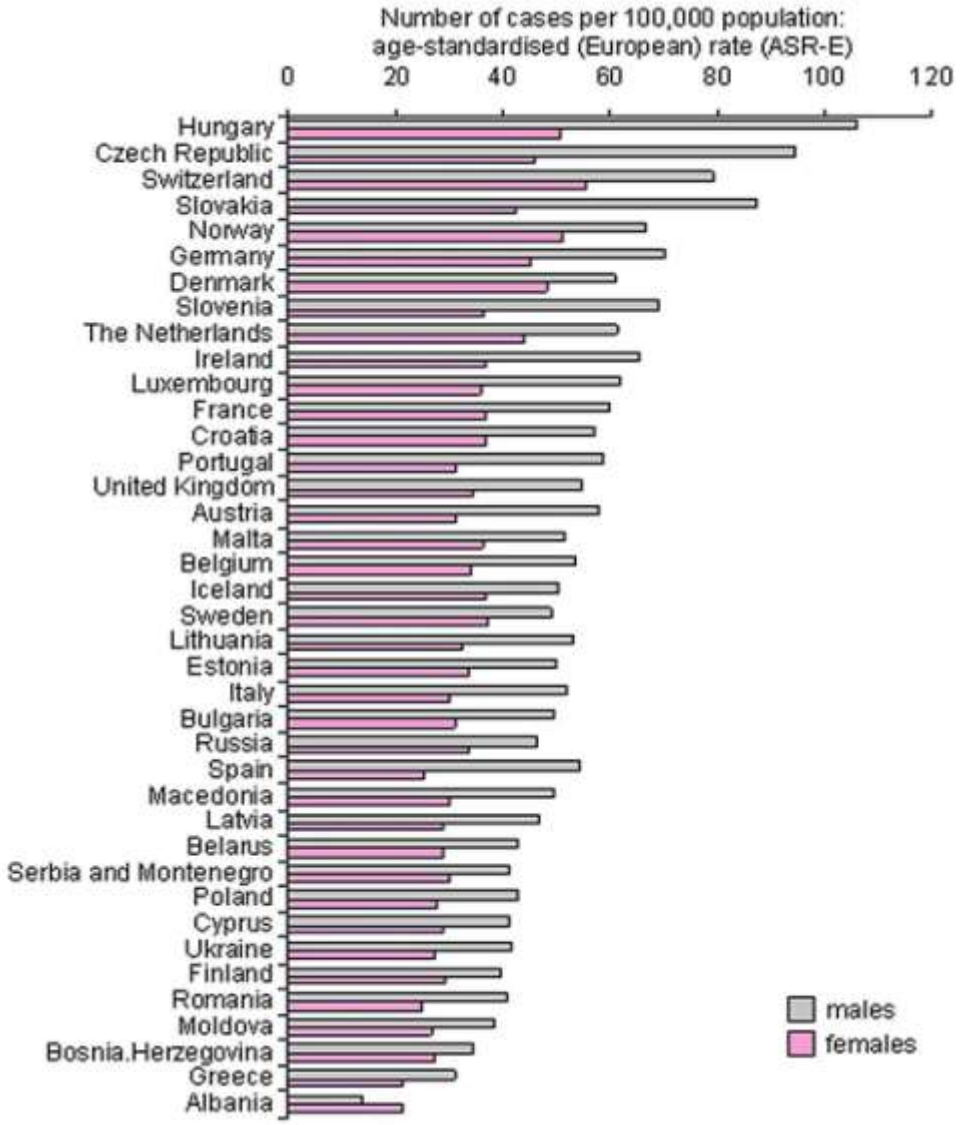
Epidemiológia kolorektálneho karcinómu v Českej republike

Kolorektálny karcinóm sa vyznačuje vysokou frekvenciou výskytu a mortalitou predovšetkým vo vyspelých krajinách v porovnaní s Indiou a Afrikou [20]. Vo veľkej miere je to zapríčinené bohatstvom a s tým súvisiacim životným štýlom a životosprávou obyvateľov týchto krajín [21]. Zdá sa, že incidencia a mortalita u mužov a žien s KRK je pred 50. rokom života rovnaká, avšak neskôr sa tento pomer obracia v neprospech mužov. KRK je ochorenie vo všeobecnosti spájané s vyšším vekom, priemerný vek v čase diagnózy je 73 rokov [22]. Predpokladá sa, že do 70. roku života dôjde minimálne u polovice populácie vo vyspelých krajinách k rozvoju určitej formy kolorektálneho nádoru, od benigného polypu až po invazívny karcinóm [23].

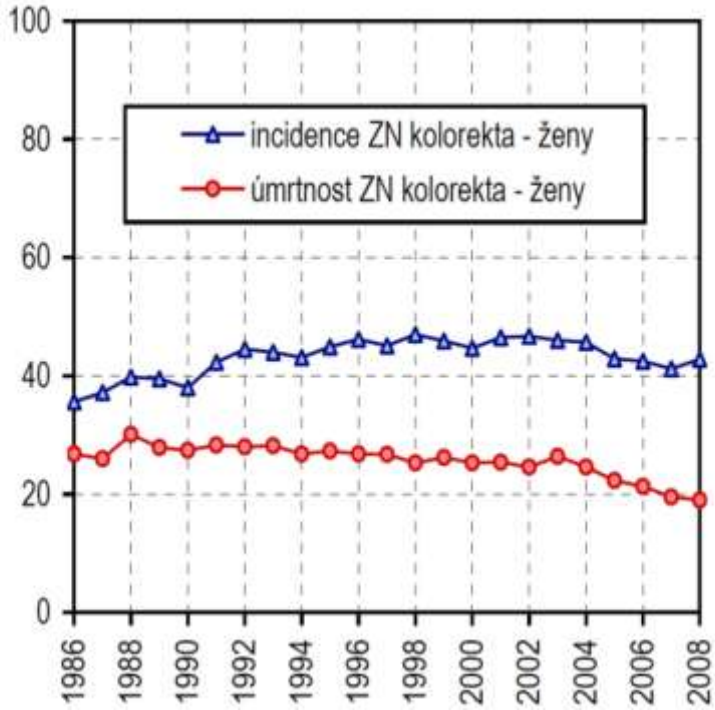
Kolorektálny karcinóm (C18-C21) je v Českej republike druhým najčastejším nádorovým ochorením u mužov a tretím u žien (Graf 1) [24].

Graf 1: Najčastejšie nádorové ochorenia u mužov a žien v ČR (2001-2005)
Zdroj: http://www.kolorektum.cz/index-en.php?pg=epidemiology--colorectal-cancer-czech-republic

Počet novo diagnostikovaných prípadov neustále rastie a v roku 2006 Česká republika zaujímala druhé miesto v počte výskytu KRK (4573 mužov a 3228 žien diagnostikovaných s KRK) v porovnaní s ostatnými štátmi v rámci Európy (Graf 2) [3]. Posledný oficiálny záznam z roku 2008 uvádza absolútny počet 4861 mužov (t.j. 95,1 prípadov na 100 tisíc mužov) a 3374 žien (t.j. 63,5 prípadov na 100 tisíc žien) diagnostikovaných s KRK [25]. Napriek zvyšujúcemu sa počtu novo diagnostikovaných pacientov s KRK má mortalita v posledných rokoch mierne klesajúcu tendenciu (Graf 3).



Graf 2: Incidencia KRK v Európe
Zdroj: Ferlay J. et al. [3]



Graf 3: Incidencia a mortalita na malígny kolorektálny karcinóm u mužov a žien (na 100 000 mužov/žien)
Zdroj: ÚZIS ČR [25]

Klasifikácia a liečba kolorektálneho karcinómu

Klasifikácia a delenie malígnych nádorov KRK má svoj nenahraditeľný význam pri stanovení prognózy a plánovaní najúčinnejšej liečby. Veľmi dôležitú úlohu pri tom zohráva TNM klasifikácia, vydávaná združením pre medzinárodnú kontrolu rakoviny (UICC), ktorá umožňuje rozdelenie nádorov do štádií (staging) na základe jednotných kritérií [26]. Okrem klinicko-patologického stagingu sa v súčasnosti začínajú využívať pri výbere liečby, určení prognózy a rýchlej detekcii nádoru aj rôzne genetické markery KRK [27]. Doposiaľ neboli zistené žiadne významné rozdiely týkajúce sa prognózy sporadických a hereditárnych KRK. Jediným známym a potvrdeným rozdielom je lepšia prognóza pre nádory s MSI (týka sa hlavne HNPCC asociovaných nádorov; či to platí aj pre nádory s MSI v dôsledku metylácie *MLH1* promótora nebolo jednoznačne overené) v porovnaní s nádormi s chromozomálnou nestabilitou [28].

Medzi základné a často prvé kroky liečby sporadických a hereditárnych KRK patrí chirurgické odstránenie nádoru. Tento prístup sa uplatňuje predovšetkým u menej pokročilých štádií, ale aj pri rozvinutejších formách ochorení. Ďalším krokom býva adjuvantná chemoterapia, ktorá u pacientov s invadovanými lymfatickými uzlinami (LU) znižuje mortalitu až o 33% [29]. Naopak zistilo sa, že adjuvantná chemoterapia na báze 5-fluorouracilu nezlepšuje, v niektorých prípradoch má dokonca negatívny efekt pri liečbe nádorov s MSI [30]. Ďalšou formou terapie býva neoadjuvantná rádioterapia, využívajúca sa pri liečbe rakoviny konečníka, ktorá znižuje riziko rekurencie o 50-60% v porovnaní so samostatným chirurgickým odstránením nádoru [31].

Ciele práce

V roku 2002 bola na Ústave biologie a lékařské genetiky zahájená analýza a riešenie problematiky hereditárneho nepolypózneho kolorektálneho karcinómu (HNPCC). V tom istom roku bol zmapovaný ďalší gén (*MUTYH*), ktorého zárodočné mutácie sú spájané s predispozíciou k *MUTYH* asociovanej polypóze (MAP). Analýza tohto génu u pacientov, ktorí nespĺňali kritéria pre HNPCC, bola jedným z prvých cieľov tejto práce. Rýchly priebeh analýzy s negatívnym výsledkom a zároveň prezentácia odborných článkov týkajúcich sa danej problematiky [10, 32] nasmerovala ďalšie štúdium k pacientom s podozrením na vzácne dedičné ochorenia s predispozíciou k rôznym formám neoplázií, predovšetkým gastrointestinálnych nádorov, a zároveň k molekulárno-genetickej analýze sporadických kolorektálnych karcinómov v českej populácií.

Jednotlivé ciele:

1. Štúdium genotypu a fenotypu u Cowdenovej syndrómu

   Cieľom bolo klasifikovať s pomocou molekulárno-genetickej analýzy vzácne prípady s podozrením na CS a detailne popísať ich minoritné fenotypové prejavy. Navrhnúť implementáciu menej častých symptómov ako diferenciačných kritérií pri iných, ťažšie identifikovateľných ochoreniach.

2. Štúdium genotypu a fenotypu u Peutz-Jeghersovho syndrómu

   Cieľom bolo previesť na základe mutačnej analýzy u pacientov s podozrením na Peutz-Jeghersov syndróm koreláciou genotypu a fenotypu a u nosičov zárodočných mutácií génu *STK11*, ktorí majú vysoké riziko vzniku nádorov GIT, stanoviť pravdepodobnosť rozvoja, vek nástupu a závažnosť ochorenia v českej populácií.

3. Štúdium genotypu a fenotypu u sporadických KRK v českej populácii

   Cieľom bolo pokúsiť sa objasniť vysokú morbiditu a mortalitu tohto ochorenia v Českej republike, identifikovať špecifické mutačné profily u českých sporadických KRK, odhaliť možnú asociáciu medzi genotypom (frekvenciou a spektrom mutácií jednotlivých génov a ich kombinácií) a fenotypom (klinickými a histopatologickými parametrami nádorov) a pokúsiť sa prostredníctvom analýzy celého génu *APC* identifikovať možných nositeľov zárodočných mutácií génu *MUTYH*, ktorí neboli a nemôžu byť detegovaní na základe rodinnej anamnézy.

4. Štúdium genotypu a fenotypu u pacienta s atypickým KRK

Cieľom bolo uskutočniť u tohto pacienta s osobitým mutačným profilom nádoru konečníka rozšírenú mutačnú analýzu, odhaliť príčinu tohto fenotypu a využiť získané poznatky v prospech pacienta, jeho rodiny alebo aj iných nepríbuzných jedincov s podobným molekulárnym a klinickým obrazom ochorenia pri liečbe, prevencii alebo sledovaní ich zdravotného stavu.

Výsledky

1. Štúdium genotypu a fenotypu u Cowdenovej syndrómu

Publikácia 1

A novel mutation of PTEN gene in a patient with Cowden syndrome with excessive papillomatosis of the lips, discrete cutaneous lesions, and gastrointestinal polyposis
Vasovcak P, Krepelova A, Puchmajerova A, Spicak J, Voska L, Musilova A, Mestak J, Martinek J. Eur J Gastroenterol Hepatol. 2007 January;19(6):513-7.

Publikácia 2

Multiple primary malignancies and subtle mucocutaneous lesions associated with a novel PTEN gene mutation in a patient with Cowden syndrome: Case report.
Vasovcak P, Senkerikova M, Hatlova J, Krepelova A. BMC Med Genet. 2011 March 15;12:38.

2. Štúdium genotypu a fenotypu u Peutz-Jeghersovho syndrómu

Publikácia 3

Mutations in STK11 gene in Czech Peutz-Jeghers patients.
Vasovcak P, Puchmajerova A, Roubalik J, Krepelova A. BMC Med Genet. 2009 July 19;10:69.

3. Štúdium genotypu a fenotypu u sporadických KRK v českej populácii

Publikácia 4

Molecular genetic analysis of 103 sporadic colorectal tumours in Czech patients
Vasovcak P, Pavlikova K, Sedlacek Z, Skapa Z, Kouda M, Hoch J, Krepelova A.
PLoS ONE, submitted.

4. Štúdium genotypu a fenotypu u pacienta s atypickým KRK

Publikácia 5

Unique mutational profile due to somatic *TDG* expression loss in a CMMR-D syndrome patient compound heterozygous for *PMS2* mutations
Vasovcak P, Krepelova A, Marra G, Puchmajerova A, Skapa P, Augustinakova A, Amann G, Wernstedt A, Jiricny J, Wimmer K. Int. J. Cancer, submitted.

Diskusia a komentáre k publikáciám

Štúdium genotypu a fenotypu u Cowdenovej syndrómu

Cowdenovej syndróm (Tabuľka 2), napriek jasne definovaným diagnostickým kritériám [33], je často mimoriadne zložité jednoznačne a správne diagnostikovať. Penetrancia tohto ochorenia je u nosičov zárodočných mutácií variabilná, klinické symptómy sú rozmanité a mukokutánne lézie nepatrné a ľahko prehliadnuteľné. Frekvencia ochorenia sa od počiatočných odhadov (1/1000000) po zavedení molekulárno-genetických analýz zvýšila na 1/200000 ale podľa najnovších odhadov môže dosahovať incidenciu až 2-3 prípady na 5000 ľudí [34].

Väčšina pacientov s CS je diagnostikovaná až v čase rozvoja malígneho ochorenia, resp. u mnohých dané ochorenie nie je diagnostikované vôbec, často z hore uvedených dôvodov ale aj pre nedostatok praktických skúseností ošetrujúcich lekárov. Pri dodatočnom komplexnom vyšetrení pacientov, prvotne indikovaných a vyšetrovaných s podozrením na iné ochorenie (napr. karcinóm prsníka, štítnej žľazy, polypózy GIT, neurologické ochorenia), bývajú zaznamenané aj ďalšie symptómy spájané s CS. Následná molekulárno-genetická analýza môže potvrdiť alebo vylúčiť nositeľa zárodočnej mutácie génu *PTEN*. Až 80% pacientov spĺňajúcich diagnostické kritéria pre CS býva nositeľom zárodočnej mutácie génu *PTEN* [35].

V publikácii 1 popisujeme prípad, ktorý názorne ilustruje tieto problémy. Jednalo sa o 36 ročného pacienta s rozsiahlou polypózou GIT. Histologické vyšetrenie potvrdilo hyperplastické a hamartomatózne polypy v žalúdku, a taktiež zápalové polypy a adenómy v hrubom čreve. Na základe histologických výsledkov bola u pacienta stanovená diagnóza Peutz-Jeghersov syndróm. Masívna papilomatóza pier pozorovaná pri kontrolnom vyšetrení GIT (14 rokov od prvého endoskopického vyšetrenia GIT) iniciovala ďalšie komplexné vyšetrenie, ktoré odhalilo glykogénnu akantózu, tri plantárne keratózy na dlani pravej ruky a makrocefáliu. Ultrasonografickým vyšetrením bolo nájdených niekoľko benígnych uzlov štítnej žľazy. Tieto klinické symptómy odpovedajúce CS viedli autora práce k zavedeniu a optimalizácií molekulárno-genetickej analýzy génu *PTEN,* ktorá u pacienta detegovala prítomnosť zárodočnej mutácie (c.825_840del) a korigovala prvotne stanovenú diagnózu. Bol to vôbec prvý prípad CS v ČR potvrdený na molekulárnej úrovni, čo umožnilo definitívne určiť presnú diagnózu a následne zahájiť odpovedajúcu formu liečby.

V publikácii 2 popisujeme 55 ročnú pacientku, u ktorej bolo zaznamenaných niekoľko primárnych nádorov. V priebehu jej života došlo k postupnému rozvoju malígneho karcinómu vaječníka, štítnej žľazy, žalúdka a hrubého čreva. Okrem týchto malignít bol u nej zaznamenaný aj rekurentný benígny meningeóm a polypóza GIT. Histologické vyšetrenie odhalilo prítomnosť rôznych neoplázií tráviaceho traktu. Potvrdené boli hlavne hyperplastické zápalové polypy, ale tiež tubulovilózne a vilózne adenómy. Nepatrné mukokutánne lézie v podobe bradavíc na predlaktiach rúk boli odhalené až po cielenom vyšetrení pre podozrenie na CS. Molekulárno-genetická analýza génu *PTEN* potvrdila, že pacientka bola nositeľkou zárodočnej mutácie (c.438delT).

Oba popísané prípady sa vyznačovali masívnou polypózou GIT, ktorá v druhom prípade viedla k rozvoju nádorového ochorenia a smrti pacienta. Podľa medzinárodných diagnostických kritérií pre CS patria gastrointestinálne hamartomatózne polypy medzi vedľajšie kritéria a riziko vzniku rakoviny GIT je u nich minimálne [35]. I keď z histologického hľadiska by pre CS mali byť typické hlavne hamartomatózne polypy, tento typ predstavuje asi len 25% spomedzi všetkých nachádzaných polypov [36]. Z literatúry tiež vyplýva, že pacienti s CS z európskych krajín majú nižší výskyt polypov resp. polypózy GIT v porovnaní s Japonskom, čo však môže byť výsledkom častejšie vykonávaných endoskopických vyšetrení u asymptomatických pacientov s CS [37]. Prevažná väčšina prác týkajúcich sa nádorových ochorení u CS nezohľadňuje doživotné riziko vzniku nádorov GIT z dôvodu relatívne nízkeho veku pacientov. Na základe našich výsledkov a niekoľkých prác iných autorov [36,38,39] predpokladáme, že polypóza GIT je u CS relatívne častá aj v európskej populácii a následné riziko vzniku nádorov je značne zvýšené, i keď s neskorším nástupom v porovnaní s nádormi prsníka a štítnej žľazy. Preto sa domnievame, že endoskopické vyšetrenie GIT by malo byť súčasťou komplexného vyšetrenia pacienta s podozrením na CS a zároveň pacienti s polypózou GIT, u ktorých bolo vylúčené iné ochorenie spájané s polypózou tráviaceho traktu (napr. FAP, PJS a ďalšie), by mali podstúpiť komplexné vyšetrenie so zameraním na odhalenie ďalších symptómov typických pre iné vzácne syndrómy. Naša analýza teda prispela k rozšíreniu známeho spektra fenotypovej variability CS a k diskusii o modifikácii súčasných indikačných kritérií pre testovanie mutácií v géne *PTEN*. Obe mutácie zistené u našich pacientov boli nové, doposiaľ u pacientov s CS nepopísané.

Štúdium genotypu a fenotypu u Peutz-Jeghersovho syndrómu

V článku 3 popisujeme pacientov s podozrením na Peutz-Jeghersov syndróm (Tabuľka 2). Toto podozrenie bolo odvodené na základe diagnostických kritérií stanovených Tomlinsonom a Houlstonom [40]. Zárodočné mutácie génu *STK11* boli nájdené u piatich pacientov z dvoch nepríbuzných rodín, ktoré spĺňali diagnostické kritériá pre PJS. Do súboru boli zaradení aj traja sporadickí pacienti nespĺňajúci kritéria, ale periorálna pigmentácia a v dvoch prípadoch aj potvrdená prítomnosť polypov rozhodli o ich začlenení do súboru. Zárodočná mutácia génu *STK11* bola nájdená len u jedného z nich. Okrem periorálnej pigmentácie a polypov bol u jednej 28 ročnej pozitívne testovanej pacientky zaznamenaný agresívne rastúci nádor žalúdka, ktorý bol pravdepodobnou príčinou jej predčasnej smrti.

Z dostupnej literatúry vyplýva, že spomedzi nádorových ochorení GIT sa rakovina žalúdka u pacientov s PJS manifestuje v priemere okolo 30. roku života, nasledovaná rakovinou tenkého (priemerne v 40 rokoch) a hrubého čreva (v 48 rokoch) [41]. U ostatných pacientov v našom súbore nebolo zaznamenané žiadne nádorové ochorenie, čo môže byť aj výsledkom nízkeho veku, lebo 4 pacienti s potvrdenou mutáciou v čase diagnózy mali menej ako 20 rokov. Kumulatívne riziko pre rôzne malignity do 70. roku života sa odhaduje na 81% a pre nádory GIT na 57% [42]. Napriek malému počtu pacientov sme sa pokúsili o koreláciu genotypu a fenotypu pacientov, pričom sme porovnávali výsledky aj s inými autormi. Pozorovali sme, že pacienti s tzv.“truncating“ mutáciami (skracujúce proteín) majú ťažší fenotypový prejav v porovnaní s deléciami celého génu. Na rozdiel od kompletnej straty génu, kedy dôjde pravdepodobne k zníženej produkcii príslušného proteínu, pri tzv. „truncating“ mutáciách sa môže uplatniť dominantne negatívny efekt, výsledkom čoho je závažnejší fenotyp. Táto hypotéza bola nedávno podporená štúdiou väčšieho počtu pacientov, u ktorých boli „truncation“ mutácie spájané so skorším nástupom ochorenia, väčším počtom polypov a nádorových ochorení [43]. Predpokladá sa, že typ mutácie by mohol slúžiť ako jeden z dôležitých faktorov kedy a v akej forme zahájiť pravidelný skríning a primeranú formu liečby. Podľa najnovších výsledkov by mali pacienti s „truncating“ mutáciou podstúpiť prvé kolonoskopické vyšetrenie v 12. roku života a v prípade pozitívneho nálezu opakovane v pravidelných ročných intervaloch [43]. Zahájenie skríningu v mladšom veku môže pomôcť pri odhalení nádorového ochorenia v rannom štádiu a zvýšiť úspešnosť následnej liečby. Aj u tohto vzácneho syndrómu teda naša analýza priniesla dôležité poznatky rozširujúce obecnú znalosť o tomto ochorenia.

Štúdium genotypu a fenotypu u sporadických KRK v českej populácii

Hlavným impulzom pre túto štúdiu bol všeobecne známy fakt, že Česká republika sa vyznačuje mimoriadne vysokým počtom nádorových ochorení GIT v porovnaní s ostanými krajinami Európy [3]. Doposiaľ však nebola uskutočnená žiadná analýza na molekulárno-genetickej úrovni u pacientov so sporadickým KRK, ktorá by pomohla odhaliť prípadné špecifiká alebo vlastnosti typické pre českú populáciu resp. možné príčiny vysokého výskytu tohto ochorenia. Mutačnou analýzou niekoľkých vybraných génov v skupine 103 sporadických KRK získaných od 102 českých pacientov sme získali niekoľko zaujímavých poznatkov.

K tým najpozoruhodnejším patrilo pozorovanie odlišného spektra a rozloženia mutácií v niektorých génoch v porovnaní s výsledkami iných autorov resp. mutačných databáz. Zistili sme, že mutácie v géne *APC* nie sú lokalizované primárne v oblasti "mutation cluster region" (MCR) [44], ale sú rozptýlené v rozsahu celého génu. Obmedzenie mutačnej analýzy na samotnú MCR by v našom súbore výrazne ovplyvnilo jednak počet nájdených mutácií a rovnako aj ich spektrum. Porovnávanie a interpretácia výsledkov mutačnej analýzy génu *APC* s obmedzením len na vybranú oblasť preto môže viesť k skresleným a nesprávnym záverom. Ďalej sme predpokladali, že analýzou celej kódujúcej oblasti génu *APC* by sme mohli odhaliť "skrytých" pacientov s *MUTYH* asociovanou polypózou (MAP), kedže frekvencia tohto ochorenia sa zdá byť v Českej republike nižšia v porovnaní s ostatnými štátmi Európy [10, 32]. Pretože MAP je recesívne ochorenie a nádory týchto pacientov sa vyznačujú charakteristickým fenotypom [9], tento postup v málopočetných rodinách (bez pozitívnej rodinnej anamnézy) sa zdal byť oprávnený. Neodhalili sme však žiadny odpovedajúci genotyp, ktorý by poukázal na pacienta s podozrením na MAP a ani následna analýza génu *MUTYH* nepotvrdila prítomnosť bialelickej mutácie. Preto sa prikláňame k názoru, že frekvencia MAP je v ČR v porovnaní s ostatnými krajinami nižšia.

Podľa niektorých prác mutačný profil génu *TP53* môže odrážať expozíciu organizmu určitým karcinogénom [45]. Porovnaním našich výsledkov s UMD p53 Mutation Database [46] sme odhalili výrazne odlišné zastúpenie najčastejšie sa vyskytujúcich mutácií v nádoroch kolorekta. Jedným z možných vysvetlení môžu byť špecifické diétne návyky typické pre českú populáciu (napr. zvýšená expozícia polycyklickým aromatickým uhľovodíkom) [47] alebo populačné odlišnosti vo frekvenciách funkčných polymorfizmov reparačných génov, ktoré by mohli modifikovať riziko vzniku KRK [48]. Ďalším zaujímavým zistením bolo, že nádory s MSI a bez metylácie promótora *MLH1* mali skorší nástup a vyznačovali sa závažnejším mutačným profilom.

To či sú tieto výsledky charakteristické pre českú populáciu alebo sú odrazom určitej selekcie pri výbere nádorov (takmer 90% pacientov bolo z hlavného mesta ČR a blízkeho okolia) je nevyhnutné overiť na ďalšom rozšírenom súbore pacientov.

Štúdium genotypu a fenotypu u pacienta s atypickým KRK

Táto práca nadväzuje na predchádzajúcu štúdiu, v rámci ktorej sme odhalili jeden nádor konečníka s osobitým mutačným profilom. Tento nádor 13 ročnej pacientky sa vyznačoval nezvyčajne vysokým počtom somatických mutácií, a preto sme pre jeho pomenovanie zvolili názov "supermutačný" fenotyp. Táto pacientka bola paralelne a nezávisle od predošlej štúdie testovaná aj pre podozrenie na Lynchov syndróm. Mutačnou a imunohistochemickou analýzou MMR génov (*MLH1, MSH2, MSH6* a *PMS2*) sa zistilo, že je nositeľkou bialelickej zárodočnej mutácie génu *PMS2*. Bialelické zárodočné mutácie MMR génov sú zodpovedné za vzácne ochorenie označované ako konštitučný mismatch reparačný deficientný syndróm (CMMR-D) [52] a táto pacientka predstavuje vôbec prvý zaznamenaný a zdokumentovaný prípad tohto ochorenia v ČR. I keď kauzálna príčina ochorenia sa zdala byť odhalená, vysoký počet bodových somatických mutácií vo viacerých génoch bol nezvyčajný a doposiaľ u CMMR-D pacientov s zárodočnou mutáciou *PMS2* nepopísaný. Mutačnou analýzou DNA získanej z KRK ďalších troch pacientov, ktorí boli známi ako nositelia bialelickej mutácie *PMS2,* sme zistili, že absencia proteínu PMS2 s veľkou pravdepodobnosťou nebude kauzálnou príčinou tohto osobitého fenotypu. Na základe typu nájdených somatických mutácií sme však vytipovali gény (*UNG2, SMUG1, MBD4* a *TDG*), ktoré by mohli byť zodpovedné za daný fenotyp. Tieto gény majú hlavnú úlohu pri odstraňovaní predovšetkým chybne inkorporovaného uracilu a tymínu v DNA, čím minimalizujú riziko vzniku tranzícií G:C>A:T, hlavného typu mutácií pri absencii resp. nefunkčnosti niektorého zo spomínanej štvorice génov [53]. Analýzou všetkých vytipovaných génov sme našli somatickú mutáciu v géne *TDG*, ktorá bola lokalizovaná vo vysoko konzervatívnej oblasti a pravdepodobne spôsobila nefunkčnosť príslušného proteínu. Imunohistochemickou analýzou sme potvrdili, že miera expresie proteínu TDG bola v nádorových bunkách v porovnaní s kontrolou znížená. Tento výsledok naznačuje mimoriadnu dôležitosť *TDG* pri zachovaní integrity genómu, avšak otázka či tento supermutačný fenotyp je dôsledok absencie defektu samotného *TDG* alebo jeho vzájomnej kooperácie s nefunkčným MMR systémom ostáva nezodpovedaná.

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Prílohy

Publikácia 1

A novel mutation of *PTEN* gene in a patient with Cowden syndrome with excessive papillomatosis of the lips, discrete cutaneous lesions, and gastrointestinal polyposis

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Cowden syndrome is an inherited disease characterized by mucocutaneous lesions, gastrointestinal hamartomatous polyposis and an increased risk of breast, thyroid and endometrial carcinomas. Despite well described phenotypic expression of this disease, it is not easy to determine correct clinical diagnosis. In this case report we present a clinical history of a patient with Cowden syndrome. When he was 22 years old, he was found to have polyposis of gastrointestinal tract. The diagnosis of Peutz–Jeghers syndrome was established. Owing to intensive belly spasms, as a 36-year-old he was sent to another gastroenterological department where the thorough gastrointestinal tract examination was performed. We found glycogenic acanthosis of the esophagus; diffuse polyposis with large polyps within the stomach, and polyposis with small polyps in duodenum, colon, and rectum. We also noted the presence of excessive mucocutaneous papillomatosis of the lips and subtle skin lesions. Possible Cowden syndrome diagnosis was suggested. The same year he underwent plastic operation of the lips. During surgery, diffuse nodularity of the trachea was also noted. After plastic operation and assessment of Cowden syndrome as a possible diagnosis, he was recommended for a genetic examination. Diagnosis of Cowden syndrome was confirmed by sequencing analysis of the *PTEN* gene (phosphatase and tensin homolog deleted on chromosome 10). We found 'c.825_840delAAATACATTCTTCATA' deletion. This case affirmed that, for establishment of a correct diagnosis, especially for rare clinically overlapping syndromes, molecular testing is usually the only reliable method. *Eur J Gastroenterol Hepatol* 19:513–517 © 2007 Lippincott Williams & Wilkins.

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Introduction

The autosomal dominantly inherited hamartoma polyposis syndromes comprise juvenile polyposis syndrome (JPS, *OMIM* 174900), Cowden syndrome (CS, *OMIM* 158350), Bannayan–Riley–Ruvalcaba syndrome (BRRS, *OMIM* 153480), and Peutz–Jeghers syndrome (PJS, *OMIM* 174900) [1]. To specify the correct diagnosis, especially because of the subtle clinical distinctions and overlapping phenotypic spectra, is a challenge. The main common clinical feature of these syndromes is the presence of hamartomatous polyps. CS was first described in a 20-year-old woman in 1963 [2]. The gene responsible for CS was mapped to chromosome 10q22–23 [3]. CS is characterized by a high risk of breast, thyroid, and endometrial carcinomas. Pathognomonic features of CS patients are mucocutaneous lesions presented in 99% of affected individuals before the age of 30 years [4]. In 1997, Marsh *et al.* found germline mutations of *PTEN* gene in BRRS patients. BRRS is characterized by hamartomatous polyposis, macrocephaly, lipomatosis, hemangiomas, and speckled penis. The mutational spectra of BRRS and CS seem to overlap. It is believed that CS and BRRS are allelic and represent a single entity [5]. It was suggested that *PTEN* would be the susceptibility gene for all inherited hamartomatous syndromes. By the identification of *STK11*, the gene responsible for PJS, and mapping to chromosome 19p13.3, this syndrome was excluded [4]. PJS is characterized by gastrointestinal (GI) hamartomatous polyps, perioral pigmentation, and risk of GI and breast cancers. The last of the hamartoma polyposis syndromes which could be diagnosed on the basis of genetic analysis was JPS. In the case of JPS, the susceptibility genes are *MADH4* on 18q21.1 and *BMPRIA* on 10q21–22. JPS is characterized mainly by the presence of GI hamartomatous polyps and an increased risk of GI malignancy. The diagnosis of JPS is made only if features classic for other syndromes are not present [1].

After the first CS case was described, more than 200 single cases have been published to date. To facilitate

diagnosis of CS, the International Cowden Consortium established operational diagnostic criteria [4].

We report a case of CS, which was initially diagnosed as PJS.

Case description

The patient, a 36-year-old man, was referred to our gastroenterology department in 2002 as having PJS. The reason for referral was severe belly spasms. The patient had been followed up for 14 years for gastric and colonic polyposis.

At admission, the patient was noted as having mild mental retardation. Proband's father (70 years) had had no clinical CS manifestation so far, mother had committed suicide at 33 years, and the patient had no siblings. The personal history was unremarkable. He was single and had no children.

On physical examination, excessive papillomatosis or cobblestone appearance of the lips (Figs 1 and 2), mild gingival papillomas, fissured tongue, and macrocephaly were identified. Also, subtle skin lesions were identified – we found only three acral keratoses on the dorsum of the right palm (Fig. 3). No acral lesions on the soles were found. Laboratory tests were normal. Imaging methods (chest and abdominal computed-tomography scans, abdominal sonography, and barium-contrast study of the small bowel) were also normal. Thyroid sonography showed multiple stationary nodules of benign appearance, which had not yet been punctured.

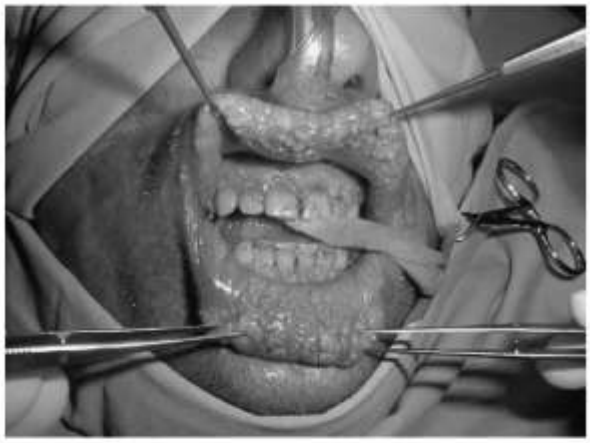
Upper GI endoscopy revealed diffuse esophageal nodularity with small whitish nodules (Fig. 4). In the stomach, polyposis with some polyps even exceeding 2 cm was seen (Fig. 5). The patient was not infected by *Helicobacter pylori*. We performed endoscopic polypectomies of several large polyps. Surprisingly, only sporadic polyps (maximum 10–13) of small size (up to 2 mm) were seen in the left colon and rectum; no polyps in the right colon were diagnosed.

Histologically, esophageal nodules showed typical glycogenic acanthosis. Gastric polyps were mostly hyperplastic (Fig. 6). They contained multiple cell types including inflammatory, lipomatous, and fibromatous cells. Colonic polyps were mainly inflammatory and some of them were adenomatous with low-grade dysplasia.

On the basis of those findings, the clinical diagnosis of CS was made. Subsequently, the genetic examination was performed and confirmed the diagnosis (Fig. 7).

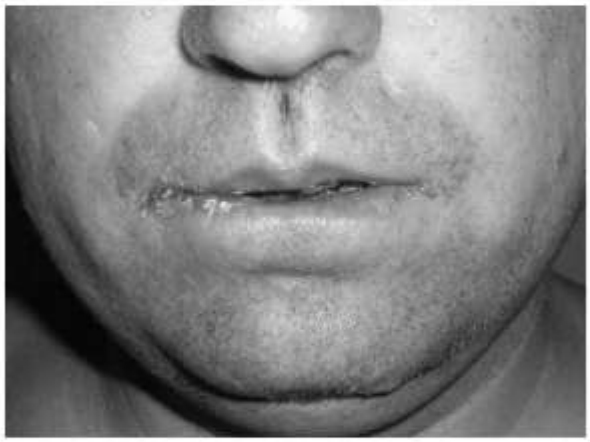
The main complaint of the patient was the excessive papillomatosis of the lips. It made it difficult for him to eat and drink and, also, created important aesthetical consequences. Therefore, he underwent plastic reconstruction of his lips (Fig. 2). The histology of the lips revealed hyperplastic polyps. During intubation, diffuse nodularity in the trachea similar to that in the esophagus (glycogenic acanthosis) was noted.

Fig. 1



Cobblestone appearance of the lips before plastic reconstruction.

Fig. 2



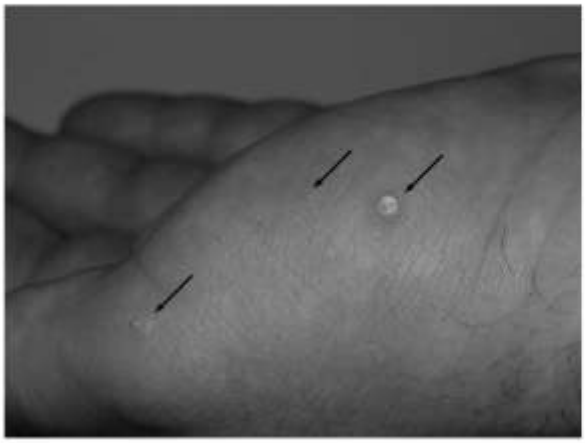
Two years after surgical reconstruction of the lips.

At present, the patient is followed up and checked regularly for cancers at risk (breast, thyroid, and colon). Regularly, we perform polypectomies of large gastric polyps but their growth is very slow.

Genetic examination

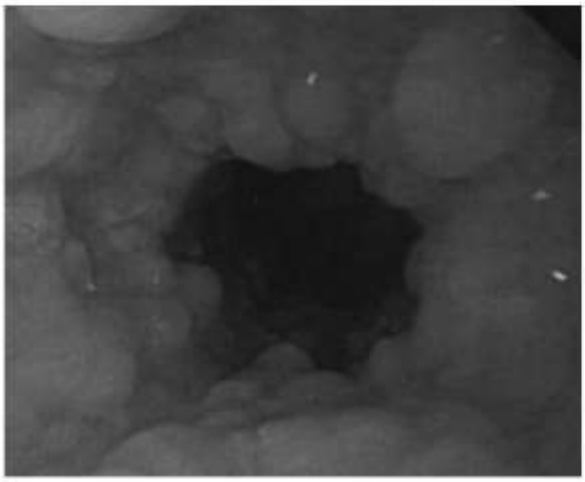
We performed PCR amplification of exons 1–9 of the *PTEN* gene and sequenced all exons bilaterally. In

Fig. 3



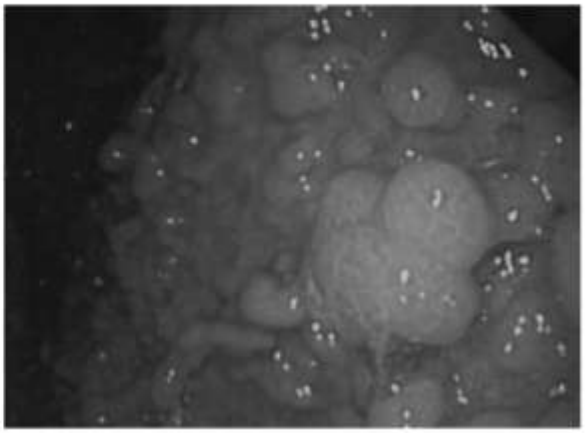
Acral keratoses on the right palm.

Fig. 4



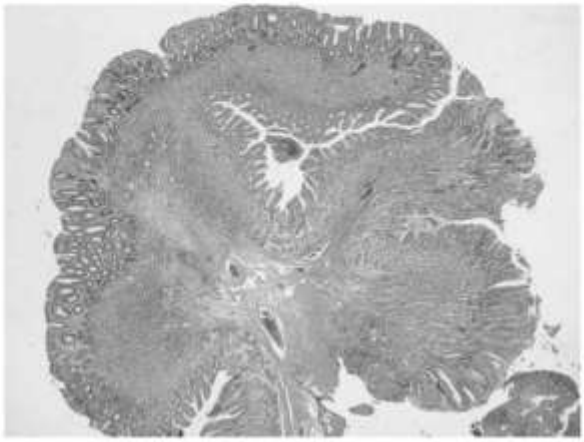
Nodular aspect of esophageal mucosa. Histology showed glycogenic acanthosis typical of Cowden syndrome.

Fig. 5



Diffuse polyposis in the stomach. The size of polyps ranged from 2–3 mm to 2 cm. Histology showed the polyps were mostly hyperplastic.

Fig. 6

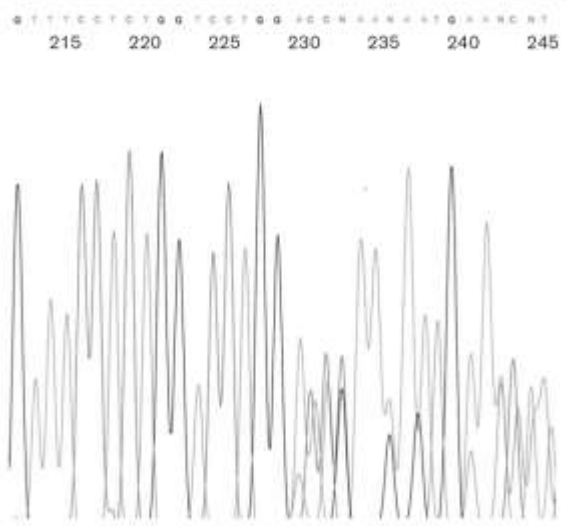


Hyperplastic gastric polyp with mildly elongated foveolar glands and intermingled smooth muscle in the arborescent stroma. Typical features of hamartomatous PJS (Peutz–Jeghers syndrome) polyps were not found.

genomic DNA of the patient, we found the heterozygous mutation 'c.825_840delAAATACATTCTTCATA' in the eighth exon (Fig. 7). The deletion of 16 nucleotides leads to frameshift mutation and premature stop codon AAC51183 (p.Gln276fsX10) in the protein.

The major role of the *PTEN* protein is to regulate phosphoinositol-3-kinase/Akt signal pathways and to effect G1 cell-cycle arrest and apoptosis. The *PTEN* protein has two major domains: the N-terminal phosphatase domain with catalytic activity and the C2 domain, which is associated with substrate binding. The C-terminal domain also contains the PEST domain, which regulates protein stability, and PDZ domain, important in protein–protein interactions. Although this novel mutation was located downstream of the phosphatase domain of the *PTEN* protein, where the majority of mutations in CS patients were identified, we assumed that the loss of the C2 domain was responsible for CS in our patient. This observation is in accordance with other truncating mutations that were found in the C2 domain of the *PTEN* gene [5–10].

Fig. 7



Sequencing chromatogram displaying the novel mutation c.825_840del in the eighth exon of the *PTEN* gene.

Discussion

In this case report, we present a 40-year-old patient with some typical features characteristic of CS, according to the International Cowden Consortium operational criteria [4]. CS is a rare autosomal-dominant disorder that is characterized by mucocutaneous lesions, GI polyposis, macrocephaly, mental retardation, and with increased risk of breast, thyroid, and endometrial malignancies [4]. Most of the CS cases published until now were presented with discernible clinical signs, which were found to have various germline mutations in *PTEN* gene. Some cases with ambiguous symptoms were also introduced, which caused a wrong diagnosis at first or it took a longer time until the correct diagnosis could be determined [4,11,12]. The incidence of CS was estimated to be at least one in 200 000 [7]. The true incidence seems to be underdiagnosed, owing to various reasons. First, revelation of CS in any patient is usually related to the finding of some neoplasia, mostly breast cancer in women and thyroid cancer in both sexes in middle age. Second, mucocutaneous marks are often discreet and palmoplantar keratoses resemble benign warts, which are relatively common in the general population. Our patient was found to have three plantar keratosis papules and no other visible mucocutaneous lesions. In contrast, he presented with excessive oral papillomatosis and he underwent plastic reconstruction.

In 1988 the patient was diagnosed with PJS owing to the presence of hamartomatous polyps. Despite repeated attempts, the older pathology specimens from that time were not available for the review. Melanin spots on the lips, buccal mucosa, and digits, which are typical features of PJS, were not present in our patient. We think that incorrect diagnosis of PJS was established because of the unavailability of genetic examinations and because there was little information about CS in 1988. Thus, the diagnosis of PJS was comprehensible at that time.

Approximately 50 to 67% of CS patients have thyroid abnormalities, including goiter, adenoma, and cancer; 25 to 76% of affected women have breast lesions, including adenocarcinoma and fibrocystic disease (two CS cases with breast cancer in men were noted as well); and 38% have macrocephaly [4,13]. Ultrasonography of the thyroid gland of our patient revealed several nodules. Goiter and carcinoma were not confirmed. Macrocephaly was determined as well. GI polyps are usually asymptomatic. They are more common in Japanese (> 90%) than in western (40–70%) CS patients. The reason for this is considered to be that GI examinations are more frequently performed in Japan [14]. From the histopathological point of view, hamartomatous, inflammatory, hyperplastic, juvenile, lymphomatous, and adenomatous polyps were found in CS patients. GI examination of our patient showed mostly hyperplastic and some hamartomatous polyps in the stomach, and inflammatory polyps and tubular adenomas in the colon. Esophageal glycogenic acanthosis was also confirmed. It was suggested that glycogenic acanthosis is a characteristic clinical manifestation of CS and should be used to diagnose this condition and to distinguish CS from other polyposis syndromes [15]. In our patient, we also identified glycogenic acanthosis of the trachea.

Over the past few years, progress has been made in the knowledge of the molecular mechanisms involved in CS and other diseases. The knowledge of the different phenotypic features also plays an important role in the diagnosis. Clinicians who are not familiar with pathognomic mucocutaneous lesions of CS will be able to distinguish CS patients through examination of breast, thyroid, and GI tract. As new genes that are responsible for different syndromes with similar phenotypic features are continually discovered, presymptomatic genetic testing of individuals at risk should be recommended.

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Declaration of authors: We hereby declare that this work has not been published previously and will not be submitted to any other journal until the final decision about acceptance/rejection is done by the editor. The authors agree with the submission of the manuscript to the *European Journal of Gastroenterology and Hepatology*. All authors contributed substantially to the manuscript and to the management of the patient described in the case report: Peter Vasovcak (genetic analysis, manuscript);

Anna Krepelova (genetic analysis, genetic counseling); Alena Puchmajerova (genetic counseling); Julius Spicak (gastroenterology, clinical examination); Ludek Voska (pathology); Andrea Musilova (plastic surgery); Jan Mestak (plastic surgery, manuscript); Jan Martinek (endoscopy, manuscript, coordination).

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Publikácia 2

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CASE REPORT Open Access

Multiple primary malignancies and subtle mucocutaneous lesions associated with a novel *PTEN* gene mutation in a patient with Cowden syndrome: Case report

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Abstract

Background: Cowden syndrome (CS) is a cancer predisposition syndrome associated with increased risk of breast, thyroid, and endometrial cancers, and is characterized by development of benign mucocutaneous lesions.

Case presentation: Here we report on a 58-year-old woman with multiple primary malignancies and subtle mucocutaneous lesions such as small polyps and wart-like papulas. Over a period of 23 years, she developed various malignant neoplasms including thyroid, ovarian, stomach, and colon carcinomas, and a benign meningioma. Direct sequencing analysis of the *PTEN* gene revealed a novel germline mutation (c.438delT, p.Leu146X).

Conclusion: This case demonstrates that Cowden syndrome is a multi-system disease that can result in the development of multiple malignant and benign tumors.

Background

Cowden syndrome (CS, OMIM 158350) is an autosomal dominant disorder characterized by multiple hamartomas, which develop in the skin, thyroid gland, breast, gastrointestinal tract, and brain [1]. Germline mutations in the *PTEN* (phosphatase and tensin homolog deleted on chromosome ten) gene have been found in 80% of patients with CS [2]. The *PTEN* gene encodes a dual-specificity protein and lipid phosphatase that regulates the phosphoinositol-3-kinase/Akt signal pathway which can result in cell cycle arrest in the G1-phase and apoptosis [3]. PTEN can directly or indirectly dephosphorylate focal adhesion kinase (FAK), resulting in inhibition of cell migration and cell spreading [3-5].

Diagnosing CS remains a challenge due to the variations in its clinical presentation. A recent molecular-genetic study estimated that the incidence of CS was 1/200 000, although the actual incidence is likely to be higher [6]. The pathognomonic features of CS include mucocutaneous wart-like lesions, which are present in 99% of affected individuals before the age of 30 years [3]. The other most commonly reported manifestations are breast carcinomas, thyroid abnormalities, macrocephaly, hamartomatous polyps and mental retardation [7,8]. In women with CS, the lifetime risk of breast cancer is estimated to be 25-50%, compared to the general female population risk of approximately 11%. The lifetime risk of epithelial thyroid cancer can be as high as 10% in both males and females with CS. Follicular carcinoma is the most common CS-associated thyroid cancer, although papillary carcinomas have also been observed [9,10].

The present report describes the identification of a novel *PTEN* germline mutation in a female CS patient who developed multiple primary tumors and subtle skin lesions.

Case presentation

A 55-year-old woman suspected to have CS was referred to the Department of Biology and Medical Genetics, University Hospital Motol, Prague, for *PTEN* gene analysis to confirm the diagnosis.

Her medical history was unremarkable until she developed a goiter at 34 years of age. At 45 years of age, she

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underwent a strumectomy. A histopathological examination confirmed a macrofollicular adenoma in the left thyroid lobe and an encapsulated thyroid tumor in the right thyroid lobe. At 54 years of age she underwent a total thyroidectomy due to a follicular carcinoma (Figure 1a).

At 43 years of age she underwent a hysterectomy for myomatosis and adnexectomy due to an adenopapillocarcinoma of the left ovary.

At the age of 49 years, the patient was admitted to hospital with epileptic paroxysms. A CT scan of the brain showed a left frontal lesion, which was the cause of the paroxysms. The lesion was surgically removed and histopathologically classified as a benign meningioma (Figure 1b). At 53 and 57 years of age, she was again surgically treated for meningioma recurrence and a steadily deteriorating condition. Despite those surgical interventions, there was a progressive decline in cognitive functions and memory.

The initial brain tumor finding together with the observed cachexia led to the suspicion of metastasis from an unknown primary cancer. Careful endoscopic examination revealed hundreds of polyps in the stomach, duodenum and colon. There were numerous mostly hyperplastic and hamartomatous polyps up to 10 mm in diameter in the stomach. Smaller and multiple sessile polyps were found in the duodenum. The esophagus was free of any polyps. Along the entire length of the colon were multiple histopathologically confirmed hyperplastic and inflammatory polyps as well as tubulovillous or villous adenomas of 4-10 mm in diameter, with low-grade dysplasia.

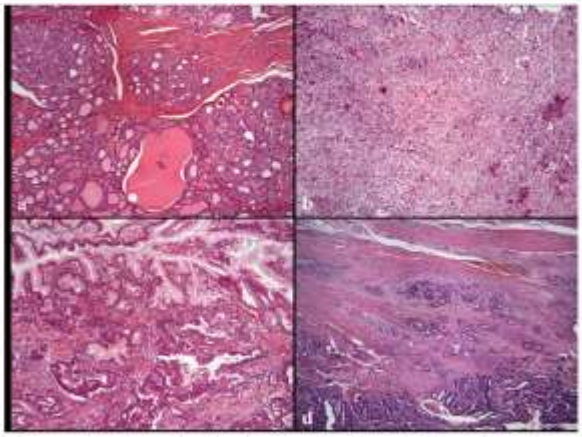

Figure 1 Histopathologic examination of the CS patient. a) Minimally invasive follicular carcinoma of the thyroid gland (H&E ×100). b) Meningotheliomatous meningioma (H&E ×100). c) Gastric hyperplastic polyp with adenocarcinoma (H&E ×200). d) Tubular adenocarcinoma of the large bowel (H&E ×100).

At the age of 55 years, the patient was examined by a clinical geneticist (MS) who observed macrocephaly, a fissured tongue and a polyp of 0.5 cm in diameter in the dorsal part of the oral cavity. A few wart-like papules on the forearms and chest were classified as senile verrucoid lesions. No other mucocutaneous lesions were found on the face or oral mucosa. The patient's mother, whose medical history was unknown to us, died of colon cancer at the age of 56 years. No other members of the family have had signs of CS or any cancer. The patient had one child who was negatively tested for mutations in the *PTEN* gene.

Several biopsies were performed throughout the gastrointestinal (GI) tract over a 9-year period, and none showed evidence of malignant lesions. Then at 57 years of age, histopathological examination of biopsy material from a hyperplastic polyp in the stomach revealed a well-differentiated intramucosal adenocarcinoma of 8 mm in diameter (Figure 1c). One year after the gastric cancer had been detected, the patient developed an aggressively growing synchronous adenocarcinoma of the anorectum (T3N0M0) and the sigmoid colon (T3N0M0) (Figure 1d). Abdominoperineal surgery (i.e., the Miles' operation) was performed. However, the patient died at post-operative 1 month due to unmanageable complications.

Methods

This study was approved by the Ethics Committee of the University Hospital Motol, Prague, according to the Helsinki Principles. After receiving written informed consent, the genomic DNA of the patient was isolated from blood leukocytes using a Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MI, USA) according to the manufacturer's guide. The genomic DNA was amplified using intronic primers (sequences are available upon request) flanking the nine exons and a promoter region of the *PTEN* gene [11]. The PCR products were purified using a SureClean PCR purification kit (Bioline, London, UK). Bidirectional cycle sequencing of the PCR products was performed using a BigDye Terminator v3.1 Cycle Sequencing kit and an ABI 3130 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA).

Results and Discussion

Examination of the genomic DNA revealed a novel heterozygous mutation, c.438delT, in exon 5 (Figure 2). That mutation is predicted to lead to a frameshift that results in formation of a premature stop codon (p.Leu146X) for PTEN protein translation. The mutation is considered to be pathogenic and causative for CS disease.

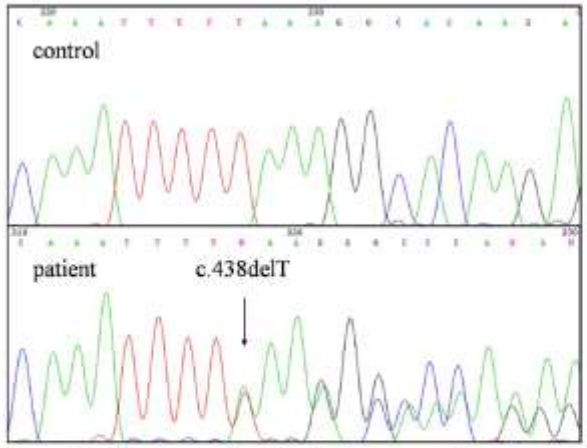

Figure 2 DNA sequencing electropherograms. Upper panel: Electropherogram of the *PTEN* gene, exon 5, from a healthy control. Lower panel: Electropherogram of the *PTEN* gene, exon 5, from the Cowden syndrome patient. Note the heterozygous deletion mutation at c.438delT (arrow).

More than 80% of patients who strictly meet the International Cowden Consortium criteria are found to harbor a *PTEN* mutation [12]. Mucocutaneous lesions are present in 99% of CS patients before the age of 30 years [3]. However, the present patient was not reported to have had mucocutaneous lesions until 55 years of age, when a targeted examination revealed subtle skin lesions. The only visible wart-like papules were found on her forearms. Such lesions are commonly found in the general population and could therefore have been overlooked or considered unremarkable.

The most common malignancies associated with CS are breast and thyroid carcinomas. Benign breast lesions (fibrocystic breast disease, fibroadenomas) and breast adenocarcinomas develop in about 76% and 25-50% of CS-affected females, respectively [3]. The average age of a breast cancer diagnosis in CS patients is between 38 and 46 years [13]. Marsh et al. observed an association between the presence of a *PTEN* mutation and malignant breast disease [12]. In our patient, a mammographic examination at 57 years of age showed no evidence of any breast lesions.

Benign thyroid lesions occur in as many as 60% and thyroid cancers in up to 10% of CS patients [3,9]. Our patient developed a follicular adenocarcinoma at 54 years of age, despite regular follow-up and treatment since her first admission to hospital due to a goiter at 34 years old.

Gynecological abnormalities are reported in 44% of females with CS [7]. Endometrial cancer is a major diagnostic criterion for CS, with an estimated frequency of 5-10% [7]. Ovarian tumors are rare in CS, with only one report documenting an ovarian dysgerminoma in a CS patient [14]. The hypothesis that a germline mutation of the *PTEN* gene was implicated in the formation of ovarian tumors in CS patients was supported by the identification of a somatic loss of heterozygosity (LOH) in the wild-type allele [14]. The present patient had myomatosis of the uterus and adenocarcinoma of the left ovary at the age of 43 years. Unfortunately, ovary tumor tissue was not available for LOH analysis.

Macrocephaly and Lhermitte-Duclos disease (dysplastic cerebellar gangliocytoma) are also major pathognomonic diagnostic criteria of CS [15]. Meningiomas (benign brain tumors) have also been described in CS patients, albeit rarely [16-19]. In our patient, the discovery of a meningioma launched an intensive and thorough follow-up which revealed additional and continually appearing symptoms. Benign brain lesions accompanied by epileptic paroxysms and a steadily deteriorating clinical condition were her main health problems.

Gastrointestinal polyps (mostly classified as hamartomatous, hyperplastic, inflammatory, juvenile, lymphomatous and adenomatous) are commonly reported in CS patients, with frequencies ranging between 35-85% and higher than 90% in Western and Japanese patients, respectively [9,20-24]. That difference in frequency is considered to reflect that GI examinations are more common in Japan [24]. In a study involving 126 CS patients, Marra et al., reported that of the 42 patients who underwent a complete GI examination, 36 (85%) had GI polyps. Non-adenomatous polyps that are generally asymptomatic may be more common in CS patients, especially when GI investigations are not part of the recommended surveillance for CS. Although an association between CS and GI cancer remains a topic of debate, reports describing malignant GI tumors in CS patients have been published [9,21-23, 25-27]. Although non-adenomatous polyps have a weak or absent malignant potential, there is evidence that carcinomas may arise within non-adenomatous polyps [26,28, and the present study]. Marra et al. stated that adenomatous polyps represent approximately 25% of CS polyps, and have a higher malignant potential than non-adenomatous polyps [22]. During a 9-year follow-up period, the present patient developed numerous GI polyps with various histopathological features (hyperplastic, inflammatory, hamartomatous and adenomatous). Ultimately, three polyps (a hyperplastic polyp in the stomach, and adenomatous polyps in the rectum and the sigmoid colon) progressed to macroscopic carcinomas.

Conclusion

Although breast and thyroid cancers are the predominant malignancies in CS patients, it should be emphasized that benign and/or malignant tumors may also develop in the GI tract from pre-existing polyps, and in the genitourinary tract or in the brain. Most of the studies that have examined cancer in CS patients do not

report the lifetime risk of development of a malignant neoplasm because the patients are relatively young. We propose that meningiomas and GI tract cancers, albeit rare, should be a component of the definition of CS. Physicians who might encounter CS patients should be aware of the possible neurological and/or GI tract manifestations.

Consent

Written informed consent was obtained from the patient for publication of this case report and for the use of the accompanying images. A copy of the written consent can be obtained from the Editor-in-Chief of this journal.

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Authors' contributions

PV carried out the molecular genetic study including the DNA sequencing, and drafted the manuscript. MS identified and diagnosed the patient. JH prepared, read and classified the histological samples. AK designed the study and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Publikácia 3

BMC Medical Genetics

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Research article

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Mutations in *STK11* gene in Czech Peutz-Jeghers patients

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Abstract

Background: Peutz-Jeghers syndrome (PJS) is an autosomal dominant hereditary disease characterized by mucocutaneous pigmentation and gastrointestinal hamartomatous polyposis. The germline mutations in the serine/threonine kinase 11 (*STK11*) gene have been shown to be associated with the disease. Individuals with PJS are at increased risk for development of various neoplasms. The aim of the present study was to characterize the genotype and phenotype of Czech patients with PJS.

Methods: We examined genomic DNA of 8 individuals from five Czech families by sequencing analysis of *STK11* gene, covering its promotor region, the entire coding region and the splice-site boundaries, and by multiplex ligation-dependent probe amplification (MLPA) assay designed for the identification of large exonic deletions or duplications of *STK11* gene.

Results: We found pathogenic mutations in *STK11* gene in two families fulfilling the diagnostic criteria of PJS and in one of three sporadic cases not complying with the criteria. The patient with the frameshift mutation in *STK11* gene developed aggressive gastric cancer. No other studied proband has developed a carcinoma so far.

Conclusion: Our results showed that a germline mutation of *STK11* gene can be found not only in probands fulfilling the PJS diagnostic criteria, but also in some sporadic cases not complying with the criteria. Moreover, we observed a new case of aggressive gastric cancer in a young patient with a frameshift mutation of *STK11* gene.

Background

Peutz-Jeghers syndrome (PJS; OMIM 175200) is an autosomal dominant disorder characterized by mucocutaneous pigmentation and gastrointestinal hamartomatous polyposis with an increased risk of cancer [1-4]. The cumulative risk of all cancers in PJS patients by the age of 60 years is 60% and is increased approximately by 8-fold as compared to general population [5]. Histopathologically, polyps in PJS are characterized as hamartomas. However, adenomatous changes may occur in polyps and they can become malignant. In addition to an elevated risk of gastrointestinal cancers, it has been described an increased risk of cancer development at other sites, particularly in the breast, ovary, uterus, cervix, pancreas, lung and testis [3,6-9]. Testicular sex cord and Sertoli cell tumors, leading to sexual precocity and gynecomastia [10-12], sex cord tumors with annular tubules and cervical adenoma malignum [13] have also been reported.

The gene responsible for PJS, denoted *STK11*, which encodes a serine/threonine kinase and mapps to chromosome 19p13.3, acts as a tumor suppressor [4,14,15]. It plays a role in the p53-dependent apoptosis pathway, in the vascular endothelial growth factor signaling pathway and in the polarization of epithelial cells [16-18].

About one-third of patients with PJS are diagnosed before the age of 10 years and up to 60% cases develop their first clinical manifestations until the third decade of life [19]. In most cases, initial symptoms are abdominal pain due to intussusceptions, obstruction and gastrointestinal bleeding with anemia [20,21]. A working definition of PJS has been suggested by Giardiello [3], where for individuals with a histopathologically confirmed hamartoma, the diagnosis of definite PJS requires two of the following three findings: a family history consistent with the autosomal dominant inheritance, mucocutaneous hyperpigmentation, or small-bowel polyposis. Tomlinson and Houlston [22] have modified the classification criteria for PJS for individuals without a family history of PJS, in whom the diagnosis depends on the presence of two or more histologically verified Peutz-Jeghers-type hamartomatous polyps.

There are some differential syndromes of PJS which could be misdiagnosed. The pigmentation of the perioral region is an external hallmark of PJS. It is not present in other hamartomatous polyposis syndromes which include Cowden syndrome (CS; OMIM 158350), Bannayan-Riley-Ruvalcaba syndrome (BRRS; OMIM 153480) and Juvenile polyposis syndrome (JPS; OMIM 174900). Laugier-Hunziker syndrome (LHS) is another differential diagnosis of PJS characterized by benign melanotic pigmentation of the oral cavity and lips, associated with spotted macular pigmentation of the fingerprints and longitudinal melanonychia. LHS is known to be a benign disease without gastrointestinal polyposis and with no systemic manifestation [23].

We report here a clinicopathological manifestation and mutational analysis of *STK11* gene in eight PJS individuals from five unrelated Czech families.

Methods

Patients

Eight patients from five unrelated families were included in the study (table 1). Four probands from two families fulfilled and three sporadic cases did not fulfill criteria to establish the diagnosis of definite PJS [3,22]. In one individual, we made a presumptive diagnosis of PJS due to a first-degree relative with PJS and the presence of mucocutaneous hyperpigmentation. All eight patients except one (A-2) underwent endoscopic procedures to examine the inspectable part of GIT.

Family A includes mother (case A-1) and her daughter (case A-2).

Case A-1 was a 29-year-old female with negative family history. The diagnosis of PJS was made at her 10 years of age due to hyperpigmentation of the lips, buccal mucosa, and perinasal region. X-ray examination of abdomen did not reveal any polyp. The patient was free from any abdominal symptoms. At her 24 years of age she underwent gastroscopy because of dyspepsia lasting for a few months. A rigid mucosa of the stomach was noted, but it was histolopathologically negative. Sixteen months later and seven months after giving birth, two hamartomatous polyps 4.5 cm and 1.5 cm in diameter and multiple small polyps 1–3 mm in diameter were found in her stomach. Colonoscopy revealed tubulous adenoma, 3 cm in diameter in her caecum. Enteroclysis did not show any pathology of the small intestine. Later on, she has been followed up every six months. During the follow up, a tubulovillous adenoma from sigmoid colon and a hamartomatous polyp from the transverse colon were removed, and at her 27 years of age, a well differentiated mucinous adenocarcinoma in her left inferior lung lobe was surgically removed. One year later, during second gestation, adenocarcinoma of the stomach was found. The patient refused termination of her pregnancy. Therefore, an operation was performed without any previous neoadjuvant therapy. Unfortunately, because of deterioration of her performance state, premature birth was induced at the 30-th week of the gestation. Three months later the patient died of gastric cancer.

Table 1: Clinical manifestations

Family	Case no	Sex	Age at onset/admission	Initial symptoms/signs	Histology of polyps	Location	Cancer	Mutation
A	*A-1	F	10	pigmentation	hamartomatous	throughout GIT	lung, stomach	+
	A-2	F	2	pigmentation	NA	NA	No	+
B	*B-1	F	36	pigmentation	hamartomatous	throughout GIT	No	+
	*B-2	M	10	pigmentation	hamartomatous	throughout GIT	No	+
	*B-3	M	6	pigmentation	hamartomatous	throughout GIT	No	+
C	C-1	F	2	pigmentation	adenomatous	small intestine	No	+
D	D-2	M	50	pigmentation	hyperplastic	colon	No	-
E	E-3	M	10	pigmentation	no polyps	no polyps	No	-

NA – not analysed, GIT – gastrointestinal tract, * – fulfillment of PJS criteria

Case A-2, a 7-year-old girl, presented with pale brown patches on the lower lip, which have been noted since her 2 years of age. Examination of her GIT was not performed.

The younger daughter, 4 years old, was not included in the study. She was free of any symptoms typical for PJS.

Family B comprises mother (case B-1) and her two sons (cases B-2 and B-3).

Case B-1, a 46-year-old female has presented with perioral and buccal pigmentation, lasting since childhood. She was found to have colonic and small intestinal hamartomatous polyps already at 36 years of age. The polyps from the stomach were histopathologically classified as hyperplastic with diffuse mixed inflammatory infiltration in stroma and focal epithelial metaplasia. Subsequently, repeated colonoscopy and enteroscopy with polypectomy have been performed. Eventually, total colectomy was performed due to excessive polyposis and recurrent GIT problems. Histopatholological examination of the polyps did not reveal any malignancy. Her family history is missing.

Case B-2 represented a 17-year-old boy with perioral brown pigmentation, mostly on the lips. At his 10 years of age, hamartomatous polyps in small the intestine were detected. Afterwards, he has been frequently examined by gastroduodenoscopy and colonoscopy and polypectomies have been performed. One polyp from the antrum of the stomach, 3 mm in diameter, was classified as hyperplastic with stromal inflammation. Three colon polyps, 3 mm in diameter, showed the typical features of hamartomatous lesions, branching strands of muscular tissue and numerous cystic dilatations of the glandular lumens of various sizes.

Case B-3, a 13-year-old boy, manifested with mucocutaneous brown to dark blue pigmentations on the lip, mostly on the lower one. At his 6 years of age, rectal bleeding due to a polyp, 55 × 35 × 20 mm, in the rectum was noted. Histopathological examination of the polyp showed a tubulovillous adenoma with mild dysplasia in the superficial colonic epithelium. Later on, he has undergone frequent gastroduodenoscopies and colonoscopies with polypectomies. Three polyps were classified as hamartomatous and one as a tubulovillous adenoma with low-grade dysplasia, 3 mm in diameter. All four polyps were excised from the sigmoid colon.

The remaining cases (C-1, D-1 and E-1) were sporadic.

Case C-1, a 20-year-old female has presented brown to dark blue pigmentations since her 2 years of age. At her 14 years of age, enteroclysis her small intestine showed one adenomatous polyp. Frequent colonoscopies and enteroclysies with negative results have been performed. At the time of the molecular analysis of her genomic DNA for germline *STK11* mutation, another capsule endoscopy examination was performed. A few (less than ten) diminutive polyps in the stomach and one polyp in the ileum were found. The polyp from the ileum showed characteristic histopathological features of adenoma. Polyps from the stomach were not biopsied. Her family history was negative.

Case D-1, a 50-year-old male with perioral and buccal pigmentation was found to have two hyperplastic polyps, one in the sigmoideum and the other one in the colon ascendens. Tubulovillous adenoma with low-grade dysplasia was excised from the ascending colon. The mother of the patient had colon cancer in 72 years of age and his father, a smoker, had lung cancer in 76 years of age. They were without hyperpigmentation.

Case E-1, a 10-year-old boy, was referred because of perioral and buccal pigmentation. Examination of his GIT did not reveal any polyp. His parents and step-siblings are without any PJS symptoms.

Genetic analysis

After receiving a written and signed informed consent, the genomic DNA of the patients was isolated from blood leukocytes using the Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MI, USA) according to manufacturer's guide. The genomic DNA was amplified using intronic primers [24,25] flanking the nine exons and the promoter region of *STK11* gene. PCR reactions were performed in a total volume of 30 μl containing 100 ng of genomic DNA, 50 pmols of each primer, 0.2 mM of each dNTP, 1 × *Taq* buffer containing 1.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase (Fermentas, Vilnius, Lithuania). The amplification was performed in 2720 Thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following protocol: an initial denaturation of 1 min at 95°C was followed by 33 cycles of 1 min at 95°C, 1 min at 53°C, 1 min at 72°C, and a final extension step of 7 min at 72°C. PCR products were purified using the SureClean PCR purification kit (Bioline, London, UK). DNA sequencing was performed using the purified PCR product, the BigDye Terminator v3.1 Cycle Sequencing kit and ABI 3130 Genetic Analyzer (both from Applied Biosystem) according to the manufacturer's instructions. Patients with negative results of DNA sequencing analysis were further examined by means of the multiplex ligation-dependent probe amplification (MLPA) method for the identification of large exonic deletions or duplications, using the P101-*STK11* MLPA kit (MRC Holland, Amsterdam, The Netherlands) and the following MLPA protocol http://www.mlpa.com/WebForms/WebForm

Main.aspx?Tag=wl2zCji\rCGANQgZPuTixtCplCA1mmwIoFo/xHPnTgc]. Samples were run and data were analyzed on ABI 3130 Genetic Analyzer in conjunction with Genotyper software (version 4.0; Applied Biosystems). Electropherograms were evaluated by visual examination of peak heights of the *STK11* fragments in relation to the adjacent control fragments and in comparison with external control DNA samples.

The study was conducted with approval by central ethical committee of Ministry of Health, Czech Republic, in accordance with the tenets of the Helsinki declaration.

Results and discussion

Excised polyps were histopathologically classified as hamartomatous in the cases A-1, B-1, B-2, and B-3, as adenomatous in the case C-1, and as hyperplastic in the case D-1. Case E-1 was free of any polyps (Table 1). All studied individuals had pigmentation of the lips and buccal mucosa, while it was most visible in the children patients. None of the probands had pigmentation of extremities. A positive family history of cancer was only noted in the case D-1. Mutation analysis revealed three different germline mutations. In the family A, a germline mutation (c.350dupT) in exon 2 (Fig. 1) was detected. This mutation is predicted to introduce a frameshift at codon Leu117, 46 novel amino acid residues, and a premature termination codon (p.Leu117PhefsX46). It was found in heterozygosity in both examined patients (A-1 and A-2).

Individuals from the family B (cases B-1, B-2, and B-3) harboured deletion of a part of the promoter region and exon 1 (Fig. 2). Case C-1 was a carrier of deletion of the whole *STK11* gene (Fig. 3). In the cases D-1 and E-1, we have not revealed any variation of *STK11* gene by using the aforementioned methods.

PJS is a relatively very well characterized disorder with a clear cut phenotype [22]. However, in sporadic cases, the diagnosis of PJS may be uncertain. Although multiple hamartomatous polyps of the GIT are pathognomonic of the PJS, hyperplastic and adenomatous polyps are commonly present [17]. Recently, it has been reported that *STK11* deletions are not a rare cause of Peutz-Jeghers syn-

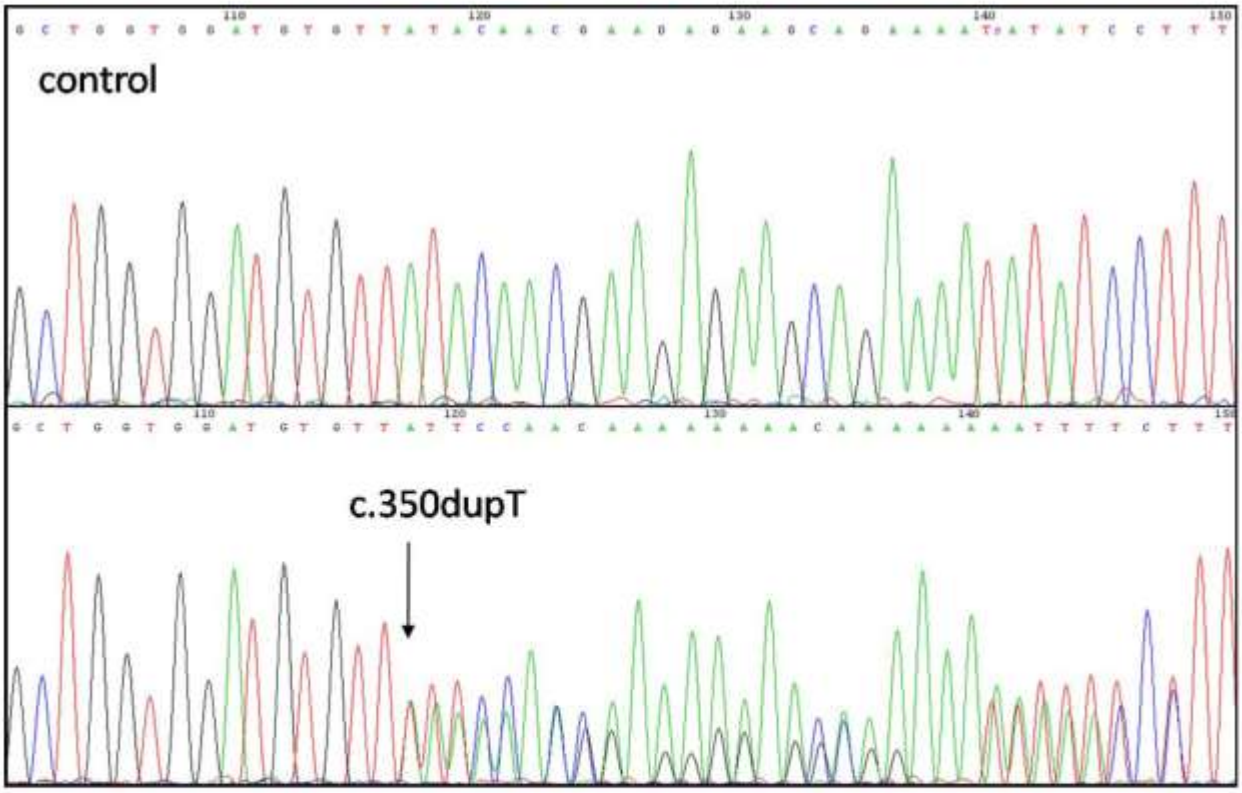

Figure 1
Sequencing chromatogram from the analysis of *STK11* gene in the family A showing a duplication of thymine in the position c.350, leading to a frameshift and a premature stop codon.

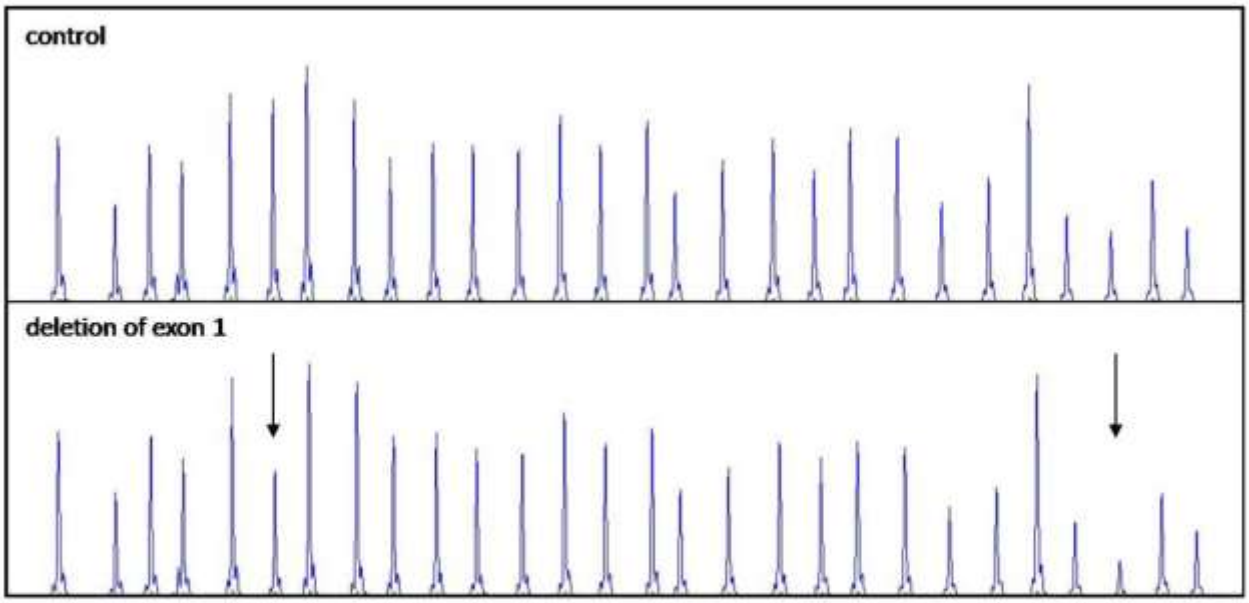

Figure 2
Representative chromatogram from MLPA analysis of *STK11* in the family B showing the relative reduction in the peak area of probes hybridising to exon 1 and a part of the promotor region (arrows mark the deleted regions).

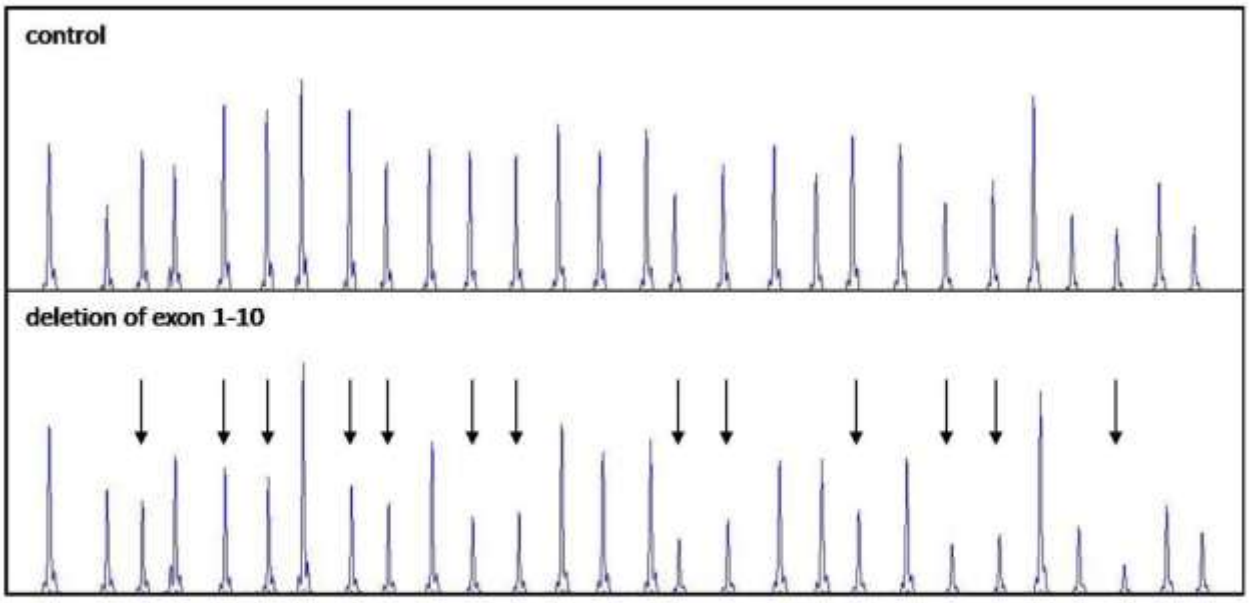

Figure 3
Representative chromatogram from MLPA analysis of *STK11* in the family C showing the relative reduction in the peak area of probes hybridising to exons 1–10 and the promotor region.

drome and account for up to 30% of patients with PJS [26,27]. There was no difference in the clinical phenotype between the patients with point mutations or with large genomic deletions [26]. However, the detailed phenotype of patients with different types of mutations was not reported.

Members of the family B (cases B-1, B-2, and B-3) had almost uniform clinical symptoms with variable age of onset of the first symptoms and detection of polyps. They are carriers of a germline mutation (deletion of a part of the promoter region and exon 1) of *STK11*. All three affected individuals had mucocutaneous hyperpigmentation predominantly on the lips and on the buccal mucosa, being the most prominent in the youngest patient (B-3) and very pale in the mother (B-1). Sons of the latter patient, i.e. the patients B-2 and B-3, were initially classified as PJS-suspected because of striking hyperpigmentation of the lips. Both of them are under careful surveillance. A few endoscopic examinations of the GIT have been performed so far, with polypectomy of hamartomatous polyps. This confirmed the diagnosis of definite PJS. Polyps were localized in the small intestine and colon. No polyps were detected in the stomach.

Deletion of the promoter region and exon 1 was reported in three independent studies in 12 PJS families overall [26-28]. It could be a recurrent mutation, probably a consequence of an unequal recombination mediated by repetitive Alu (SINE elements) sequences. In accord with UCSC Genome Browser, the region of chr19:1,129,999–1,196,665, where *STK11* gene is located, is rich for these repetitive sequences, which can be involved in large chromosome rearrangements. The implication of Alu repetitive elements in unequal genomic recombinations were described for another tumor suppressor gene, *MSH2*, implicated in Lynch syndrome (HNPCC) [29].

We failed to find any variation of *STK11* gene in sporadic cases D-1 and E-1, which would explain their phenotype. On the other hand, they did not fulfill criteria for the diagnosis of definite PJS [3,22]. We included these cases to the study on the basis of the result from the case C-1. Especially in case E-1 PJS polyps could develop later on. Studies with more individuals not fulfilling PJS diagnostic criteria were reported. None of the patients harboured a germline mutation of *STK11* gene [26,30]. Some studies suggested there could be another locus responsible for PJS phenotype [31,32]. Other authors stated according to their results that another locus is unlikely and the causative variation could be in regulatory regions such as promoter, enhancers, or splicing sites deep in introns, which are not detectable by conventional methods [26,27,33].

The risk of developing various types of GIT cancers (in the esophagus, stomach, small bowel and colon) was determined in several studies [3,5,7,8,34]. The cumulative risk for stomach cancer was 29% [8]. Amos et al. noted that gastric polyps are very common among individuals with PJS [30]. However, they did not specify the proportion of patients with a detectable PJS germline mutation and the gastric polyps/cancer. There are several case-reports and reviews reporting gastric cancer in PJS patients [3,20,35-44]. In our group of probands, the case A-1 had developed gastric cancer at 28 years of age and died one year later. No genotype-phenotype correlations were published in PJS patients with gastric cancer [7,30,33]. Konishi et al. reviewed 103 PJS patients with malignancy from literature and found out that the mean age of 8 cases with gastric cancer was 31.2 years as compared to 39.7 years in duodenal carcinoma (9 cases), and 48 years in colorectal carcinoma (13 cases). According to the literature and our results we suppose that gastric cancer has very aggressive course in some individuals with PJS and despite the very frequent endoscopic examinations with relevant treatment the next course is usually poor. Therefore, more attention should be paid to patients with molecularly confirmed PJS, especially those who have polyposis of the stomach. It would be particularly interesting to find out if there is a correlation between the genotype and phenotype in relation to the development of gastric cancer. There have been only two reports dealing with gastric cancer in PJS patients and mutational analysis of *STK11* gene so far [24,43]. Shinmura et al. described two PJS females (sisters) with gastric cancer in whom a *STK11* germline mutation (c.890delG) was identified [24]. Takahashi et al. reported a 14-year-old girl with sporadic PJS and early-onset gastric cancer harboring a frameshift (c.757_758insT) *STK11* mutation [43]. Similarly, as in our family A, the mutations led to a truncated protein lacking the kinase domain. These results suggest that the truncation mutations leading to loss of *STK11* kinase domain could act in a dominant negative fashion and be responsible of tumor development. Schumacher et al. summarized clinical and mutational data from 132 PJS cases (83 without and 49 with cancer) to find correlation between the type/site of mutation and cancer. They proposed two different mechanisms of tumor development. One is based on the loss of *STK11* functions due to truncation mutations and subsequent LOH as a second hit. This hypothesis is not in accord with findings of other authors, where a second hit was not a requisite condition for tumor development [24,43].

Conclusion

In summary, we found germline mutations of *STK11* gene in three families. One patient (C-1) with the germline mutation did not fulfill the criteria for establishing the diagnosis of PJS. Therefore, the variability in time of onset of symptoms should be always kept in mind when establishing the diagnosis of PJS and managing this disease.

Abbreviations

STK11: serine/threonine kinase 11; PJS: Peutz-Jeghers syndrome; MLPA: multiplex ligation-dependent probe amplification; BRRS: Bannayan-Riley-Ruvalcaba syndrome; JPS: Juvenile polyposis syndrome; LHS: Laugier-Hunziker syndrome; GIT: gastrointestinal tract; SINE: short interspersed element; UCSC: University of California Santa Cruz; *MSH2*: mutS homologue 2; HNPCC: hereditary nonpolyposis colon cancer; LOH: loss of heterozygosity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PV carried out molecular genetic studies including DNA sequencing, MLPA analysis for all the families, and drafted the manuscript. AP identified and diagnosed the patients. JR performed GIT examinations and provided histopathological information. AK designed the study and revised the manuscript. All authors read and approved the final manuscript.

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Molecular genetic analysis of 103 sporadic colorectal tumours in Czech patients

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Abstract

The Czech Republic has one of the highest incidences of colorectal cancer (CRC) in Europe. To evaluate whether sporadic CRCs in Czech patients have specific mutational profiles we analysed somatic genetic changes in known CRC genes (*APC*, *KRAS*, *TP53*, *CTNNB1*, *MUTYH* and *BRAF*, loss of heterozygosity (LOH) at the *APC* locus, microsatellite instability (MSI), and methylation of the *MLH1* promoter) in 103 tumours from 102 individuals. The most frequently mutated gene was *APC* (68.9% of tumours), followed by *KRAS* (31.1%), *TP53* (27.2%), *BRAF* (8.7%) and *CTNNB1* (1.9%). Heterozygous germline *MUTYH* mutations in 2 patients were unlikely to contribute to the development of their CRCs. LOH at the *APC* locus was found in 34.3% of tumours, MSI in 24.3% and *MLH1* methylation in 12.7%. Seven tumours (6.9%) were without any changes in the genes tested. The analysis yielded several findings possibly specific for the Czech cohort. Somatic *APC* mutations did not cluster in the mutation cluster region (MCR). Tumours with MSI but no *MLH1* methylation showed earlier onset and more severe mutational profiles compared to MSI tumours with *MLH1* methylation. *TP53* mutations were predominantly located outside the hot spots, and transitions were underrepresented. Our analysis supports the observation that germline *MUTYH* mutations are rare in Czech individuals with sporadic CRCs. Our findings suggest the influence of specific ethnic genetic factors and/or lifestyle and dietary habits typical for the Czech population on the development of these cancers.

Introduction

Colorectal cancer (CRC) is the second most common form of cancer in Europe, and the Czech Republic has the second highest CRC incidence and mortality among 38 European countries [1]. The reasons for this are unknown and can include both genetic and environmental factors. Hereditary cancer susceptibility syndromes account for no more than 5% of CRC cases [2]. The major autosomal dominant disorders with a high risk of CRC include Lynch syndrome (hereditary non-polyposis colorectal cancer, HNPCC), familial adenomatous polyposis (FAP), Peutz-Jeghers syndrome (PJS) and juvenile polyposis (JP) [3]. *MUTYH* associated polyposis (MAP) is an autosomal recessive hereditary CRC predisposition [4]. The incidence of germline *MUTYH* mutations in Czech FAP negative sporadic CRC patients is lower compared to other European countries [5,6], and there seems to be no increased incidence of the autosomal dominant forms either.

About 75% CRCs are sporadic, occurring in individuals with no remarkable family history of the disease. Dietary and other lifestyle-related and environmental factors are supposed to play an important role in the aetiology of this form of CRC. Sporadic CRCs have even more biological variables compared to hereditary CRCs. Most sporadic CRCs have mutations in the *APC* gene [7], and an increased rate of G:C>T:A transversions in *APC* can also reveal "hidden" MAP patients [4]. Similarly defects in several other pathways result in other specific mutation signatures [8]. *APC* negative tumours can carry *CTNNB1* gene mutations [9,10]. The mutation status of the *KRAS* and *TP53* genes, two other key players in CRC [11], can reflect carcinogen exposure and reveal the tumour aetiology [12]. Microsatellite instability (MSI) is found in about 15-20% of CRCs; 3-5% are associated with Lynch syndrome and the remaining are sporadic [13]. MSI is associated with *MLH1* promoter methylation, somatic *BRAF* mutations, and has an inverse relationship with *APC* mutations [14,15]. Thus, the molecular genetic landscape of CRC is rather complex. However, its exploration is a prerequisite for personalized molecular medicine and identification of biomarkers for early detection of tumours, risk stratification, prognosis and prediction of treatment responses [16].

The aim of our study was to contribute to the understanding of CRC tumorigenesis by the analysis of most genes known to be implicated in CRC, and by correlating the molecular genetic profiles of the tumours with their clinical and histopathological data. We also focused on the molecular genetic features of the Czech CRC patients, because we hypothesized that their high

incidence and mortality could be accompanied by specific mutation profiles, which could reflect possible specific ethnic, geographical, dietary or lifestyle factors. To this aim we analysed the complete coding region of the *APC* gene and loss of heterozygosity (LOH) at the *APC* locus, the *CTNNB1, MUTYH, KRAS* and *TP53* genes, as well as MSI, methylation status of the *MLH1* promoter, and *BRAF* mutations in CRCs from 102 Czech patients.

Material and methods

Ethics Statement

The study was based on informed consent and approval of the local ethics committee.

Patients

The samples were obtained from unselected consecutive patients who had undergone curative surgical resection for primary colorectal cancer at the Department of Surgery, University Hospital Motol, Prague, Czech Republic. Because of our focus on sporadic CRC patients, we excluded individuals with family history of CRC disease and/or presence of polyps. Also excluded were patients who received preoperative radiotherapy, patients with low quality of the DNA sample, and patients in whom no matching mucosa sample was available. Finally 103 tumours and matching normal tissues were collected from 102 CRC patients (51 males and 51 females, age at tumour onset 13-86 years, median 64 years) at the Department of Surgery, University Hospital Motol, Prague, Czech Republic. Fifty-six patients were from Prague and the rest were from all regions of the Czech Republic. All tumours were fresh-frozen at -70°C at colectomy. A minimum of 85% of neoplastic tissue was present in each resected sample as assessed by a pathologist. The characteristics of the patients and clinical and histopathological features of their tumours are shown in Table S1.

Mutation analysis

DNA was prepared using the Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MI, USA) according to the manufacturer instructions. *APC* exons 1-15, *TP53* exons 2-10, *CTNNB1* exon 3 and *KRAS* exons 1-2 were amplified in PCR reactions containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 0.5% (v/v) Nonidet P40, 0.5% (v/v) Tween 20, 50% (v/v) glycerol, 200 μM dNTPs, 1 U of Taq Polymerase (Fermentas, Glen Burnie,

MD, USA), 3 pmol of each primer, 1 µl of 10x LCGreen Plus Dye (Idaho Technology, Salt Lake City, UT, USA) and 20 ng of DNA in a total volume of 10 µl for 1 min at 95°C, 45 cycles of 1 min at 95°C, 30 s annealing, 30 s at 72°C, and then 7 min at 72°C. Heteroduplexes were formed by heating the PCR products to 95°C for 2 min and cooling down to 4°C, and subjected to high resolution melting (HRM) analysis using LightScanner (Idaho Technology). *BRAF* exon 15 and *MUTYH* exons 6-8, 12 and 13-14 were sequenced directly from PCR amplicons prepared as above but with 10 pmol of each primer, no dye, in a total volume of 30 µl for 1 min at 95°C, 32 cycles of 1 min at 95°C, 1 min annealing, 1 min at 72°C, and then 7 min at 72°C. Annealing temperatures, $MgCl_2$ concentrations and primer sequences are available upon request. PCR products with suspected variations identified by HRM were purified using the SureClean PCR purification kit (Bioline, London, UK) and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Somatic mutations found in tumours were also analysed in the corresponding mucosa to assess their germline status. The functional impact of APC mutations was predicted using Polyphen [17]. Bioinformatic analysis also used the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/snp), the UMD p53 Mutation Database (UMD, http://p53.free.fr/Database/p53_database.html, The p53 Handbook 2.0), and the Leiden Open Variation Database (LOVD, v.2.0 Build 29, http://www.insight-group.org/mutations/). In tumours with 2 or more *APC* mutations separated by a distance of ≤ 5 kb the phase of the mutations was analysed using allele-specific PCR and sequencing. The primer sequences are available upon request.

LOH analysis

LOH at the *APC* locus was tested using the microsatellite marker D5S346 and capillary electrophoresis on an ABI 3130 Genetic Analyser. Allelic loss was scored if the area under one allelic peak in the tumour was reduced by 50% or more relative to the other allele, after correcting for the ratio of allelic peak areas in normal DNA. Samples with constitutional homozygosity at D5S346 or showing MSI in tumours were scored as non-informative.

MSI analysis

MSI was assessed at five microsatellite loci (Bat-25, Bat-26, D2S123, D5S346, and D17S250) as described previously [18]. Matching normal and tumour DNA samples were compared, and tumours showing instability at one locus were scored as MSI-low (MSI-L), at two or more loci as MSI-high (MSI-H).

DNA methylation assay

DNA methylation of the *MLH1* promoter was analysed using methylation specific multiplex ligation-dependent probe amplification (MS-MLPA). SALSA MS-MLPA Kit ME011-A1 (MRC-Holland, Amsterdam, The Netherlands) with 6 probes in the *MLH1* gene was used according to the manufacturer instructions. PCR products were analysed using an ABI 3130 Genetic Analyser. Data analysis was performed with the Genemapper and Coffalyser software (Applied Biosystems and MRC-Holland, respectively). The relative peak area of the signal from a specific probe was calculated by dividing the peak area by the combined areas of peaks of the control probes and multiplying the value by 100. The relative peak areas of probes from the Hha I digested sample were compared with those from the corresponding undigested sample, giving the percentage ratio of methylation at CpG sites. The cut-off value for aberrant methylation was set to 25% or higher.

Immunohistochemistry (IHC)

A portion of each tissue sample was formalin fixed, embedded in paraffin and processed using standard histopathologic procedures. Representative blocks containing enough of tumour and normal tissue were cut to 4 μm sections, deparaffinised and rehydrated. Target Retrieval Solution, High pH (DakoCytomation, Glostrup, Denmark) was used for epitope retrieval at 96 ºC for 30 min. The sections were incubated overnight at 4 ºC with primary monoclonal mouse anti-human *MLH1* and *MSH2* antibodies (clones G168-15 and G219-1129, BD Biosciences, NJ, USA) diluted 1:100. The *MLH1* antibody complexes were visualized using the streptavidin-biotin detection kit LSAB+, Dako REAL Detection Systems, HRP/DAB+, Rabbit/Mouse (DakoCytomation) and 3,3´-diaminobenzidin tetrahydrochlorid (DAB, Fluka Chemie, Buchs, Switzerland). The *MSH2* antibody complexes were localised using N-Histofine Simple Stain MAX PO (MULTI) (Nichirei Biosciences, Tokyo, Japan) and DAB. All sections were stained with hematoxylin, dehydrated and mounted. Nuclear staining only was considered for both

antibodies, and the normal tissue in the same section was used as an internal positive control. Only cases with complete negativity of all tumour cells and positivity of the internal control were interpreted as negative and suspicious of *MLH1* or *MSH2* gene dysfunction. This IHC analysis was performed in 39 tumours (see Table S1).

Statistical analyses

Statistical analyses were carried out using GraphPad InStat 3.10 (GraphPad Software, La Jolla, CA, USA) and IBM SPSS Statistics version 18 (IBM Corporation, New York, USA). Fisher's exact, Chi-square or Exhaustive CHAID tests were used where appropriate. All P values were two-tailed, and P values less than 0.05 were considered statistically significant. Mutations of the genes tested and their combinations, LOH, MSI and *MLH1* promoter methylation were correlated with age at tumour onset, gender, location of the tumour, and its histopathological characteristics.

Results

Details of mutation profiles, LOH, MSI, DNA methylation and IHC of individual tumours are shown in Table S1.

Distribution of genetic defects in tumours

Tumours were scored as *APC* mutated if they carried at least one clearly deleterious *APC* mutation (mutations leading to premature termination and missense mutations found in CRC but not in the corresponding mucosa, absent from dbSNP and predicted to be pathogenic - a total of 103 mutations). Overall, 71 tumours (68.9%) had the *APC* gene mutated (Table 1): 45 CRCs had 1 deleterious mutation, 23 had 2 mutations, 2 had 3 mutations and 1 had 6 mutations. Seven additional tumours (6.8%) showed no mutation but had LOH at the *APC* locus. Twenty tumours had neither a mutation nor LOH. The remaining 5 non-mutated tumours were not informative. Three missense *APC* variants (R382S, N813S and A1366V) were absent from mucosa and dbSNP but were predicted to be benign, totalling the number of somatic *APC* variants observed to 106.

LOH at the *APC* locus was found in 35 of 90 informative tumours (38.9%). Of 63 informative tumours with the *APC* gene mutated, 28 had LOH at the *APC* locus (44.4%). Of 43 tumours with

only one deleterious *APC* mutation, 27 had LOH (62.8%), while of 20 tumours with 2 and more mutations, only 1 had LOH (5%, $P < 0.0001$). Mutant allele specific amplification was possible in 12 of 26 tumours with 2 and more *APC* mutations to show their phase. Fourteen other samples could not be assessed due to a large distance between the mutations (≥ 10 kb, 10 tumours; ≥ 5 kb, 4 tumours). In all 12 tumours tested the mutations were in trans configuration. Out of the 27 tumours with LOH and one *APC* mutation, 15 had LOH of the wildtype allele based on the relative signal intensity of the mutated and wildtype alleles, 6 had LOH of the mutant allele and 6 cases could not be unequivocally resolved. However, any admixture of normal mucosa can partly mask the LOH of the wildtype allele.

TP53 mutations were considered deleterious if they were absent from mucosa and dbSNP, and were not listed as polymorphisms in UMD. Tumours with any variation in codons 12, 13 or 61 of *KRAS* and in exon 15 of *BRAF* were classified as mutated. In contrast to *APC*, no tumours contained more than one mutation in any of these 3 genes. Simultaneous occurrence of mutations in the *APC*, *TP53*, *KRAS* and *BRAF* genes is shown in Figure 1. The most common combination of mutated genes was *APC* and *KRAS* (35.6%), *APC* and *TP53* (31.1%), whereas no tumours had mutations in *TP53* and *KRAS* only. *BRAF* mutations were mutually exclusive with *KRAS* mutations, and four *BRAF* mutations were found in tumours with MSI. Fifteen tumours lacked mutations in any of these 4 genes; however, 3 of them had LOH in *APC*. Of the remaining 12 tumours, 2 were not informative for LOH and 10 were lacking any detectable defect in these 4 genes.

Only 2 tumours had mutations in the *CTNNB1* gene. One of them was the tumour with 6 deleterious *APC* mutations mentioned above. No other mutations or LOH were found in the two *CTNNB1* mutated tumours. Two other CRCs carried heterozygous *MUTYH* mutations which were also present in the mucosa, thus excluding the presence of MAP patients in our cohort.

MSI was found in 25 tumours (24.3%), out of which MSI-H in 21 cases (20.4%) and MSI-L in 4 cases (3.9%). Twelve tumours with MSI (all MSI-H) had the *MLH1* promoter methylated. None of the patients with a MSI tumour had a relative with a HNPCC-related cancer. Thirteen MSI tumours without *MLH1* methylation displayed more severe mutation profiles (11 had an *APC* defect, 4 *KRAS*, 2 *CTNNB1* and 3 *TP53* mutations, and only 1 showed no other genetic defect than MSI). This contrasted to 12 tumours with MSI and *MLH1* methylation, of which 7

had no other genetic defect or a *BRAF* mutation (P = 0.0099). Six of 9 *BRAF* mutated tumours showed MSI, and all of those had the *MLH1* promoter methylated and were proximally located.

Mutation spectra

Considering all 106 variants found in *APC*, point substitutions (46 nonsense, 9 missense and 3 splice, 54.7%) were slightly more frequent than frameshift (FS) mutations (29 deletions, 18 insertions/duplications and 1 indel, 45.3%, Figure 2). However, in the mutation cluster region (MCR) [7], FS mutations occurred more often than point substitutions (34 FS (64.2%) and 19 point substitutions (35.8%) out of the total of 53 mutations in MCR, P = 0.0003, Figure 2). Of the total of 31 *APC* mutations in MSI tumours, 15 were FS (48.4%) and 16 point substitutions (51.6%). This was similar to tumours without MSI, where out of the total of 72 mutations 33 were FS (45.8%) and 39 point substitutions (54.2%, P = 0.8326).

Of 28 *TP53* mutations observed, 15 (53.6%) were missense and 13 (46.4%) were FS and nonsense, which was significantly different from CRC mutations listed in UMD, where missense mutations were highly predominant (81% missense, 19% FS and nonsense, P = 0.0005, X^2 test). *TP53* mutations were located in exons 4-10, with 64% of them in exons 5-8. Interestingly, only 1 *TP53* mutation (4%) belonged to the 10 most common CRC mutations listed in UMD, where out of 3584 mutations 1566 (43.7%) were in these hotspots (P = 0.004, X^2 test, Figure 3). Compared to UMD, we could observe less G:C>A:T but more G:C>C:G, G:C>T:A and FS events (Figure 4).

The majority of *KRAS* mutations, 23 (71.9%), were found in codon 12. Codons 13 and 61 were involved in 5 (15.6%) and 4 (12.5%) tumours, respectively. The most common substitution was G>A (16, 50%), G>T (11, 34.4%) and A>C (3, 9.4%), while G>C and A>T substitutions were present once each (3.1%).

No difference in tumour spectra was observed between males and females (Table S1).

Correlation of molecular findings with clinical and IHC data

APC and *KRAS* mutations did not show any significant correlation with tumour location, stage, grade and lymph node involvement, age at onset or sex of the patient (Table S1). *BRAF* mutations were correlated with proximal tumour location: 7 of 36 proximal (19.4%) and 2 of 58 distal (3.5%) tumours were mutated (P = 0.0323). *TP53* mutations were more frequent in distal

tumours: 21 of 60 distal tumours (35.0%) were mutated compared to only 7 of 43 proximal tumours (16.3%, P = 0.0440), and in invading tumours: 17 of 43 invading tumours (39.5%) were mutated compared to only 9 of 56 non-invading tumours (16.1%, P = 0.0114, 4 tumours lacked information). Exhaustive CHAID test revealed that tumours with *TP53* mutations had a tendency to skip stage II or progress through it very quickly to stage III (P = 0.002, Figure 5).

The number of *CTNNB1* mutated tumours was too low for any correlation. Both were in distal colon and showed lymphatic invasion. Concerning *MUTYH,* one mutation carrier was an 83 year old woman with stage II, grade 2, proximally localized CRC without lymphatic invasion and MSI but with 2 FS *APC* mutations and one splice *TP53* mutation. The other *MUTYH* mutation carrier was a 58 year old man with proximally localized stage III, grade 3 CRC, with no lymphatic invasion, showing MSI-H and *MLH1* methylation but no other genetic defects.

Both MSI and *MLH1* promoter methylation were significantly more frequent in proximal tumours (MSI in 21 of 43 proximal tumours but only in 4 of 60 distal tumours, P = <0.0001; *MLH1* methylation in 13 of 30 proximal tumours but in none of 60 distal tumours, P = <0.0001, Table 1). The mean age at tumour onset was similar in patients with and without MSI (62.7 and 64.7 years, respectively, P = 0.4723). However, the mean age at onset in 13 patients with MSI without *MLH1* methylation was lower (58.2 years) compared to that in 12 patients with MSI and *MLH1* methylation (67.6 years, P = 0.0894). The tumour stage was comparable in both groups. The tumour grade was predominantly I+II in the first group and III in the second (P = 0.0820).

Discussion

We analysed mutation profiles in sporadic CRCs of Czech patients, in whom CRC incidence and mortality is one of the highest in Europe and is still increasing [1,19].

APC mutations are the key player in CRC tumorigenesis. However, mutation analysis of the *APC* gene is time-consuming and expensive, and is often limited to MCR which covers about 10% of the APC coding region [7]. In several studies where the entire gene was sequenced the frequency of mutations in CRCs was 60% [15,20], similar to our results (68.9%). If we restricted our analysis to MCR only, we would miss 59 mutations (55.6%) including 33 mutations in 26 tumours (25.2%) which would be classified as non-mutated. Some reports indicated an interdependence of two hits in *APC* both in sporadic and in FAP associated CRCs: *APC* mutations in the MCR were predominantly associated with LOH while mutations outside the

MCR with another mutation [21,22]. In our cohort, mutations in the MCR and outside the MCR were equally associated with LOH, and many mutations outside the MCR were coupled with at least one mutation in the MCR. Point substitutions occurred more often outside the MCR compared to FS mutations (Figure 2), and commonly included C>T transitions at CpG sites mainly changing arginine codons to STOP as reported previously [15,20]. The frequency of *APC* defects rose to 75.7% if LOH at the *APC* locus was included (7 of our CRCs had LOH only). LOH at the *APC* locus was reported in 30-40% of CRCs [22], similarly to our results (35%). Significantly increased LOH in our tumours with just 1 somatic *APC* mutation, the trans position of 2 pathogenic *APC* mutations confirmed in tumours where the mutation phase was tested, and preferential loss of the wildtype allele in tumours with LOH and an *APC* mutation support the two-hit model.

CRCs with intact *APC* may carry mutations in *CTNNB1*, a critical downstream gene of the WNT signalling pathway [23], although these are rather rare in sporadic CRCs [9,10]. *CTNNB1* mutations may be more frequent in MSI-H tumours [9]. Both our tumours with *CTNNB1* mutations were MSI-H. One carried p.S45F, a likely activating mutation located in one of the hotspots and supposed to deregulate the WNT signalling instead of *APC*. Indeed, this tumour carried no *APC* mutation. The other CRC had the p.A20V mutation, which is not located in any of the critical sites, does not change amino acid polarity and may not be disease-causing. This tumour had several inactivating *APC* mutations.

Germline *MUTYH* mutations have lower incidence in the Czech Republic [5,6]. The frequency of biallelic carriers of 2 most prevalent Caucasian mutations, p.Y179C and p.G396D, among *APC* negative patients with polyposis was 2-40%, and the frequency of carriers of monoallelic *MUTYH* mutations among CRC patients was 0.9-4.2% [5]. Cases of biallelic *MUTYH* carriers with sporadic CRC without polyps are rare [24], and our study included primarily individuals without polyps. Of the monoallelic *MUTYH* mutations observed in our patients, the p.R182H mutation is pathogenic [25], while p.Q479L is of an unknown effect. An elevated risk of CRC was proposed for carriers of monoallelic *MUTYH* mutations [26], but two large studies did not confirm this conviction [27,28]. A retrospective analysis of pathological reports revealed 3 diminutive tubulovillous adenomas with moderate dysplasia in the carrier of the first mutation. However, neither of the 2 CRCs in our patients had the characteristic mutation profile [4], and therefore they were not likely caused by the germline *MUTYH* mutations.

In addition to "hidden" MAP patients the mutation analysis of the whole *APC* gene could also reveal other specific mutation profiles typical for Czech patients. We identified a remarkable patient with 8 somatic variants in the *APC* gene. Two of these point variants were nonsense, two splice, three missense (one of them predicted to be benign) and one silent, and, interestingly, the tumour was MSI-H. The patient was analysed in detail and the results will be published separately (manuscript in preparation).

MSI can be detected in up to 15% of sporadic CRCs and in almost all HNPCC-associated CRCs. While MSI in HNPCC tumours is caused by germline mutations in mismatch repair (MMR) genes, MSI in sporadic tumours is often associated with *MLH1* promoter methylation and accompanied by somatic *BRAF* mutations [14]. These changes were rarely if ever seen in HNPCC tumours, and may be mutually exclusive with *KRAS* mutations [29]. In our sample 24% of tumours showed MSI. This higher incidence can be explained by the exclusion of rectal tumours with neoadjuvant therapy that biased the distribution towards proximal tumours where the MSI frequency is higher [30]. Similarly also the exclusion of tumours with no available matching mucosa might have biased the sample against aggressive and rectal cancers. In our cohort the frequency of *MLH1* promoter methylation decreased with the distance of the tumour from caecum, and was completely absent in tumours of the distal colon (Table S1). Rare *MLH1* promoter methylation in rectal cancers was described in one study, but it was accompanied by high rate of MMR protein deficiency, possibly due to the inclusion of Lynch-associated tumours [31]. The rectal MSI-H tumours had worse prognosis compared to those without MSI, which could be caused by pre-operative irradiation or chemotherapy which had no effect or might even be harmful for MSI-H cancer patients [31]. In another study, distal MSI CRCs had lower incidence of *MLH1* methylation and worse prognosis compared to proximal MSI CRCs [32]. Our data suggest that proximal CRCs without *MLH1* methylation could have similar clinicopathological and molecular features as distal CRCs. Although we did not perform IHC analysis of all MMR proteins in all MSI tumours and therefore cannot exclude germline mutations in these genes, we suppose that tumours without *MLH1* methylation represent a different subgroup. The reason for the uneven localization of the MSI tumours, differential *MLH1* promoter methylation and earlier onset of MSI CRCs without *MLH1* methylation is unknown and can be caused by dietary habits, different environment (e. g. varying pH) in different parts of the colon, different genes involved or the combination of all of the above.

Our data confirmed the notion that *BRAF* mutations are frequently found in sporadic MSI tumours [29], and support the previous observation of *BRAF* mutations in about 5% of CRCs without MSI [29,33]. In accordance with other reports [34,35], we have found the inverse association between *BRAF* and *APC* mutations. Six of 9 tumours with a *BRAF* mutation had no somatic *APC* mutation. None of these 9 tumours had a *KRAS* mutation either, which is in accord with others [33,34]. We did not observe any differences in clinicopathological features of these tumours. MMR deficiency, irrespective of its genetic or epigenetic origin, leads to the mutator phenotype, and FS *APC* mutations, predominantly in mononucleotide tracks, are more frequent in MSI tumours [36]. The mutational spectrum of our MSI tumours was not different from that in tumours without MSI, but FS mutations were more frequent in proximal MSI tumours without *MLH1* promoter methylation.

The increased incidence of CRC in the Czech Republic can be partly explained by the joint effect of elevated smoking prevalence and obesity [19,37]. Mutation signatures in *TP53* can reflect DNA damage induced by specific carcinogens, ethnicity or lifestyle habits [12]. For example, exposure to ultraviolet light is correlated with *TP53* transitions at dipyrimidine sites (CC>TT) [12]; aflatoxin B_1 exposure with G:C>T:A transversions in codon 249 in hepatocellular carcinoma; and exposure to cigarette smoke with G:C>T:A transversions in lung carcinoma [12]. *TP53* mutations observed in our sample included very few hotspot codons, and their pattern and distribution was distinct from that of CRC mutations listed in UMD (Figure 3, 4). Czech CRC patients have less G:C>A:T transitions and more FS, G:C>C:G and G:C>T:A events, although no predominant mutational event or specific hot spot can be observed. These mutations are caused by polycyclic aromatic hydrocarbons (PAHs) [38,39]. One of the main sources of PAHs except tobacco smoke and environmental pollutants is high-fat diet rich for smoked red meat [40]. PAHs are formed on the surface of meat at high temperatures [41]. Home production of smoked food and high consumption of red meat products is characteristic of Czech households, especially in rural areas [42]. It remains to be verified on a larger set of CRCs if these dietary habits are the cause of the *TP53* mutation signature observed. Another explanation could involve population differences in the frequency of functional polymorphisms in DNA repair genes, which could modify the risk of CRC [43]. Further research is needed to address this scenario.

Frequency and spectrum of *KRAS* and *APC* mutations did not differ compared to most other reports. This could be partly explained by the nature of mutations in these genes and their ability to give the cell a growth advantage leading to positive clonal selection. Only *KRAS* mutations in codons 12, 13, and 61 and nonsense *APC* mutations are considered to give such advantage, and therefore the investigation of mutational spectra of these genes is of limited use [44].

No tumours had concurrent *TP53* and *KRAS* mutations in context of non-mutated *APC* (Figure 1). Similar findings were noted in 2 other studies [15,45]. Concurrent *TP53* and *KRAS* mutations could be disadvantageous for tumour progression and may arise only on the *APC* mutation background.

Nowadays two main independent molecular pathways of colorectal tumorigenesis have been proposed: the conventional adenoma-carcinoma pathway characterised with the initial inactivation of the *APC* gene, accumulation of mutations in other genes and chromosomal instability [46]; and the serrated pathway with microsatellite instability, a relatively high frequency of *BRAF* mutations and increased level of DNA methylation [47]. Although most of CRCs could be clearly classified into one of these pathways, they overlap and the mutational profile of a CRC may show evidence of both. Thus, the classification of many tumours remains ambiguous, e.g. of MSI tumours without *MLH1* promoter methylation but with severe mutational profiles and earlier onset of the disease compared to Lynch associated tumours, as reported here and elsewhere [31,32]. Although we cannot exclude the possibility that among 13 MSI tumours from our cohort without *MLH1* promoter hypermethylation there may be a hidden Lynch associated tumour, the selection criteria and the relatively low percentage of true Lynch tumours among unselected CRCs [48] stand against it.

There is an increasing effort to assess individual specific molecular alterations for personalized diagnosis, prognosis and/or treatment. As can be seen from our results, tumours with mutations in multiple genes often had better staging or grading compared to tumours with no or only very few genetic defects. This implies that focusing on a single gene or defect or interpretation of the findings using too simple rules may be misleading. Systematic sequencing of cancer genomes reveals the diversity of cancer as to the number and pattern of mutations arising probably due to DNA repair defects, mutagenic exposure and cellular metabolism [49]. It has been shown that a single CRC can harbour up to 76 point mutations and 9 copy number changes, and that rather

whole pathways than individual genes govern the process of carcinogenesis [50,51]. High-throughput methods like next-generation sequencing or copy number variation arrays can therefore be more helpful in managing cancer patients.

In summary, the molecular genetic analysis of CRCs in Czech patients confirmed the data from other studies but also yielded potentially novel findings. First, MSI tumours with unmethylated *MLH1* promoter have earlier onset and more severe mutational phenotype. Second, the Czech pattern and distribution of *TP53* mutations differ significantly from published data. Third, mutational analysis of the whole coding region of the *APC* gene significantly increases the yield of the analysis, but did not pinpoint any MAP patient, confirming that germline *MUTYH* mutations are rare in the Czech population.

Supporting Information

Table S1 The characteristics and clinical features of the patients and mutation profiles, LOH, MSI, MLH1 methylation, IHC and histopathology of their tumours.

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Table 1. Numbers of tumours with genetic defects studied

(n)	*APC* mutated (%)	*KRAS* mutated (%)	*TP53* mutated (%)	*BRAF* mutated (%)	*CTNNB1* mutated (%)	with *MLH1* methylation (%)	with MSI (%)
all CRC (103)	71 (68.9)	32 (31.1)	28 (27.2)	9 (8.7)	2 (1.9)	13 (12.6)	25 (24.3)
Sex of the patient							
CRC in females (51)	36 (70.6)	14 (27.5)	15 (29.4)	4 (7.8)	1 (2)	7 (13.7)	11 (21.6)
CRC in males (52)	35 (67.3)	18 (34.6)	13 (25)	5 (9.6)	1 (1.9)	6 (11.5)	14 (26.9)
Tumour location							
proximal CRC (43)	26 (60.5)	10 (23.3)	7* (16.3)	7* (14.9)	0 (0)	13** (30.2)	21** (48.8)
distal CRC (60)	45 (75)	22 (36.7)	21* (35)	2* (3.3)	2 (3.3)	0** (0)	4** (6.7)
Tumour stage							
I (18)	14 (77.8)	5 (27.8)	7 (38.9)	1 (5.6)	0 (0)	1 (5.6)	4 (22.2)
II (38)	24 (63.2)	12 (31.6)	2 (5.3)	5 (13.2)	0 (0)	9 (23.7)	14 (36.8)
III (32)	23 (71.9)	8 (25)	12 (37.5)	2 (6.3)	2 (6.3)	2 (6.3)	4 (12.5)
IV (12)	7 (58.3)	4 (33.3)	5 (41.7)	1 (8.3)	0 (0)	1 (8.3)	2 (16.7)
n.a. (3)	3	3	2	0	0	0	1
Lymphatic invasion							
0 (56)	38 (67.9)	17 (30.4)	9* (16.1)	6 (10.7)	0 (0)	10 (17.9)	18 (32.1)
I+II (43)	29 (67.4)	11 (25.6)	17* (39.5)	3 (7)	2 (4.7)	3 (7)	6 (14)
n.a. (4)	4	4	2	0	0	0	1

n.a., information not available; * significant (P<0.05); ** significant (P<0.0001, Fisher's exact)

Figure Legends

Figure 1: Distribution of mutations in the *APC*, *TP53*, *KRAS* and *BRAF* genes in 103 tumours studied. Nineteen tumours carried no mutations in these genes. [a] this group includes 1 tumour with a *CTNNB1* mutation; [b] this group includes 1 tumour with a germline *MUTYH* mutation; [c] this group includes 1 tumour with 8 point substitutions in *APC*.

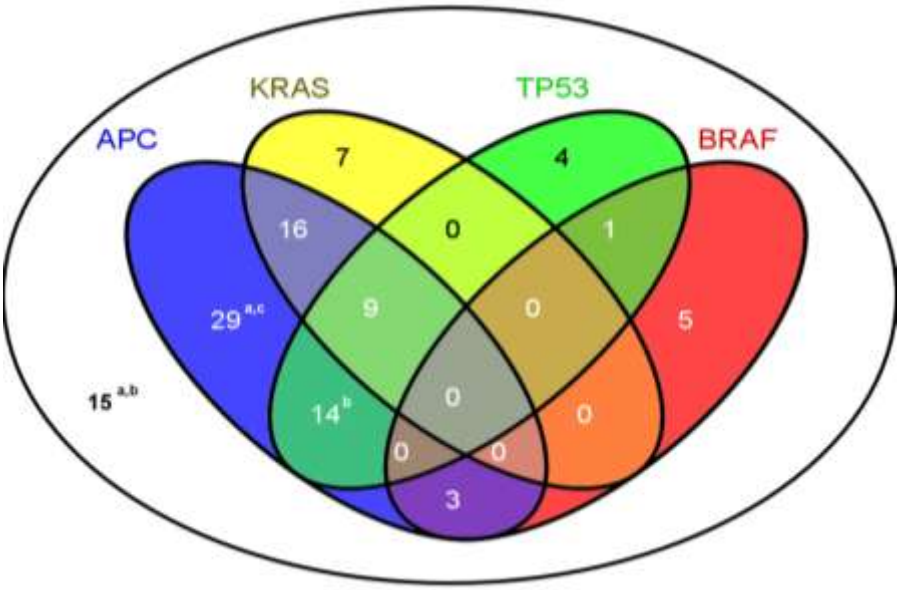


Figure 2: Comparison of the type of mutations in the whole *APC* coding region (A), in MCR (B), and outside of MCR (C). Light grey, FS mutations; dark grey, point substitutions. FS mutations are more frequent in MCR while point substitutions, especially C>T resulting in Arg>STOP, are more common outside of MCR.

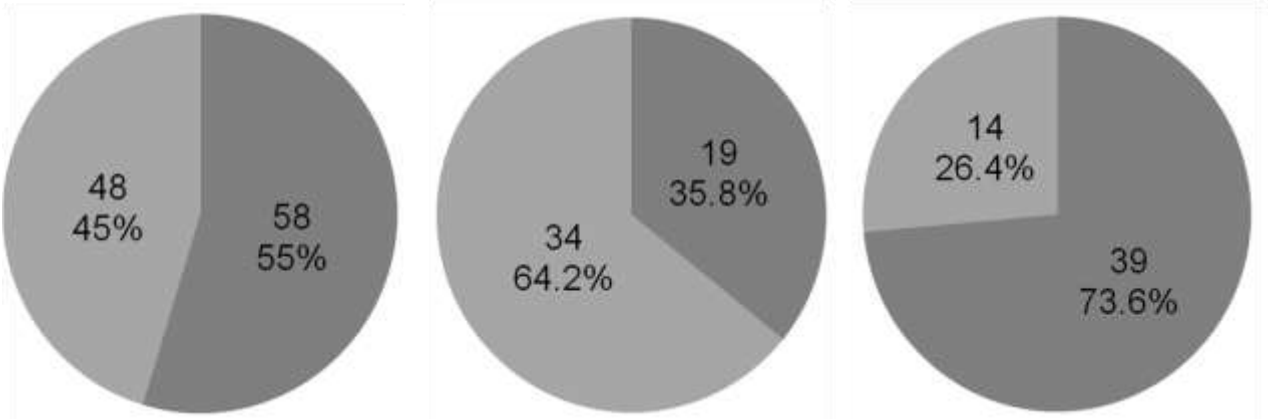


Figure 3: Comparison of the distribution of mutations along the *TP53* coding region in CRCs from the UMD database (top) and in our sample (bottom). The length of the bars reflects the number of mutations. Seven hot spot positions (representing 10 most common substitutions in UMD) are indicated by codon numbers. Blue colour indicates two different frequent substitutions at the same position. In our sample, two different point substitutions were observed in codon 245, and only one of them belonged to the 10 hot spot variants. Asterisks indicate FS mutations in our cohort.

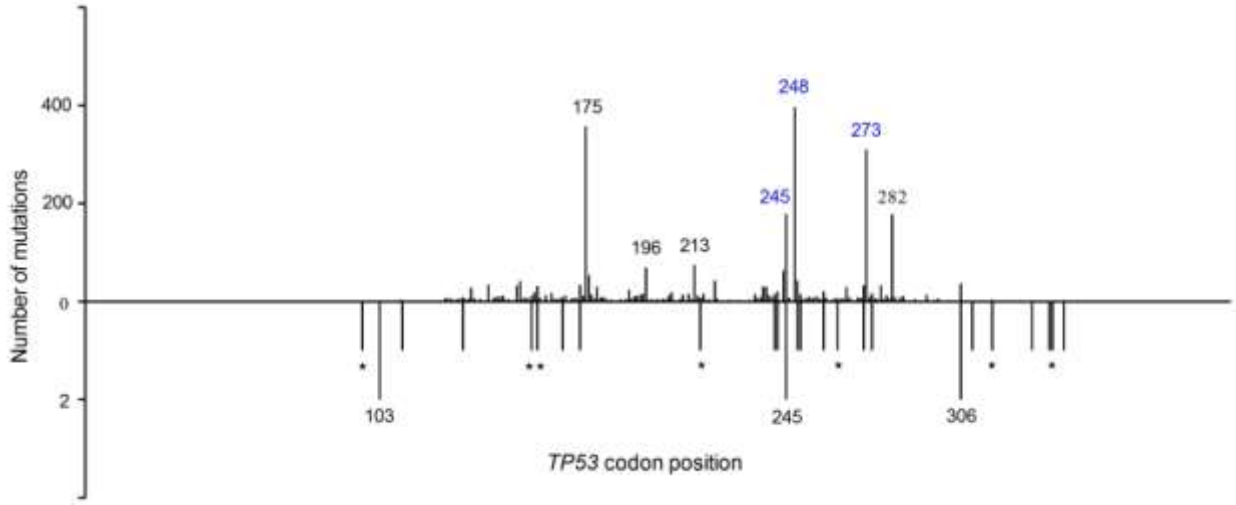


Figure 4: Mutational events in the *TP53* gene in CRCs listed in the UMD database (left) and observed in our CRCs (right). The CRCs of Czech patients had more frameshift mutations and transversions, while transitions, especially in CpG sites, were less frequent.

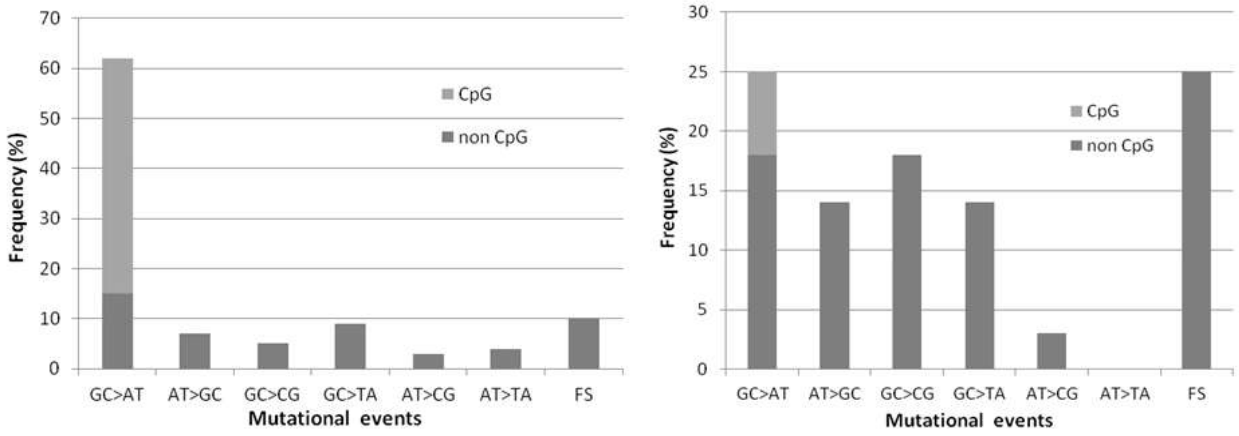
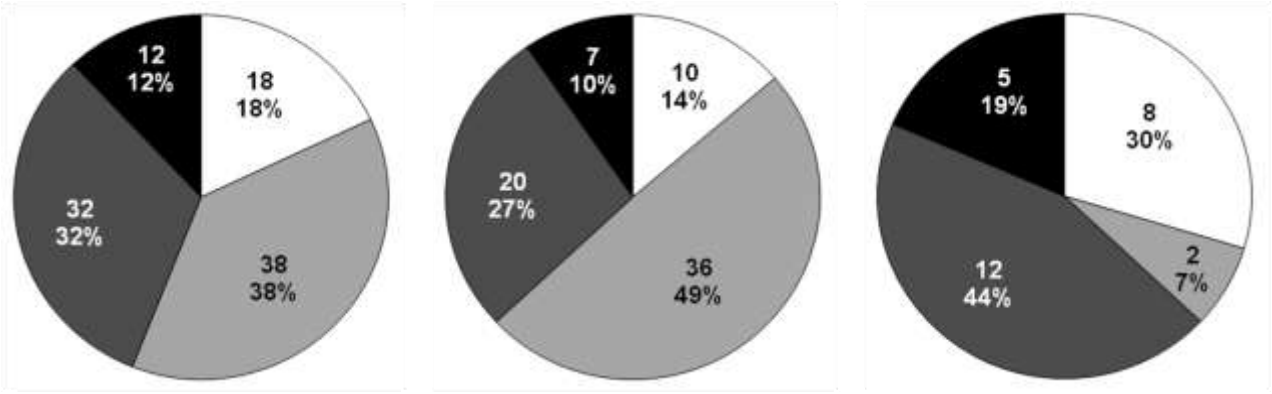


Figure 5: Comparison of the stage of tumours without and with *TP53* mutations. White, stage I; light grey, stage II; dark grey, stage III; black, stage IV. Segments show the number and percentage of tumours (3 tumours lacked the information). Tumours with *TP53* mutations may have a tendency to skip stage II or progress through it very quickly compared to tumours without *TP53* mutations (P = 0.002).



all tumours without TP53 mutation with TP53 mutation

Publikácia 5

Unique mutational profile associated with *TDG* expression loss in a rectal cancer of a patient with constitutional *PMS2* defect.

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Abstract

Germline defects of various DNA repair mechanisms have been shown to be associated with several human cancer predisposing disorders. Absence or malfunction of the repair proteins due to germline or somatic mutations is usually accompanied by a specific mutational signature of selected genes critical for tumour development. We have analysed the colorectal tumour of a patient with constitutional mismatch repair deficiency due to biallelic germline *PMS2* mutations for somatic mutations in a number of tumour suppressor genes. In addition to microsatellite instability (MSI), the tumour contained an excess of point mutations, mostly C:G>T:A or G:C>A:T transitions. This intriguing finding previously reported neither in hereditary nor sporadic colorectal tumours prompted mutation analysis of the base excision repair genes *UNG2*, *SMUG1*, *MBD4*, and *TDG*. A heterozygote mutation in the thymine-DNA glycosylase (*TDG*) gene was uncovered in tumour DNA. This somatic missense mutation affecting a highly conserved domain of the protein was associated with protein expression loss in the neoplastic tissue. Our finding indicates that absence or malfunction of the repair enzyme TDG contributed to the particular *supermutational* signature in tumour DNA of the CMMR-D patient supporting a role of TDG in preventing mutability of 5-methylcytosin deamination in the human genome.

Introduction

Maintenance of the genomic integrity is of crucial importance for all living organisms. DNA is constantly attacked by many various endogenous and exogenous DNA damaging agents that lead to mispairings and other deviations from Watson-Crick base-pairing. Equally, replication errors lead to mismatches and small nucleotide insertion/deletion loops (IDLs). Unrepaired, mispairings and IDLs are pro-mutagenic DNA aberrations that may lead to deleterious effects. There is a

variety of DNA repair mechanisms to prevent damage. The two main pathways implicated in the correction of mispairings and IDLs are postreplicative mismatch repair (MMR) and base excision repair (BER) (1). Germline mutations of genes involved in these repair processes give rise to human hereditary conditions associated with an increased cancer risk (1). Hereditary colorectal cancer (CRC) can be caused by defects in MMR and BER (2;3). Heterozygous germline mutations in the MMR genes *MLH1*, *MSH2*, *MSH6,* and *PMS2* cause an autosomal dominant cancer predisposition syndrome named hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome (2). Colorectal and endometrial cancers are the most prevalent cancers for this syndrome (4). Lynch syndrome associated tumours display loss of the remaining wild type *MLH1*, *MSH2*, *MSH6* or *PMS2* allele and, hence, somatic MMR deficiency (MMR-D). Somatic MMR-D is also observed in 15% of sporadic CRCs mainly due to *MLH1* gene silencing by promoter hypermethylation (5). Individuals with biallelic germline mutations in one of the MMR genes are referred to as constitutional MMR-deficiency (CMMR-D) patients. They develop childhood malignancies, mainly haematological malignancies and/or brain tumours as well as very early-onset CRC. Most CMMR-D patients show also some signs reminiscent of neurofibromatosis type 1 (NF1), mainly café au lait spots (CLS) (6). The hallmark of CRCs deficient in MMR is microsatellite instability (MSI) manifested by an accumulation of somatic frameshift mutations in the repetitive sequences of microsatellites. Frameshift mutations are frequently found also in the coding sequences of genes involved in tumour development such as growth regulatory genes (*TGFßRII*, *IGF2R*, *BAX*) as well as in DNA repair genes (*MSH3*, *MSH6*) in MSI tumours. A preponderance of frameshift mutations was shown also in the *APC* gene (7), a gene thought to play a central role in initiating colorectal tumorigenesis (8). Other reports could not confirm this observation and, therefore, suggest that at least in sporadic MSI tumours *APC* mutations, rather than genomic instability, are the initiating event in tumorigenesis.

Up to now, the only BER gene known to be implicated in CRC development is *MUTYH*. Homozygous or compound heterozygous *MUTYH* germline mutations cause *MUTYH*-associated polyposis (MAP) syndrome (3). A characteristic feature of MAP-associated CRC is prevalence of somatic G:C>T:A transversions in the *APC* gene (3).

In this report we present a patient with biallelic germline *PMS2* mutations who developed an early-onset CRC with a particular *supermutational* phenotype. Intriguingly, the microsatellite instable (MSI) tumour DNA revealed excess of somatic C:G>T:A transitions many of them at

CpG dinucleotides but no FS mutations in several analyzed tumour suppressor genes, particularly in the *APC* and *NF1* gene.

Material and methods

Case report

A 13 year old female patient with a so far insignificant medical history was admitted to hospital due to intermittent bellyache and diarrhoea. Ultrasound examination revealed pseudotumour of the left ovary. Infiltration of a rectal tumour was found during adnexectomy. The rectal tumour was histopathologically classified as pT3N2Mx with moderate differentiation. After a surgical excision of the tumour the patient underwent adjuvant chemotherapy regimen based on capecitabine plus oxaliplatin and after disease progression cetuximab plus irinotecan as a second choice treatment was used. Finally, panitumumab was indicated, unfortunately with no effect on disease progression. Chemotherapy was interrupted and two months later radiotherapy was initiated. The patient received 15 doses within two weeks. Tumour growth was suppressed and patient is 17 months in remission now.

Immunohistochemical analysis of the mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 and of the base excision repair protein TDG

Expression of MSH2, MSH6, MLH1, and PMS2 was studied by immunohistochemistry as described previously (9)

Immunohistochemical staining for TDG protein was performed on fresh excision wich were frozen in liquid nitrogen and evaluated by a standard frozen section technique for the presence of representative vital tumour and non-neoplastic control tissue. Following the blocking of endogenous peroxidase activity, the 4 μm thick cryosections were incubated overnight at 4 ºC with primary polyclonal rabbit anti-human TDG antibody (clone AP06727PU-N, Acris Antibodies GmbH, Germany) diluted 1:100. The antibody complexes were visualized using the streptavidin-biotin detection kit LSAB+, Dako REAL Detection Systems, HRP/DAB+, Rabbit/Mouse (DakoCytomation) and 3,3´-diaminobenzidin tetrahydrochlorid (DAB, Fluka Chemie, Buchs, Switzerland). All sections were stained with hematoxylin, dehydrated and mounted. Nuclear and/or cytoplasmatic staining was considered for a positive result of the immunoreactions. The non-neoplastic tissue was used as a positive control.

Microsatellite instability analysis

Five quasi-monomorphic mononucleotide repeat markers, i.e. BAT-26, BA-T25, NR-21, NR-24 and MONO-27 were investigated to asses microsatellite instability (MSI) employing a fluorescence-based pentaplex-PCR assay (Ingenetix, Vienna, Austria) according to the manufacturer's recommendations.

Germline mutation analysis

After informed consent blood samples were obtained from the patient and her parents for molecular analysis. Screening for *MLH1* and *MSH2* mutations was performed by denaturing gradient gel electrophoresis (DGGE) as previously described (10). The *MSH6* gene was analyzed by sequencing all exons from gDNA using published primers (11). A previously described RNA-based mutation analysis protocol (12) was used to identify mutations in the *PMS2* gene. *PMS2* exon 11 was amplified and subsequently sequenced with published primers (13). Improved *PMS2* MLPA was performed with SALSA kits P008-A1 and P008-X1, the latter being the beta version of the new kit P008-B1 (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's instructions using a set of six control DNAs containing each two copies of *PMS2*- and two copies of *PMS2CL*-specific sequences (Wernstedt et al. manuscript in preparation). *NF1* mutation analysis included *NF1* cDNA sequencing as described in (14) and MLPA analysis using SALSA kits P081-B1 and P082-B2 (MRC-Holland, Amsterdam, The Netherlands).

Mutation analysis in tumour tissue

DNA was extracted also from fresh frozen tumour of the index patient and corresponding mucosa tissue at -70°C, snapped during colectomy using Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MI, USA) according to manufacturer's recommendations. Mutation analysis of *APC, KRAS, TP53, BRAF*, *CTNNB1, MUTYH* genes, MSI and *MLH1* promoter methylation status was performed as described previously (Vasovcak et al. submitted). Denaturing gradient gel electrophoresis (DGGE) method was used for pre-screening of *MLH1* and *MSH2* genes also in tumour DNA. PCR fragments showing an aberrant profile were checked by sequencing. The *MSH6* and *NF1* genes as well as the BER genes, *UNG2*, *SMUG1*, *MBD4* and *TDG*, were sequenced directly from tumour DNA (primers for amplification of BER genes are listed in Supplementary Table 1). Somatic mutations found in tumour DNA were analysed also in corresponding mucosa to exclude possibility of germline mutations.

For mutation analysis in the three additional CMMR-D patients' tumours DNA was isolated from paraffin-embedded tumour tissue using Puregene Tissue Kit (Gentra Systems, Minneapolis, MI, USA) according to the manufacturer's recommendations. Tumour DNA was amplified using intronic primers as published in (3;15;16) and sequenced bidirectionally.

Mutant allele-specific PCR amplification (17) was performed to show that two somatic stop mutations are located *in trans* in *APC* exon 6. A forward primer (5′ GTTTCTTGTTTTATTTTAGT 3′) with terminal 3′ nucleotide specific for first mutation (c.646C>T, p.R216X) and a reverse primer (5′ CTACCTATTTTTATACCCAC 3′) positioned downstream of the second mutation (c.694C>T, p.R232X) were used for PCR and subsequent sequencing of the generated PCR product.

Cycle sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA) and the Applied Biosystem 3130 Genetic Analyser according to manufacturer's instructions.

All mutations are described in accordance with the recommendations of the human genome variation society (http://www.hgvs.org/mutnomen/). Reference sequence NM_000535 was used for *PMS2*.

Results

Identification of two compound heterozygous germline PMS2 mutations in a young colorectal cancer patient

The patient presenting with a colorectal cancer at the age of 13 years was initially suspected to suffer from HNPCC despite of a negative family history of Lynch syndrome-associated tumours. The tumour showed microsatellite instability (MSI) at all tested markers, but routine immunohistochemical analysis of MLH1 and MSH2 revealed nuclear staining of both proteins. Equally, mutation analysis performed for the MMR genes most frequently involved in Lynch syndrome, *MLH1*, *MSH2* and *MSH6* rendered negative results. Subsequent detailed physical examination by the clinical geneticist revealed two café au lait maculae (CALMs) on the back of the patient raising the suspicion of CMMR-D syndrome which is frequently caused by biallelic *PMS2* germline mutations (6). This was confirmed by immunohistochemistry showing isolated PMS2 expression loss in the tumour and in the surrounding non-neoplastic mucosa tissue (Figure 1). Direct cDNA sequencing revealed heterozygous loss of exons 12, 13 and 14

(r.2007_2445del439) in the *PMS2* transcripts and subsequently performed improved MLPA analysis confirmed an intragenic deletion (c.2007-?_2445+?del) affecting these exons. Furthermore, mutation c.1687C>T leading to the premature stop codon p.Arg563X was identified by cDNA sequencing and subsequently confirmed by sequencing of exon 11 from gDNA. Analysis of parental DNA confirmed that the two pathogenic mutations were located *in trans* since the mother of the patient carries the stop mutation p.Arg563X and the father is heterozygous carrier of the intragenic deletion (c.2007-?_2445+?del) affecting exons 12 to 14.
NF1 mutation analysis was negative in the blood lymphocytes of the patient who showed two CALMs on her back.

The colorectal cancer of the CMMR-D patient has a unique mutational phenotype

Independently from the diagnostic work-up of the young colorectal cancer patient, a sample of her tumour was included in an unselected series of 103 anonymized apparently sporadic CRCs which were examined for the mutational profile of *APC*, *KRAS*, *TP53*, *BRAF*, *CTNNB1*, and *MUTYH* as well as for LOH at the *APC* locus, MSI, and *MLH1* promoter methylation status (Vasovcak et al. submitted). Within this series, the tumour of the patient showed a unique mutation pattern with microsatellite instability and nine somatic alterations, eight in the *APC* and one in the *CTNNB1* gene. Of note, none of the alterations were frameshift mutations, although more than half of the mutations found in other sporadic MSI colorectal cancers were of this type (Vasovcak et al. submitted). The majority (7/9) of the mutations found in the tumour of the CMMR-D patient were C:G>T:A or G:C>A:T transitions of which six affected CpG dinucleotides (Table 1). Two of the *APC* alterations are recurrent pathogenic stop mutations (see LOVD Insight database: http://www.insight-group.org/mutations/) and were found to be located *in trans* position to each other by mutant allele-specific PCR amplification and subsequent sequence analysis showing the wildtype nucleotide at the other mutated site. LOH assessment at the *APC* locus was not determined due to the uninformativity of the examined marker. Since the tumour showed MSI, mutation analysis of the MMR genes *MLH1*, *MSH2* and *MSH6* was initiated in the neoplastic tissue independent from the search for germline mutations in these genes in the blood lymphocytes of the patient. Furthermore, mutation analysis of the *NF1* gene, which is a known target of MMR deficiency induced mutations (18), was performed by exon sequencing in the neoplastic tissue. This lead to the detection of further seven C:G>T:A or

G:C>A:T transitions five of which affected CpG dinucleotides (Table 1). Neither of the detected somatic changes found in the tumour was present in the non-neoplastic tissue. Taken together, a total of 16 somatic single nucleotide substitutions, 12 of them affecting Cs at CpG dinucleotides, were identified among 34000 nucleotides analyzed.

Identification of a heterozygous TDG mutation and concomitant TDG expression loss in the neoplastic tissue.

This mutational phenotype with an excess of C:G>T:A or G:C>A:T transitions but no frameshift alterations in the MSI tumour of a CMMR-D patient compound heterozygous for germline *PMS2* mutations was intriguing. In the literature we found one report of a Turcot syndrome patient (19) who developed in addition to an astrocytoma grade IV, a malignant lymphoma and a fibroma three independent colon carcinomas and multiple adenomas at the ages of 13 and 16 years. This patient carried an inferred biallelic *PMS2* germline mutation although only one heterozygote mutation was identified at that time. All colonic neoplasms in this patient exhibited MSI. Analysis of the three colorectal carcinomas for the presence of somatic mutations in the *APC* and *TP53* genes revealed a total of 11 mutations. The tumour with the highest number of alterations carried six mutations, four of them in the *APC* and two in the *TP53* gene, respectively. One of these six mutations was a frameshift mutation and five single-nucleotide substitutions of which only one was a C:G>T:A transitions at a CpG dinucleotide. These data from the literature indicate that colorectal cancers in CMMR-D patients with biallelic *PMS2* germline mutation may have a high frequency of somatic mutations in their tumours, but do not render evidence for a preponderance of C:G>T:A or G:C>A:T mutations as seen in the index patient. Therefore, we aimed at analyzing more colorectal cancers of patients with germline *PMS2* defects. We analysed the *APC* and *CTNNB1* genes in tumour DNA of three other *PMS2* deficient CMMR-D patients. Since all somatic *APC* mutations of the index patient were found in exons 1 to 14 and within the first ~ 5000 bp of the large exon 15, this region as well as the critical *CTNNB1* exon 3 was analyzed in the tumour of a so far unpublished patient who developed a colonic adenocarcinoma at the age of 12 years and was found to be homozygous carrier of a truncating *PMS2* mutation (patient 2 in Table 2). This tumour showed MSI at 4/5 analyzed markers. Secondly, the tumour of a previously published CMMR-D patient (20) which showed MSI at 5/5 analysed markers was analysed. Only a limited amount of tumour DNA was

available from this patient (patient 3 in Table 2). Therefore, only the *APC* mutation cluster region (MCR) (21) and the *CTNNB1* exon 3 could be sequenced. Finally, we were able to investigate the tumour tissue of a patient (patient 4 in Table 2) who developed at the age of 15 years a colon cancer. Although this tumour did not display MSI, it is assumed to be a CMMR-D patient due to a biallelic *PMS2* germline mutation since the tumour showed isolated PMS2 expression loss by immunohistochemical staining. We found only a single alteration, i.e. an *APC* frameshift mutation (c.4666dupA), in this latter patient while no somatic sequence change was found in the tumours of the two other patients. Comparing the frequency of mutations per analysed nucleotides among the four *PMS2* deficient tumours analysed in this study (Table 2), the tumour of our index case has the highest frequency in any comparison.

Since we found the unusual preponderance of C:G>T:A or G:C>A:T transitions in the somatic mutation spectrum in the index patient, we reasoned that a defect in one of the four DNA glycosylases, UNG2, SMUG1, MBD4, and TDG could explain this observation. These proteins show activity against bases that arise through deamination of C (uracil) or against deaminated 5-MeC residues (thymine) (22). Mutation analysis of these genes in the tumour DNA revealed a heterozygote *TDG* gene substitution c.850G>T which was not present in DNA of non-neoplastic tissue (Table 1). This substitution leads to a non-synonymous amino acid change p.D284Y which is located in the highly conserved central domain of the protein (Figure 2) (23). It is predicted to be damaging according to different *in silico* tools, i.e. Polyphen (24), SIFT (25), and Grantham scores (26). The identification of this most likely pathogenic *TDG* mutation initiated immunohistochemical (IHC) analysis with a polyclonal anti-TDG antibody in the patient's tumour and the tumour of CMMR-D patient 2 (see above) as control. The IHC analysis showed reduced nuclear TDG staining in the neoplastic cells of the index patient 1 when compared to surrounding normal mucosa as well as to the control tumour (Figure 1). This result indicates loss of/lowered TDG expression in the tumour tissue of the patient. A second mutation in the tumour was not found and analysis of the tumour DNA even from a microdissected sample carrying nearly 100% neoplastic cells did not reveal loss of the wild type allele. Hence, we suspect that an assumed second somatic TDG inactivating hit either escapes the detection by the applied methods or that the mutation p.D284Y has a dominant negative effect reducing also the expression of wild-type protein.

Discussion

We have encountered a CMMR-D patient with a constitutive *PMS2* defect who's MSI tumour harboured 16 nucleotide substitution mutations in five of ten analysed tumour suppressor genes and proto-oncogenes with a striking preponderance (14/16 mutations) of C/G>T/A and G/C>A/T transitions. Of the 14 transitions 12 were located at CpG dinucleotides eight affecting a C on the sense and two at the anti-sense strand, respectively. This mutational phenotype is unique, since it was not observed in CRCs from three further patients with a constitutive *PMS2* defect analysed in this study for somatic *APC* mutations. Also, although a high somatic *APC* mutation rate was previously described in three CRCs of an inferred CMMR-D patient (19), the spectrum of somatic mutations in this patient showed no obvious preponderance of C/G>T/A and G/C>A/T transitions. Equally, the *NF1* mutation spectrum previously found in several *MLH1*-, *MSH2*- and *MSH6*- deficient cancer cell lines, primary tumours and mouse embryonic fibroblasts differed with only one G/C>A/T transition affecting a CpG dinucleotide among 10 mutations (five being frameshifts) from the mutational spectrum found in the here described patient (18). Hence, it appeared unlikely, that the constitutional MMR defect due to the identified biallelic germline *PMS2* mutations (alone) is responsible for the mutation phenotype observed in the tumour of the index patient and to which we would like to refer to as *supermutational*.

The MMR system processes most base-base mismatches and small insertion-deletion loops although with different efficiency (27) and acts mainly as a backup of polymerase proofreading with the ability to discriminate nascent from template strand to prevent accidental excision of the correct template nucleotide (28). Somatic genetic or epigenetic inactivation of the MMR genes is characterised by MSI in the associated tumours (5) and a prevalence of frameshift mutations in various analysed genes which are thought play a role in tumorigenesis (7). In contrast to the MMR system, BER enzymes act irrespective of whether a damaged or mismatched base is located on the nascent or template strand (1). Until the discovery that inactivation of a single BER protein, *MUTYH*, may increase mutational rate with specific mutation signature reflecting its particular activity (3), the importance for keeping up genome integrity of each individual BER genes were considered low due to their functional redundancy. Co-evolution of the four BER proteins with similar enzymatic properties implies the existence of non-redundant exclusive biological functions that are coordinated. The major enzyme for removal of deaminated cytosine in replication associated repair is UNG2, with SMUG1 acting as a broad specificity back up,

especially in non-replicating chromatin (29;30). TDG serves as the predominant G:T glycosylase in mammalian cells, whereas MBD4 is a minor glycosylase for this type of mutational event (1). Coordination between different repair mechanisms or enzymes of the same mechanism can be achieved by spatiotemporal regulation (1). For example, TDG- and UNG2-dependent base excision repair alternates throughout cell cycle, where UNG2 is active during DNA replication and TDG functions in non-replicating DNA (31). Methylated CpG dinucleotides in mammalian genome are hotspots for mutations. Owing to its ability to excise thymidine when mispaired with guanine, TDG was proposed to act against the mutability of 5-methylcytosine (5-mC) deamination in the mammalian DNA (32). The identification of a most likely pathogenic somatic *TDG* mutation concomitantly with severely reduced expression of this BER gene in the index patient's tumour showing a *supermutational* phenotype appears to confirm this notion. However, we cannot discern whether the *supermutational* phenotype is caused mainly or exclusively by the absence/reduced expression of TDG or whether a defect in TDG-dependent BER and a defective MMR system work together. Of note, *TDG* gene was recently shown to be critically important for mouse embryonic development, promoting proper epigenetic modification of developmental genes (33;34). These recent data (34) suggest that TDG may not only have a role as critical DNA repair enzyme in the mammalian genome working especially against mutations arising from deamination of 5-methylcytosine, but also in initiating the DNA demethylation process, thus controlling the potentially mutagenic deaminase activity of AID and also the methylation status of gene promoters. Hence, Cortellino et al. speculate that somatic TDG inactivation or altered relative expression of TDG may play a role in tumour formation not only by increasing deamination-induced transition mutations but also by promoting hypermethylation of critical tumor suppressor gene promoters (34). It may be hypothesised that various TDG expression level (due to a different spectrum of mutations, epigenetic modification and/or nature of mutation) may have different impact on a mutational/epigenetic phenotype of tumour cells.

It is widely accepted that aberrant activation of the Wnt signalling pathway is considered a common denominator in the onset of nearly all CRCs. Inactivating *APC* or activating *CTNNB1* mutations are thought to be also the initiating event in Lynch syndrome-associated tumours preceding somatic mismatch repair deficiency that subsequently accelerates the adenoma-carcinoma transformation by increasing the mutation rate in pre-cancerous lesions. In contrast to Lynch syndrome patients who develop *non-polyposis* colorectal cancer, the presence of multiple

polyps/adenomas has been reported in several CMMR-D patients (6;35). It is conceivable that genes such *APC* and *CTNNB1* which are critical for tumour initiation are targets of the constitutional defect in the mismatch repair system. Hence, in CMMR-D patients, not only the transformation process of pre-cancerous lesions is accelerated but also the formation of these lesions is increased. Of note the two clearly truncating *APC* mutations found to be located *in trans* as well as most of the other mutation in the here described tumour are C:G>T:A transitions. Hence, it may be speculated that in the context of this tumour, possibly also in other CRCs, TDG has a function as a caretaker tumour suppressor that's inactivation helped to initiate neoplastic cell transformation by increasing the mutation rate of the *APC* gene.

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Table 1: Somatic mutations found in tumour DNA of the patient.

APC GenBank Ref.Seq NM_000038.4	NF1 GenBank Ref.Seq NM_000267.2	MLH1 GenBank Ref.Seq NM_000249.3	MSH6 GenBank Ref.Seq NM_000179.2	CTNNB1 GenBank Ref.Seq NM_001904.3	TDG GenBank Ref.Seq NM_003211.4
c.646C>T, p.R216X **c.694C>T**, p.R232X c.730-15A>G **c.1242C>T**, p.R414R c.1312+4T>G **c.3313C>T**, p.R1105W c.4097C>T, p.A1366V **c.4901C>T,** p.P1634L	**c.862G>A**, p.V288M **c.1154G>A**, p.R385H **c.5601C>T**, p.I1867I c.8245C>T, p.L2749F	**c.1490G>A**, p.R497Q **c.2040C>T**, p.C680C	**c.2195G**>A, p.R732Q	**c.59C>T,** p.A20V	c.850G>T, p.D284Y

Transitions affecting CpG dinuclotides are highlighted in bold.

Table 2: Comparison of the somatic mutations found in the colorectal cancer of the index patient and of three other *PMS2-/-* patients

Patients	MSI [1]	APC [2] (~7000 bp)	APC-MCR [3] (~700 bp)	CTNNB1 [4] (~300 bp)
Patient 1	MSI 5/5	8 6x C>T/G>A 1x T>G 1x A>G	1 1x C>T	1 1x C>T
Patient 2	MSI 4/5	0	0	0
Patient 3	MSI 5/5	n.a.	0	0
Patient 4	MSS 0/5	1 (c.4666dupA)	0	n.a.

Patient 1 is the index patient, patient 2 is a so far unpublished CMMR-D patient carrying a homozygous *PMS2* stop mutation, patient 3 has previously been published (20) and patient 4 is a inferred CMMR-D patient who developed at the age of 15 years a CRC which showed isolated PMS2 expression loss. [1] The number of instable markers per analyzed markers is given. [2] Analysis of the entire coding and flanking intronic sequences of exons 1-14 and the first 5kb of exon 15. [3] Analysis of only the mutation cluster region (MCR) in *APC* exon 15. [4] Analysis of exon 3 and flanking intronic sequences.

Figure 1. Immunohistochemical staining of colon cancers with antibodies against MLH1, PMS2, and TDG.

(A), (B), and (C): Colon cancer of the index CMMR-D patient; (D): Colon cancer from an unrelated patient with a homozygous germline *PMS2* mutation (patient 2). While tumour cells expressed normally MLH1 **(A)**, the expression of the MLH1 heterodimeric partner PMS2 was lost in the proband's colon cancer **(B)**. PMS2 was absent also in normal stromal cells **(B)** and in the normal mucosa (not shown). **(C)** In the same cancer, the expression of the DNA repair enzyme TDG was markedly reduced or completely absent in the nuclei. (The cytoplasmic staining may be either background or residual expression of TDG in this cellular compartment.). **(D)** TDG was normally expressed in the colon cancer of the unrelated CMMR-D patient (patient 2). Inset: staining of this cancer with the TDG pre-immune serum (negative control).

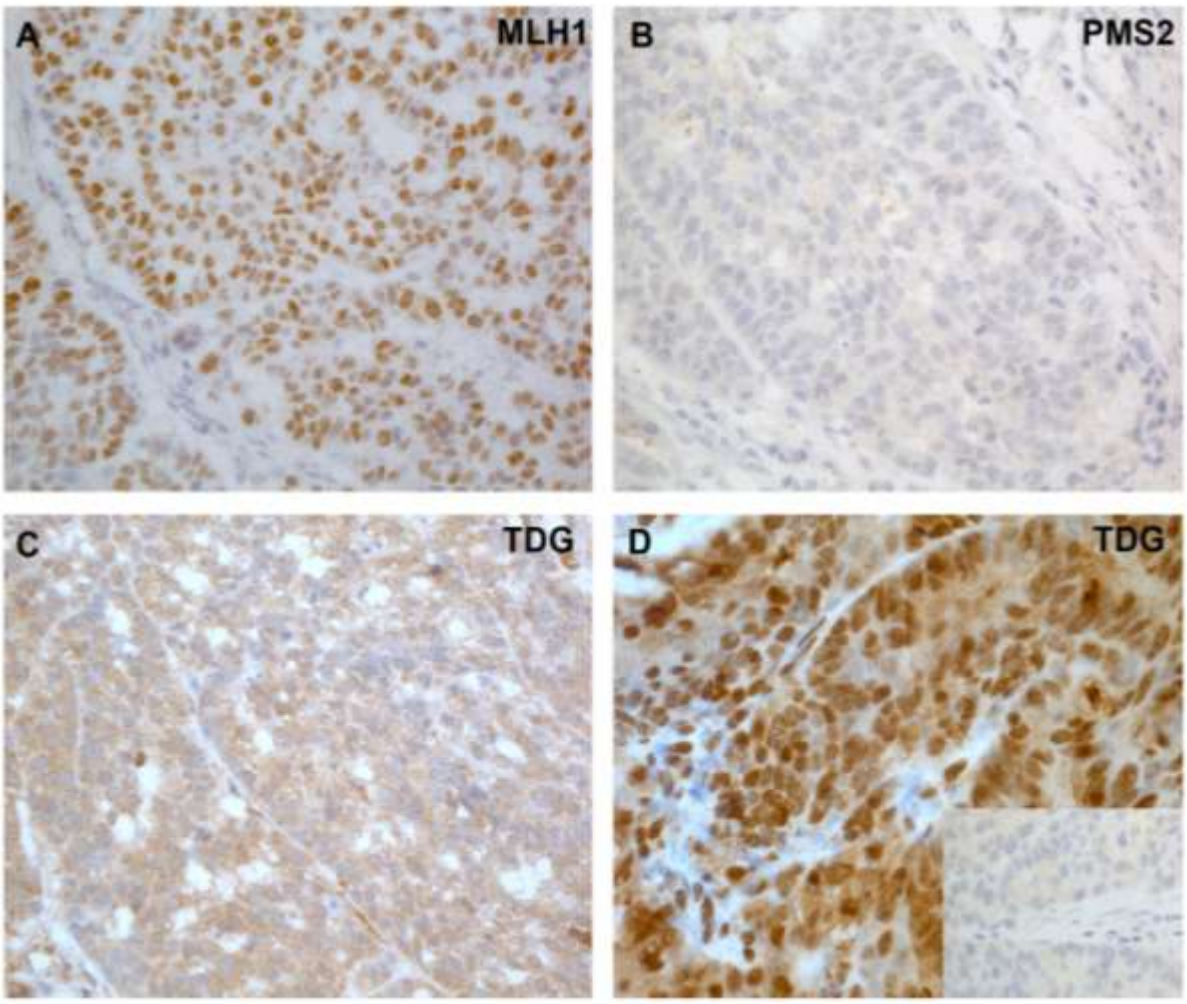

Figure 2 Evolutionary conservation of the amino acid residue of *TDG* which was found mutated in patient.

Comparison of the G/T glycosylase domain of the TDG protein in various organisms. The highlighted aspartate residue at 284 in the human sequence is conserved among many organisms. A mutation encoding the D284Y variant at this position was identified in the rectal cancer of our patient.