UNIVERZITA KARLOVA V PRAZE, LÉKAŘSKÁ FAKULTA V PLZNI

ŠIKLŮV ÚSTAV PATOLOGIE

PRAKTICKÁ APLIKÁCIA IMUNOHISTOCHEMICKÝCH A MOLEKULOVO GENETICKÝCH METÓD V DIFERENCIÁLNEJ DIAGNOSTIKE LÉZIÍ UROGENITÁLNEHO A GYNEKOLOGICKÉHO TRAKTU.

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Doktorská dizertačná práca

Plzeň 2018
Obor: Patologie
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Albrecht Dürer, Apocalypsis cum figuris, X. (Ap. XII, 7-12)
Drevoryt zo zbierok grafiky a kresby Národní galerie v Praze.

Abstrakt

Dizertačná práca je komentovaným súborom siedmich publikácií uverejnených v anglicky písaných impaktovaných časopisoch. Hlavnou témou je hľadanie optimálnej stratégie využitia imunohistochemických metód a metód molekulovej genetiky pri diagnostike zriedkavých a nových nádorov či podtypov nádorových jednotiek v gynekopatológii a aj v patológii urogenitálneho traktu. Ťažiskovou témou je gynekopatológia. Všeobecnejší úvod je výberovým náčrtom spôsobov akými patológovia môžu aplikovať metódy molekulovej genetiky vo výskume a stručne popisuje stav poznania v jednotlivých oblastiach záujmu. Ciele práce stručne charakterizujú výskumné zámery jednotlivých projektov. Prvý z nich sa zaoberá histomorfologickou „mulleriánskou" variabilitou kožnej endometriózy. Nasleduje raritná kazuistika serózneho papilárneho borderline tumoru fimbrií tuby s analýzou prítomnosti mutácií génov KRAS, BRAF a p53. Prospektívna longitudinálna štúdia ťažkej dysplázie dlaždicového epitelu (HSIL) krčka maternice u HPV vakcinovaných žien sa snaží odhaliť príčiny tohoto fenoménu. Ďalšie dve štúdie sa zaoberajú incidenciou *fumarát hydratáza (FH)* deficientných leiomyómov maternice a syndrómu hereditárnej leiomyomatózy a renálneho karcinómu (HLRCC) s cieľom prispieť k zlepšeniu diagnostiky a zvýšiť záchytnosť tohoto syndrómu. Napokon je v podobe dvoch publikácií predstavená bizarná dysplázia krčka maternice ako varianta HSIL. Záver je zamyslením nad miestom molekulovej genetiky v práci patológa.

Abstract

This thesis focuses on gynecopathology. It consists of a collection of seven papers published in pathology journals with impact factor. Introduction section contains selection of examples showing scientific application of molecular genetic methods and basic information available in respective fields of interest. Further on the aims of individual research projects are described. The first project comprises histomophologic study of skin endometriosis addressing „mullerian" differentiation. A case report of a rare tumor namely borderline papillary serous tumor of the fimbriated end of the fallopian tube follows with molecular genetic analysis of KRAS, BRAF and p53 gene mutation status. Prospective longitudinal study on high grade squamous dysplasia (HSIL) of the cervix in HPV vaccinated women, so called DAV (dysplasia after vaccination) aims to elucidate pathogenesis of this phenomenon. Two other studies focus on incidence of fumarate hydratase deficient leiomyomas of the uterus and hereditary leiomyomatosis and renal cell carcinoma syndrome (HLRCC). The aim of those studies is to improve our diagnostic capability and increase detection rate of the patients with HLRCC syndrome. Finally a new subtype of HSIL namely bizarre cell dysplasia is described in two separate studies. Conclusion remarks contemplate the role of molecular genetics in surgical pathology.

Predhovor

Aplikácia metód molekulovej genetiky za účelom diagnostickým, prognostickým alebo prediktívnym sa v dnešnej dobe búrlivo rozvíja. Veľké množstvo jednotlivých metodík a povaha sledovaných genetických zmien spôsobuje, že hľadanie optimálneho využitia najvhodnejšej metódy v každom konkrétnom prípade je procesom veľmi zdĺhavým. Dizertačná práca je malým príspevkom do tohoto snaženia.

Prehlásenie

Čestne vyhlasujem, že dizertačnú prácu som vypracoval samostatne, na základe štúdia odbornej literatúry, ktorej zoznam je kompletne uvedený na príslušnom mieste.
Súhlasím s trvalým uložením elektronickej verzie práce v databáze UK LF Plzeň ako aj s možnosťou zapožičania tlačenej verzie tejto práce.

V Plzni, 15 júna 2018

Ondrej Ondič

Poďakovanie

Prof. MUDr. Ondřeji Hesovi, Ph.D. – vynikajúcemu školiteľovi, pri ktorom som si znovu uvedomil, aký podiel v našich životoch má náhoda a ako veľmi záleží na učiteľovi.

Prof. MUDr. Michalovi Michalovi – za dôveru a veľkorysosť.

Československým kolegyniam a kolegom patológom za mnohé priateľské a inšpiratívne stretnutia.

Kolektívom Šiklovho Ústavu Patologie a Bioptické laboratoře s.r.o. za milé a profesionálne pracovné prostredie.

Obsah

Zoznam použitých skratiek

2SC	S-(2-sukcino) cysteín
ASC-H	Atypie dlaždicových buniek, nemožno vylúčiť HSIL
ASCUS	Atypie dlaždicových buniek neistého významu
BCD	Dysplázia obsahujúca bizarné bunky
BPSTFT	Borderline papilárny serózny tumor fimbrií vajcovodu
CD	Skupina determinantov
CIN	Cervikálna intraepiteliálna neoplázia
CMV	Cytomegalovírus
DAV	Dysplázia po HPV vakcinácii
DNA	Deoxyribonukleová kyselina
EBV	Epstein-Barrovej vírus
FFPE	Fixovaný vo formole a zaliaty do parafínu
FH	Fumarát hydratáza
HC2	Hybrid capture
HE	Hematoxylín – eozín
HHV4	Ľudský herpes vírus 4
HIER	Teplom indukované odhalenie epitopov
HLRCC	Syndróm hereditárnej leiomyomatózy a renálného karcinómu
HPV	Ľudský papillomavírus
HSIL	Dlaždicová intraepiteliálna neoplázia s vysokým stupňom dysplázie
HSV	Ľudský herpes vírus
ID	Identifikačné číslo
IHC	Imunohistochemické vyšetrenie
IM	Intramurálny
ISH	In situ hybridizácia
IVF	In vitro fertilizácia
LAST	Terminológia dlaždicových lézií dolnej anogenitálnej oblasti
LBC	Liquid based cytology (Cytológia z tekutého média)
LGL	Veľké granulované lymfocyty
LLHSIL	Ťaždká dysplázia podobná ľahkej dysplázii dlaždicového epitelu
LOH	Strata heterozygozity
LSIL	Dlaždicová intraepiteliálna neoplázia s nízkym stupňom dysplázie
LSIL-H	Ľahká dysplázia dlaždicového epitelu, nemožno vylúčiť ťažkú dyspláziu
MLPA	Na väzbe závislá amplifikácia próby
MPS	Masívne paralelné sekvenovanie
NA	Nie je k dispozícii
Neg	Negatívny
NILM	Bez intraepiteliálnej lézie a bez malignity
NK	Prirodzený zabíjač
NP	Nevykonaný
PAP smear	Náter gynekologickej skríningovej cytológie

PCR	Polymerázová reťazová reakcia
Pos	Pozitívny
RCC	Karcinóm z renálnych buniek
RHA	Systém reverznej hybridizácie
RNA	Ribonukleová kyselina
S	Subserózny
SM	Submukózny
STM	Transportné médium
STUMP	Hladkosvalový tumor s neistým malígnym potenciálom
TMA	Tkanivový mikročip
VZV	Varicella-zoster vírus
WHO	Svetová zdravotnícka organizácia
WT	Prirodzený typ

1 Úvod

1.1 Úvodné poznámky k endometrióze

Endometrióza je definovaná ako prítomnosť endometriálneho tkaniva mimo maternicu (1). Kožná endometrióza je dobre známa a obyvke nepredstavuje diagnostický problém aj keď sa vyskytuje relatívne zriedkavo. Po úvodných publikáciách Specka a Helwiga (2,3), ktoré popisujú spolu 82 prípadov nasledovali iba ojedinelé kazuistiky a popisovali neobvyklé histologické rysy i anatomické lokalizácie (1,4-6). Niketoré lézie boli označované ako atypická endometrióza a predstavovali diagnosticky obtiažne prípady. Normálny mulleriánsky epitel má sklon k metaplastickým zmenám , ako to môžeme vidieť v endometriálnych žliazkach (7-12). Závažné sú tiež zmeny v stromálnej komponente kde bola popísaná hladkosvalová metaplázia a degeneratívne zmeny priečne pruhovanej svaloviny, ktoré môžu vyvolávať diagnostické rozpaky. Navyše v blízkosti ložísk endometriózy boli popísané neobvyklé zmeny v tukovom tkanive - išlo o adipocyty s intranukleárnymi inklúziami (Lochkern, Kerbenkern a Ringkern) ako ich prvýkrát popísal Unna v roku 1895 (13).

1.2 Borderline papilárny serózny tumor fimbrií tuby s peritoneálnymi implantmi

Označenie borderline papilárny serózny tumor vajcovodu poprvýkrát použil Zheng v roku 1996 (14) a prvé review prípadov bolo publikované v roku 2005 (15). Tento nádor splňuje histologické kritériá epitelovej stratifikácie a jadrových atypií tak, ako sú popísané v jeho ovariálnej variante. V čase publikácie bol známy popis iba 8 prípadov. V nedávnej dobe sa objavili prvé molekulovo genetické charakteristiky ovariálnych SBOT a to alelické straty chromozómov 17,18q, 20q a zisky na chromozómoch 12p, 13-q23 (16) a alelické zmeny chromozómov 1p, 5q, 8p, 18q, 22q, Xp a mutácie génov KRAS (50%) a BRAF (20%) (17,18). Donedávna bola problematická i otázka stagingu týchto nádorov (19).

1.3 Ťažká dysplázii (HSIL) u HPV-vakcinovaných žien

Vakcinačný program HPV vakcinácie začal v Českej republike v decembri 2006. Kvadrivalentná a bivalentná vakcína bola dostupná pre mladé ženy v doporučovanom vekovom rozmedzí 15 až 26 rokov. Nad touto vekovou hranicou bola indikácia tiež možná, ale až po individuálnej konzultácii s ošetrujúcim gynekológom. Vakcinácia nebola hradená zo zdravotného poistenia až na výnimku, keď je na žiadosť rodičov od apríla 2012 možná

vakcinácia dievčat vo veku 13 rokov s plnou úhradou z verejného zdravotného poistenia. Sporadicky od roku 2009 sme v našom laboratóriu konfrontovaní s prípadmi ťažkej dysplázie (HSIL) u HVP vakcinovaných žien s riadne ukončenou vakcináciou. Tento fenomén nebol nikdy systematicky študovaný aj keď ho spomína Szarewski (20), ktorý popisuje 8 pacientiek s HSIL, z toho 6 asociovaných s HPV typom 16 a dva prípady asociované s typom HPV 18. Metodologicky išlo o štúdiu založenú na vyhodnocovaní nálezov LBC skríningovej cytológie. Autori tento fenomén nijako nekomentovali. Recentnejšia študia (21) Castla et al., bola tiež založená na hodnotení LBC nálezov s následnou HPV detekciou a typizáciou u 617 pacientiek. Autori konštatujú, že väčšina identifikovaných prípadov HSIL bola diagnostikovaná v dvojročnom časovom intervale po ukončení vakcinácie a jednalo sa o „prevalentné prípady" t.j. postvakcinačný LBC odber obsahoval HPV typ zhodný s typom identifikovaným v LBC materiáli pred vakcináciou. Cielená a histologická štúdia neexistuje.

1.4 Úvodné poznámky k diagnostike syndrómu hereditárnej leiomyomatózy a renálneho karcinómu (HLRCC)

Germinálna heterozygotná mutácia génu pre fumarát hydratázu bola nedávno idenifikovaná ako hereditárny predisponujúci faktor pre niektoré uterinné a kožné leiomyómy a renálne karcinómy v rámci syndrómu HLRCC (22,23). Tento syndróm bol prvýkrát popísaný fínskymi autormi ako zostava 25 probandov (24). Najzávažnejším nálezom u časti pacientov je vznik biologicky agresívnych renálnych karcinómov. To je dôvod, prečo sa novoidentifikovaným pacientom s HLRCC syndrómom ponúka vstup do programu pravidelného USG sledovania obličiek 1x ročne. Prepoklad je, že prípadný karcinóm by tak bol zachytený vo časnom štádiu vývoja. Vzhľadom na raritnosť syndrómu a neúplnú penetranciu je stále otvorená otázka efektívnej identifikácie pacientov v populácii. Doteraz bolo navrhnutých niekoľko prístupov. Sanz-Ortega navrhuje histomorfologický skríning s nasledujúcou genetickou testáciou pre zárodočnú mutáciu FH génu (23). Bardella navyše navrhuje používanie protilátky 2SC (25). Reyes tiež navrhuje kombináciu histologických charakteristík a imunohistochemickú pozitivitu expresie 2SC pred molekulovo genetickou analýzou (26). (Pozn. autora - kolektív A. Abbasa v máji 2018 na základe skúseností s 22 prípadmi FH deficientných leiomyómov navrhuje ako efektívny prvý krok vyhodnocovanie prítomnosti histomorfologických znakov (27).

1.5 Zriedkavé leiomyómy s deficitom fumarát hydratázy (FH deficientné leiomyómy) v rutinnej biopsii

HLRCC syndróm, známy aj ako Reedov syndróm, je hereditárny autozómovo dominantne dedičný nádorový syndróm a je podrobnejšie popísaný v predošlej kapitole. Jedným z jeho prejavov sú leiomyómy maternice. U väčšiny žien s HLRCC syndrómom vzniknú symptomatické leiomyómy vyžadujúce chirurgickú intervenciu v mladom veku v čase pred vznikom renálneho karcinómu (23,24,28). Podľa jednej štúdie až u 98% žien s kožnými leiomyómami boli súčasne prítomné leiomyómy maternice, z ktorých bolo až 91% operovaných. Je významné, že takmer 60% z týchto pacientiek podstúpilo hysterektómiu vo veku menej ako 30 rokov. Všeobecne sú leiomyómy maternice veľmi časté. Pravdepodobne 20% až 50% žien vo veku do 30 rokov má leiomyóm maternice. Vo vekovej skupine do 50 rokov je to 80% (29). Preto akýkoľvek skríningový test na identifikáciu pacientiek s HLRCC syndrómom musí byť veľmi špecifický. V minulosti sa zistilo, že leiomyómy asociované so zárodočnou mutáciou FH génu môžu prezentovať kombináciu zvlášnych histomorfologických charakteristík (23,26,30). Ich senzitivita a špecificita však ešte nie je jednoznačne potvrdená (30). V prvých publikáciách na tému HLRCC syndrómu (31) sa ukázalo, že imunohistochemická strata expresie FH v spojení s morfologickými charakteristikami môže svedčiť pre FH-deficientné leiomyómy. FH protilátka je komerčne dostupná.

1.6 Bizarná dysplázia krčka maternice

Väčšinu prípadov HSIL je možné jednoznačne diagnostikovať v súlade s WHO klasifikáciou 2014 (32). Malá časť prípadov je diagnosticky problematická z dôvodu metaplastického fenotypu, alebo v prípade, že sa jedná o nízky dysplastický epitel (33-35). Asi 10 % lézií sa však tejto klasifikácii môže vymykať a to najmä v prípadoch s prítomnosťou mnohojadrových buniek s bizarnými jadrami v dysplastickom epiteli. V súlade so závermi štúdie LAST (36) bloková pozitivita expresie antigénu p16 dovoľuje léziu jednoznačne označiť ako HSIL napriek tomu, že na úrovni histomorfológie nespĺňa kritéria konvenčnej lézie typu HSIL. Časť týchto lézií skôr patrí k léziám „blandného typu", ktoré v minulosti čiastočne popísali Park (35) a Kitahara (34) a označovali ich ako „low grade skvamózna intraepiteliálna lézia s výraznými cytologickými atypiami"(ktoré potom preklasifikovali na HSIL - osobná komunikácia, USCAP 2015, poster session, Boston, USA) a ako „zradná dysplázia".

1.7 Význam bizarných buniek v skríningovej LBC cytológii

LBC cytologické preparáty niekedy obsahujú výrazné veľké bunky dlaždicového pôvodu vzhľadu superficiálnych buniek s objemnou cytoplazmou, ktoré charakterizuje výrazne zväčšené jadro (viac ako trojnásobok plochy jadra intermediálnej bunky). Príčinou je fenomén

dvojjadrovosti, alebo mnohojadrovosti, ktorá vedie k bizarným tvarom jadier. Podľa aktuálnej Bethesda klasifikácie z roku 2014 tieto bunky patria do kategórie LSIL. Vzhľadom na paralelný projekt popisu bizarnej dysplázie z histologického uhla pohľadu (37) na úrovni cytológie sa nám zdalo vhodné pozorovať tento fenomén v prospektívnej štúdii (38). Tento zámer možno vnímať v kontinuite s pozorovaniami Parka (35), ktorý popísal „low grade skvamózna intraepiteliálna lézia s výraznými cytologickými atypiami". Neskôr Washija v roku 2013 (39) hodnotil význam dvojjadrovosti spojenej s kompresiou jadier a prítomnosť mnohojadrových dlaždicových buniek v LBC vzorkách. Napriek tomu, že tento autor použil trochu iný prístup a bolo to v čase platnosti Bethesda klasifikácie 2001 (40) a pred štúdiou LAST (36) a pred WHO klasifikáciou cervikálnej dysplázie z roku 2014, štúdia zistila silnú asociáciu medzi prítomnosťou dvojjadrovosti, alebo mnohojadrovosti a vysokou pravdepodobnosťou HPV pozitivity s následnou histologickou diagnózou CIN 1 až CIN 2. Nedávno Masand (41) popísala LLHSIL ako léziu, ktorá korelovala s high-risk HPV genotypom. Autori konštatovali, že LLHSIL morfologicky imituje LSIL a že u týchto lézií je plne indikovaná analýza expresie antigénu p16. Z hľadiska manažmentu pacientiek, ktorý je popísaný a diskutovaný nižšie, je významná informácia z databázy Bioptické laboratoře a to, že pravdepodobnosť existencie záznamu o histologickej verifikácii lézie pri cytologickej diagnóze LSIL je 10%. Pre diagnózu ASC-H je to 22 % a pre diagnózu HSIL 32 %. Z toho vyplýva, že pacientka s diagnózou ASC-H má dvojnásobne vyššiu šancu na histologické overenie cytologického nálezu než pacientka s diagnózou LSIL. (Tieto údaje vyplývajú z databázy cytologicko-histologických korelátov, ktoré sa generujú automaticky a z faktu, že abnormálny cytologický nález nie je jediným indikačným kritériom pre histologické overenie lézie. Okrem toho klinici nemajú povinnosť dohlásiť histologický nález cytologickému laboratóriu.)

2 Ciele práce

1. Popis histomorfologickej „mulleriánskej“ variability kožnej endometriózy.
2. Popis zvláštneho nádoru – borderline serózneho papilárneho tumoru fimbrií tuby s analýzou prítomnosti mutácií génov KRAS, BRAF a p53.
3. Prospektívna longitudinálna štúdia ťažkej dysplázie dlaždicového epitelu (HSIL) krčka maternice u HPV vakcinovaných žien so snahou odhaliť príčiny tohoto fenoménu.
4. Zlepšenie diagnostiky a zvýšenie záchytnosti HLRCC syndrómu pomocou navrhovaného selektívneho molekulovo genetického skríningu.
5. Posúdenie incidencie *fumarát hydratáza (FH)* deficientných leiomyómov maternice a syndrómu hereditárnej leiomyomatózy a renálneho karcinómu (HLRCC).
6. Histologický popis bizarnej dysplázia krčka maternice ako varianty HSIL.
7. Cytologický popis bizarnej dysplázia krčka maternice ako varianty HSIL.

3 Výsledky

3.1 Histomorfologická variabilita endometriózy kože a povrchových mäkkých tkanív vznikajúcej spontánne a v súvislosti s jazvou: Štúdia 71 prípadov s dôrazom na atypické rysy a typy mulleriánskej diferenciácie.

(Morphological variations of scar-related and spontaneous endometriosis of the skin and superficial soft tissue: A study of 71 cases with emphasis on atypical features and types of mullerian differentiations)

Práca podáva podrobnú histomorfologickú analýzu atypických rysov a rôznych typov „mulleriánskej“ diferenciácie prítomnej v ložiskách endometriózy kože a povrchových mäkkých tkanív u 71 pacientiek vo veku 22 až 65 rokov (priemerný vek 32 rokov). Plzeňský nádorový register a register konzultácií autorov obsahovali 71 prípadov endometriózy kože a mäkkých tkanív, ktoré boli revidované. Formou telefonicky vyplňovaného dotazníka sa u každej pacientky zisťovali klinické a anamnestické údaje, a to najmä lokalizácia lézie, klinické symptómy a zvolený operačný postup. Histologické hodnotenie bolo zamerané na prítomnosť reaktívnych atypií (23%). Atypické mitózy v žľazovom epiteli boli prítomné v 6% prípadov, ďalej rôzne typy metaplázií: tubálna (61%), oxyfilná (15%), cvočkovitá (10%), mucinózna (4%), papilárna syncytiálna (3%). V stromálnej komponente sme sa zamerali na hľadanie reaktívnych zmien, zmien súvisiacich s graviditou a na zmeny asociované so zvýšenou hladinou progestínu. Identifikovali sme hladkosvalovú metapláziu (31%), stromálnu endometriózu a elastózu (do 1%), decidualizáciu (1%). Z iných nálezov to boli bunky podobné lipoblastom (15%), degenerované myocyty (10%), prítomnosť špirálových artérií (4%) a prítomnosť veľkých granulovaných lymfocytov (LGL). Záver: Kožná endometrióza môže indukovať neobvyklé reaktívne zmeny v okolitom tkanive a to najmä v tukovom tkanive (bunky podobné lipoblastom) a vo svalovine (degenerácia myocytov), ktoré môžu imitovať malignitu. Novým poznatkom je popis špirálových arteriol endometriálneho typu a veľkých granulovaných lymfocytov (LGL).

Morphological variations of scar-related and spontaneous endometriosis of the skin and superficial soft tissue: A study of 71 cases with emphasis on atypical features and types of mullerian differentiations¨

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Background: Seventy-one cases of scar-related and spontaneous endometriosis of the skin and superficial soft tissue were studied, with a focus on atypical features and types of mullerian differentiation. All patients¨ were women, whose ages ranged from 22 to 65 years (median, 32 years).

Methods: Histological, immunohistochemical, and electronmicroscopic studies were performed. Clinical information was ascertained via a questionnaire solicited by the referring physicians.

Results: All types of metaplastic changes of mullerian epithelium were found, including tubal (61%),¨ oxyphilic (15%), hobnail (10%), mucinous (4%), and papillary syncytial (3%) metaplasia. Atypical features included reactive atypia (23%) and atypical mitoses in glandular epithelium (6%). Stromal changes included smooth muscle metaplasia (31%), decidualization (\1%), stromal endometriosis (\1%), and elastosis (\1%). Other features recognized included lipoblast-like cells (15%), some with intranuclear inclusions; atypical/degenerative myocytes (10%); spiral arteries (4%); and perineurial invasion (\1%). CD56 staining identified large granular lymphocytes in 15 of 20 studied specimens. Ultrastructurally, these cells showed cytoplasmic granules, some with a delimiting membrane.

Limitations: This study utilizes tissue specimens that mainly were received as consultations; therefore some inherent selection bias exists. Specimens were randomly selected for CD56 immunostaining, leading also to potential sampling error.

Conclusions: All types of mullerian metaplasia can be encountered in cutaneous endometriosis. In addition,¨ so-called atypical features described in endometriosis affecting other anatomic sites may be seen in the skin. Some features may represent a diagnostic pitfall. (J Am Acad Dermatol 2007;57:134-46.)

Endometriosis is defined as the presence of endometrial tissue outside the endometrium and myometrium.[1] Cutaneous endometriosis is a well-known condition, usually presenting no problem for dermatopathologists, despite its relatively infrequent occurrence in dermatopathology practice. After the seminal papers by Steck and Helwig[2,3] comprising 82 cases, only a few small series were published, whereas the majority of the reports on cutaneous endometriosis in the literature appeared as isolated case reports. In recent years, a number of unusual microscopic features have been described in endometriosis in various anatomic sites.[1,4-6] Some of them have been called atypical and

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Funding sources: None.
Conflicts of interest: None declared.

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0190-9622/$32.00

doi:10.1016/j.jaad.2006.11.036

shown to represent potential diagnostic pitfalls. To our knowledge, these features have not been systematically analyzed in cutaneous endometriosis. Therefore we undertook the current study and herein present a detailed histopathologic analysis of a large series of cutaneous and subcutaneous endometriosis, with an emphasis on atypical features and types of mullerian differentiation, documenting¨ some hitherto undescribed microscopic features in this location.

MATERIAL AND METHODS

A search in our consultation and routine institutional files in Pilsen, Czech Republic between 1993 and 2006 yielded 73 cases coded as endometriosis involving the skin or superficial soft tissue, including the subcutis and that occurring either within surgical scars or spontaneously. Hematoxylin-eosinestained slides were reviewed to confirm the diagnosis, and the histopathologic findings were correlated with the clinical data to confirm the location and appropriate clinicopathological context. Two cases were excluded, with one matching the description of presumptive endometriosis (it exhibited fibrotic granulation tissue, large numbers of pseudoxanthoma cells, and totally lacked endometrial epithelium and stroma), and the second case representing cutaneous endosalpingiosis. Thus 71 cases were included in the study. Questionnaires seeking clinical details were sent to all patients' physicians. Specific inquiries regarding the type of operation, location, symptoms were included.

Histologic parameters were estimated mainly in accordance with the summary of atypical features detailed by Clement and Young[6] in 2000. The following features concerning the glandular component were assessed: reactive atypia, metaplasia (tubal, hobnail, squamous, mucinous, oxyphilic), pregnancy or progestin-related changes (Arias-Stella reaction, optically clear nuclei), hyperplasia (typical, atypical). For the stromal component, the following features were evaluated: pregnancy or progestin-related changes (decidual change, signet ringelike cells), myxoid change, smooth muscle metaplasia, absence of glandular component (stromal endometriosis, including micronodular stromal endometriosis), and elastosis.[6-8] We also looked for other rare changes seen in endometriosis, such as necrotic pseudoxanthomatous nodule formation, blood vessel and lymphatic invasion, perineural invasion, and Liesegang rings.[9-11] Lastly, we recorded other unusual features, if present. The number of tissue blocks available for review varied from 1 to 18.

In 20 randomly selected cases, immunostaining with an antibody against CD56 (clone 1B6, dilution 1:100, microwave antigen retrieving, Novocastra) was performed. CD56 was used to identify so-called endometrial large granular lymphocytes (LGL), known ¨also in the early literature as Kornchenzellen ¨(German; Kornchen = small grains, Zellen = cells). Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue according to standard protocols using an alkaline phosphatase-antiealkaline phosphatase method or avidin-biotin complex labeled with peroxidase or alkaline phosphatase. Appropriate positive and negative controls were applied.

For electronmicroscopic investigations wet formol-fixed tissue was available in two cases. It was postfixed in 4% paraformaldehyde, followed by 1% osmium tetroxide, and embedded in epoxy resin (Durcupan-Epon). Sections 1 m in thickness were cut, stained with toluidine blue, and examined by light microscopy. Appropriate areas were selected, and thin sections were cut and stained with uranyl acetate and lead citrate, and examined with a Philips EM 208S electron microscope (Eindhoven, Netherlands).

RESULTS

Clinical data

All patients were women, whose ages ranged from 22 to 65 years (median 32 years, mean 33.9 years). Sixty-seven patients presented with scar-related endometriosis, which occurred on the abdomen, in the perineum, inguinal area, or vulva within cutaneous scars of the following types of surgical operation: cesarean section, n = 36; laparotomy, 5; episiotomy, 4; appendectomy, 1; laparoscopy, 1; Bartholin gland cyst removal, 1. In 19 cases the type of surgical operation remained unknown. In the remaining 4 women, endometriosis was spontaneous and the lesions were located in the navel. The period between the surgical operation and the development of endometriosis in the scars, as specified in 46 cases, varied from 8 months to 19 years (median 4 years, mean 5.5 years). The lesions were usually solitary; however, in 5 cases, there were 2 or 3 nodules. Pain was specifically commented in 11 cases, but this may be an underestimate as not all clinicians addressed this issue. Two patients presented with recurrent endometriosis, and one of them also had inguinal lymph involvement. One woman presented simultaneously with cutaneous endometriosis involving the navel and peritoneal endometriosis. One patient had tubal pregnancy at presentation. All patients were treated by surgical resection.

Gross features

Adequate gross description was available in 29 cases, among which a majority represented

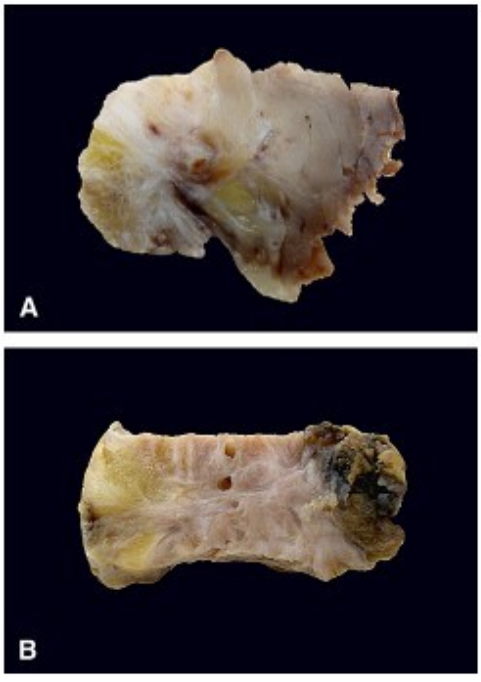

Fig 1. A and B, Gross appearance of scar-related endometriosis: white scar tissue, small hemorrhages and tiny cystic spaces can be recognized.

scar-related endometriosis described, on cut section, as a gray-white scarlike tissue, with or without evidence of hemorrhage containing tiny cysts when viewed under a magnifying glass (Fig 1).

Histopathologic data

See Table I. All cases but two (which corresponded to stromal endometriosis and stromal micronodular endometriosis) revealed typical endometriotic glands associated with a characteristic stroma. The glands and stroma varied in size, configuration, luminal content, and composition reflecting changes of the proliferative phase, secretory phase, or that reminiscent of menstruation of the normal uterine mucosa (Fig 2, A, B, C, and D). Rarely, telescopic glands or glands with the invagination of the stroma in to the lumen with an appearance reminiscent of a miniature polyp were seen (Fig 2, E). The endometriotic foci were randomly distributed, but in one case the growth pattern was that of complex (adenomatous) hyperplasia without atypia (Fig 3).

The most common type of metaplasia in the endometrial glands was ciliated (tubal), with cilia and intercalated (''peg'') cells (Fig 4, A and B). To mark the epithelium as tubal type we required the presence of both ciliated cells and ''peg'' cells, although, in reality, many ''transitional'' tubal-endometrial glands were seen. Tubal metaplasia was

Table I. Histopathologic features in cutaneous and superficial soft tissue endometriosis (N = 71)

Histopathological features	Frequency
Glandular component	
Metaplasia	
Tubal	43/71 (61%)
Hobnail	7/71 (10%)
Mucinous	3/71 (4%)
Oxyphilic	11/71 (15%)
Squamous	0/71
Papillary syncytial	2/71
Reactive atypia	15/71 (3%)
Atypical mitoses in glands	4/71 (23%)
Arias-Stella reaction	0/71 (6%)
Optically clear nuclei	0/71
Hyperplasia	1/71*
Stromal component	
Decidual change	(\1%)
Signet-ring-like cells	
Myxoid change	1/71 (\1%)
Smooth muscle metaplasia	0/71
Stromal endometriosis	49/71 (69%)
Micronodular stromal endometriosis	22/71[y] (31%)
Elastosis	1/71 (\1%)
Other features	1/71 (\1%)
Necrotic pseudoxanthomatous nodule formation	1/71 (\1%)
Blood vessel invasion	
Lymphatic invasion	0/71
Perineural invasion	
Liesegang rings	0/71
Lipoblast-like cells	0/71
Intranuclear inclusions in adipocytes	1/71 (\1%)
Atypically appearing myocytes	0/71
Spiral arteries	11/71 (15%)
CD56[1] large granular lymphocytes	7/71 (10%)
	7/71 (10%)
	3/71 (4%)
	15/20[z] (75%)

*Complex hyperplasia without atypia.
[y]Smooth muscle metaplasia was seen as well-demarcated fascicles of well-differentiated smooth muscle cells (n = 10) or as a desmoid-like myofibroblastic proliferation (n = 12).
[z]Identified on immunohistochemistry.

followed by oxyphilic and hobnail metaplasia (Fig 4, C and D). In 3 cases, mucinous metaplasia with formation of endocervical glands was identified (Fig 4, E), and in one of these 3 cases there were also goblet cells (Fig 4, F). Two lesions exhibited glands with incipient papillary syncytial metaplasia (Fig 4, G).

Reactive atypia of the endometrial glands characterized by mild pleomorphism of epithelial cells with some crowding was identified in 16 cases (Fig 5, A), and in 4 of these, additionally, there were rare atypical mitoses in the glandular epithelium (Fig 5, B).

The glands were invariably associated with the stroma, with exception of one case of authentic

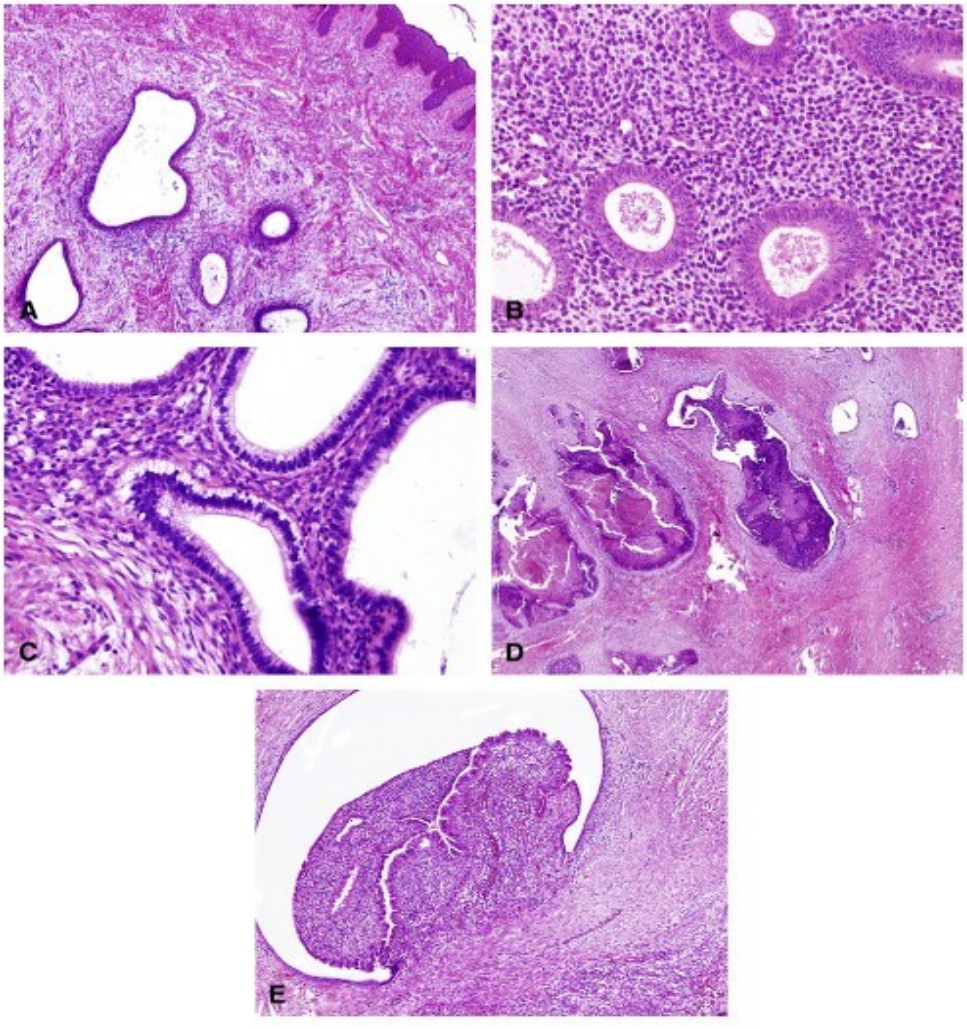

Fig 2. A, Endometriotic glands and stroma in the skin. B, Epithelial and stromal changes reminiscent of a proliferative phase. C, Subnuclear vacuoles with dense chromatin nuclei corresponding to early secretory phase. D, Glands containing cellular debris. E, Invagination of stroma into glandular lumen with appearance reminiscent of a miniature polyp. (Hematoxylin-eosin stain; original magnification: A, 340; B, 3100; C, 3200; D, 340; E, 310.)

stromal endometriosis in which only the stroma was present throughout the lesion, and another case matching the description of so-called micronodular stromal endometriosis.[6] However, it was not uncommon to find in a specimen small areas of the stroma not associated with glandular tissue. When embedded in a fibrous tissue in scar-related cases, these stromal islands devoid of the glandular epithelium sometimes had a resemblance to tumorous elements seen in plexiform fibrohistiocytic tumor, especially to the areas in the latter entity in which there are no giant cells, a situation encountered at times.[12]

Smooth muscle metaplasia was seen in 22 cases and appeared either as well-formed, well-demarcated fascicles of well-differentiated smooth muscle cells, often with cigar-shaped nuclei (Fig 6, A) or the appearance was that of a fascicular myofibroblastic proliferation with a similarity to desmoid (Fig 6, B). Smooth muscle metaplasia was also seen in the lymph node specimen involved by recurrent endometriosis.

Decidualization of the stroma was observed only in one case (Fig 7).

In 11 specimens, we observed a peculiar change in adipose tissue in the vicinity of the endometrial foci: vacuolization of lipocytes with the vacuole(s) indenting the nucleus, which resulted in a lipoblast-like appearance. When collected in clusters or small sheets these lipoblast-like cells, including signet-ring

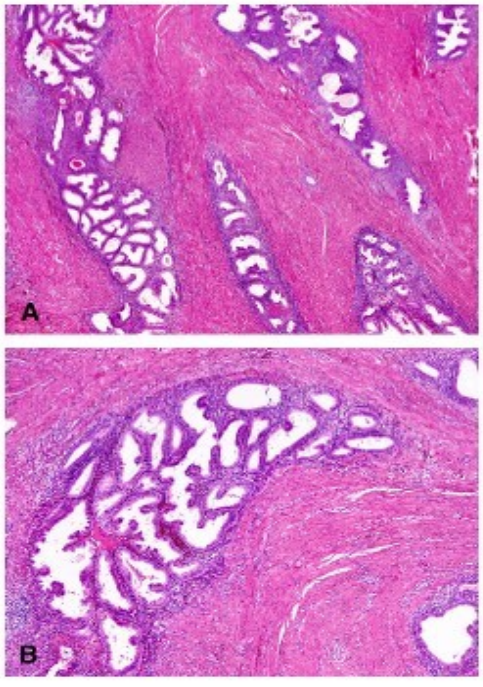

Fig 3. A and B, Complex (adenomatous) hyperplasia without atypia. Crowded glands with complex outlines and projections into lumens arranged ''back-to-back'' with little intervening stroma. (Hematoxylin-eosin stain; original magnification: A, 340; B, 3100.)

forms scattered among variably sized adipocytes, often in a myxoid background produced an appearance very similar to a well-differentiated liposarcoma (Fig 8, A). In addition, adipocytes with intranuclear vacuoles were identified in 7 cases (Fig 8, B).

In 7 cases in which skeletal muscle was incorporated in the specimen, there were degenerative and often multinucleated myocytes (Fig 9). When lying in close approximation to endometriotic glands, they simulated a sarcomatous change in the stroma (Fig 9).

One case exhibited perineural invasion. There was no intravascular invasion of endometriotic elements, but in two cases endometriotic glands and stroma were found in the vicinity of the thick-walled vessels, almost wrapping them around. These vessels resembled spiral arteries of the normal endometrium by virtue of a small, even, round lumen and a thick wall containing several concentric layers of smooth muscle cells (Fig 10). Overall, such vessels were detected in 3 specimens.

Elastosis of the stroma was seen in one case and confirmed by elastic staining.

In 4 cases, eccrine glands displayed a peculiar change characterized by irregularly branching lumens in the secretory parts and enlargement of the outer layer cells that contained ample round cytoplasm, were round with a well-delineated cellular membrane, thus imitating a pagetoid spread (Fig 11).

In the case with lymph node involvement, besides endometriotic glands and stroma, there was focal muscle cell metaplasia and areas resembling an incipient mullerian adenofibroma by virtue of leaf-¨ like formations of the glands with adjacent bland, cellular stroma (Fig 12).

Immunohistochemical findings

Immunostaining for CD56 performed in 20 cases revealed intraepithelial and stromal cells with granular cytoplasm, representing endometrial LGL in 15 specimens (Fig 13). On hematoxylin-eosinestained slides, these cells were often difficult to recognize, except for the case with decidualization of the stroma (Fig 7).

Ultrastructural findings

Electronmicroscopic investigation was aimed at identifying LGL. They were seen as cells that, in addition to usual organelles, contained round cytoplasmic granules, some with a delimiting membrane (Fig 14).

DISCUSSION

Normal mullerian epithelium is apt to show a¨ broad spectrum of metaplastic changes, and these have been demonstrated in endometriotic glands.[13-18] In our series, the most common type was tubal. To mark the epithelium as tubal type we required the presence of both ciliated cells and ''peg'' cells, although, in reality, many ''transitional'' tubal-endometrial glands were seen. Comparison of our findings with those in the previously published material suggests that tubal metaplasia is underrecognized in cutaneous endometriosis. For example, Steck and Helwig,[2,3] having studied 82 cases of cutaneous endometriosis, which remains the largest series on this condition reported to date, did not mention a single case of tubal metaplasia. In addition to ciliated metaplasia, we also detected all other types of metaplasia seen in mullerian epithelium, namely hobnail,¨ oxyphilic, papillary syncytial, and mucinous metaplasia. The latter is considered a change toward endocervical type epithelium and sometimes designated as endocervicosis.[19]

In contrast to the data of Steck and Helwig, who identified no single case of smooth muscle metaplasia, we observed this feature in quite a number of cases, even if one may discard the myofibroblastic desmoid-like variety. Steck and Helwig[2] ''. . . found only the smooth muscle of vascular structures and

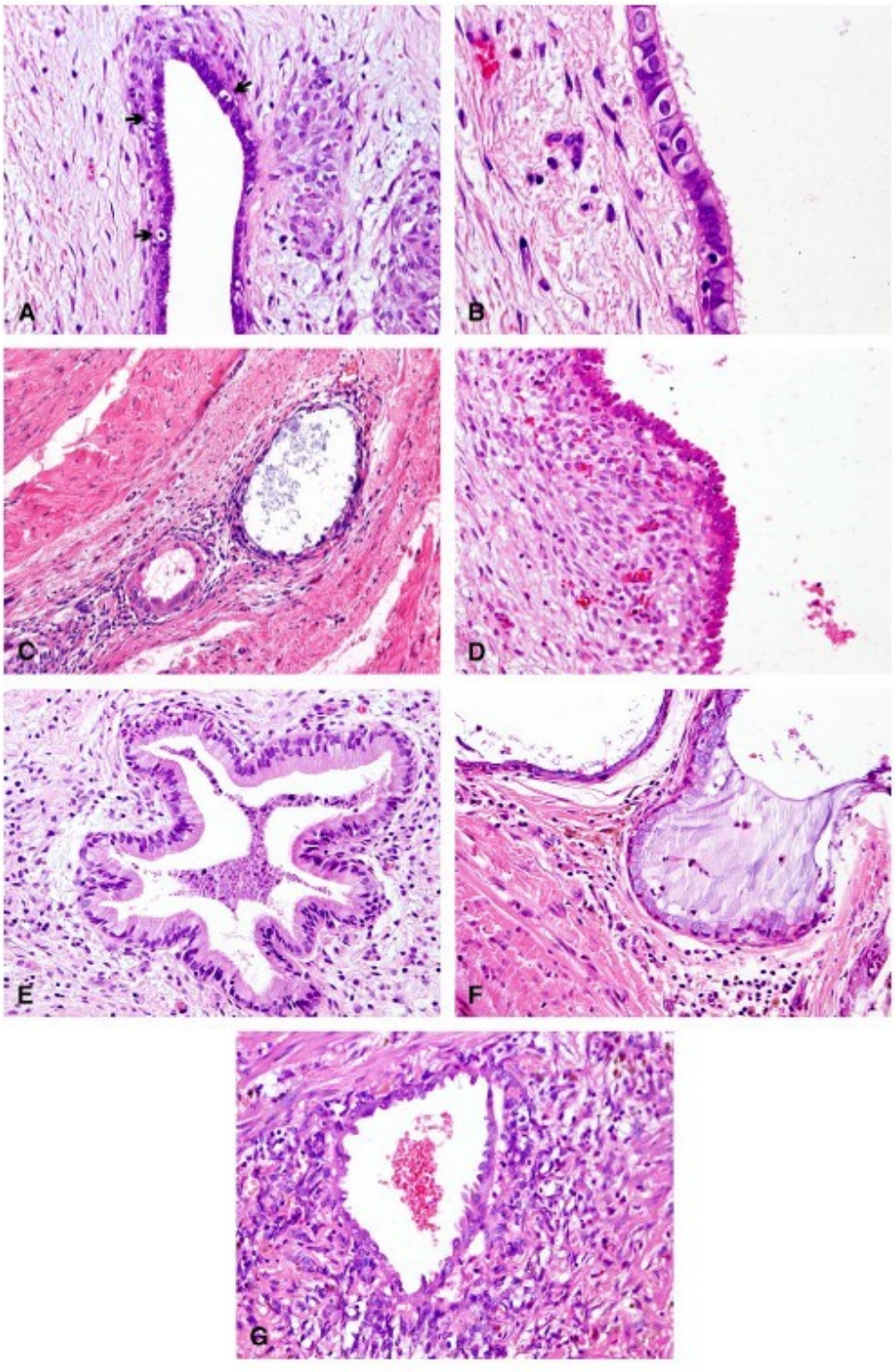

Fig 4. Types of mullerian metaplasia. A, Tubal metaplasia; note ciliated epithelium and ‘‘peg’’¨ cells (arrows). B, Well-developed cilia. C, Oxyphilic metaplasia in gland at left. D, Hobnail metaplasia; cells with enlarged rounded nuclei. E, Mucinous (endocervical) metaplasia; epithelial lining of this gland resembles normal endocervical mucosa with small basilar nuclei above which mucin-filled cytoplasm can be seen. F, Goblet cells; mucin-containing cells with nuclei displaced to base of cell by cytoplasmic mucin. G, Incipient papillary syncytial metaplasia. (Hematoxylin-eosin stain; original magnification: A, 3100; B, 3400; C, 3100; D, 3200; E, 3400; F, 3200; G, 3200.)

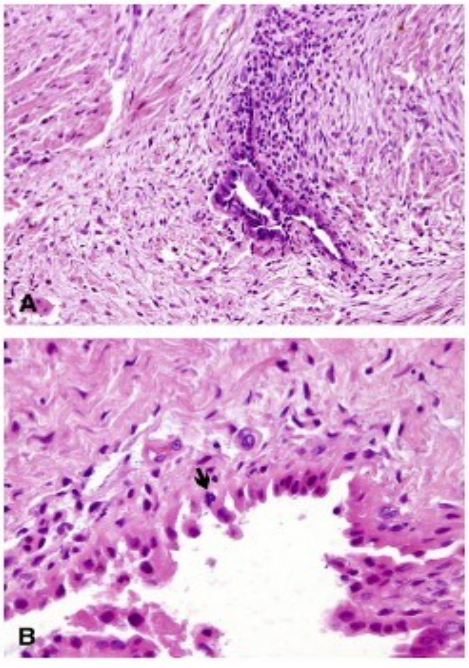

Fig 5. Glands with reactive atypia (A) and atypical mitotic figure (arrow) (B). (Hematoxylin-eosin stain; original magnification: A, 3100; B, 3400.)

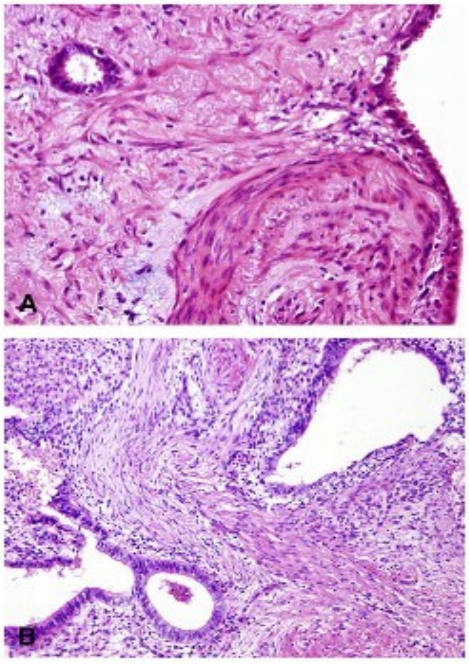

Fig 6. Smooth muscle metaplasia. A, Well-formed, well-demarcated fascicles of well-differentiated smooth muscle cells, some of which have cigar-shaped nuclei. B, Fascicular myofibroblastic proliferation with similarity to desmoid. (Hematoxylin-eosin stain; original magnification: A, 3100; B, 3100.)

the arrector pili.'' We observed the foci of smooth muscle tissue both in superficial cutaneous specimens and in those that came from deeper tissue, where no hair follicles are normally found, excluding the possibility of the arrector pili being the source for the smooth muscle tissue. In addition, smooth muscle metaplasia was noted in a lymph node specimen clearly excluding the arrector pili as the origin. The presence of foci smooth muscle is explained by the myofibroblastic nature of the endometrial stromal cell. In terms of the incidence of smooth muscle metaplasia in various anatomic sites affected by endometriosis, it is most often seen within the walls of ovarian endometriotic cysts, but occasionally may be found elsewhere.[1,20-23] On occasion, extensive amount of smooth muscle tissue can result in endomyometriosis that takes the form of uterus-like masses. This has been reported in various locations including omentum, ovary, broad ligament, lumbosacral region, obturator lymph node, and even the paratesticular regions in male patients.[20-24] We think that smooth muscle differentiation around cutaneous endometriotic glands represents an abortive and incomplete differentiation to the myometrium.

Cutaneous deciduosis is a well-recognized feature, both in scar-related and spontaneous endometriosis of the skin.[25-28] Its incidence in the Steck and Helwig series[2] was 9 of 82, while we have observed only one case among our 71 patients. Decidualized endometrium is due to the influence of ovarian and placental hormones, principally progesterone; thus it is commonly encountered in histologic specimens obtained during pregnancy or progestin therapy. Other pregnancy- or progestin-related changes seen

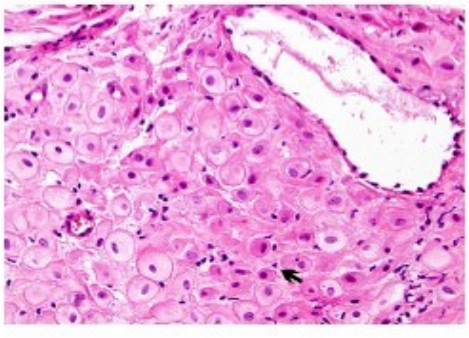

Fig 7. Decidual reaction. Cells with oval, bland nuclei and large amount of pale eosinophilic granular cytoplasm and prominent cytoplasmic membranes. Note an endometrioid gland with atrophic epithelial lining and scattered granular cells likely representing large granular lymphocytes (arrow). (Hematoxylin-eosin stain; original magnification: 3400.)

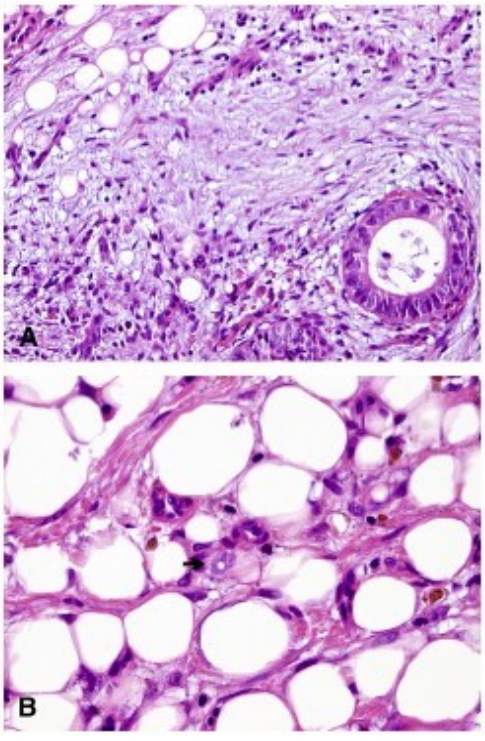

Fig 8. A, Lipoblast-like cells, including signet-ring forms scattered among variably sized adipocytes in a myxoid background resemble a well-differentiated liposarcoma. B, Intranuclear inclusion (arrow) can be seen. (Hematoxylin-eosin stain; original magnification: A, 3200; B, 3400.)

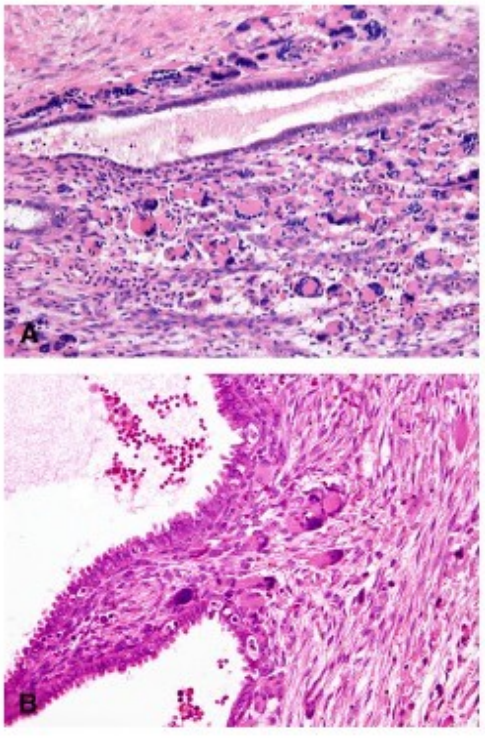

Fig 9. A and B, Degenerative multinucleated myocytes lying in close approximation to an endometriotic gland and simulating a sarcomatous change in the stroma. (Hematoxylin-eosin stain; original magnification: A, 3200; B, 3200.)

in the endometrium and endometriotic glands include the Arias-Stella reaction and optically clear nuclei, features we did not encounter in our cases. Interestingly, there was a woman in our series who presented with tubal gravidity and, simultaneously, scar-related endometriosis of the abdominal wall after a previous cesarean section, and there were neither decidual changes nor the Arias-Stella phenomenon in the skin specimen. In addition, of note is one of our patients with simultaneous presentation of cutaneous and peritoneal endometriosis in which the histologic sections of the peritoneal lesion disclosed decidual changes of the endometrial stroma, whereas this was absent in the skin specimen. This infers that, although endometriotic tissue is hormone dependent, it does not necessarily respond the same way in different anatomic sites in the same person. Likewise, the clinicopathologic correlation of histologic features in cutaneous endometriosis with respect to the main phases of the menstrual cycle and the phase of the menstrual cycle itself revealed no consistent relationship.[29]

Detection of granular lymphocytes recognizable on hematoxylin-eosinestained slides in the decidualized stroma in one of our cases prompted further investigations along this line. As these cells, admittedly, are poorly visible in general and may easily be confused with neutrophils or debris, we used an antibody against CD56 and, therewith, were able to detect $CD56_1$ cells in 15 of 20 studied specimens, both in the stroma and intraepithelially. We believe that these cells do represent endometrial LGL, present in the normal human endometrium throughout the menstrual cycle and reaching a peak in the decidua in early pregnancy. Endometrial LGLs are considered a subset of natural killer (NK) cells exhibiting an unusual phenotype $CD3_e$, $CD16_e$, $CD56_1$, $CD57_e$ and thus differing from peripheral blood NK cells, which are $CD16_1$, $CD56_1$, $CD57_1$.[30] It has been suggested that decidual LGLs are derived from peripheral blood LGLs that home in the uterine mucosa.[31] In addition to normal eutopic endometrium, these cells have been detected in ectopic endometrium,[32,33] but as far as we know their presence in cutaneous endometriosis has not been specifically addressed. Pellegrini[25] commented on ''mononuclear cells'' present in cutaneous decidualized endometriosis; Fig 4 in that article depicted cells likely representing LGL. Tidman and MacDonald[29] mentioned small clear cells that were

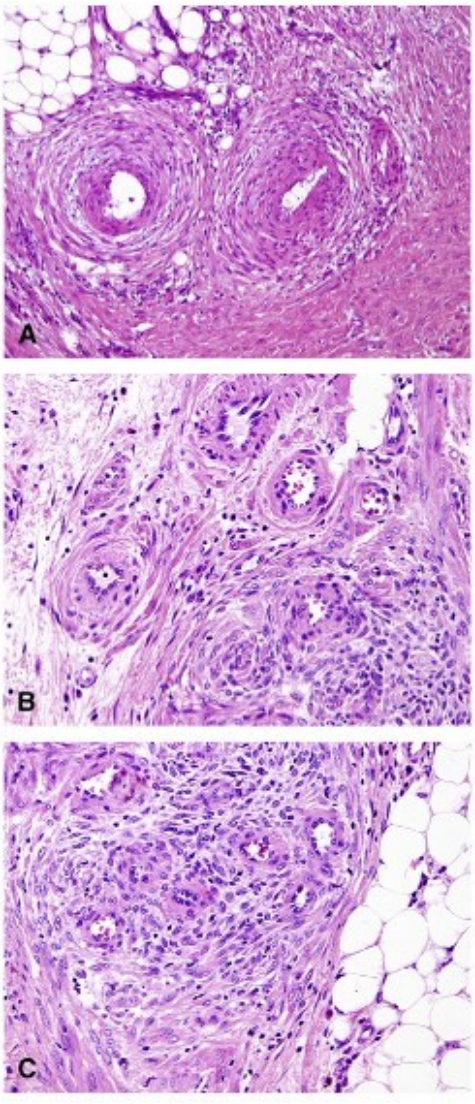

Fig 10. A, B, and C, Thick-walled vessels with small, even, round lumen and concentric layers of smooth muscle cells resembling spiral arteries of normal endometrium. Note endometrioid stroma around these vessels (C). (Hematoxylin-eosin stain; original magnification: A, 3100; B, 3100; C, 3100.)

''. . . morphologically similar to the 'predecidual cell' and endometrial granulocytes . . .''; however, the clear cell illustrated in their Fig 4 seems to be a ''peg'' cell. Our electronmicroscopic investigation identified cells that contained intracytoplasmic granules, and their appearance corresponded to that in LGL reported in the endometrium, both in humans and monkeys.[34,35] The granules differed from those seen in mast cells and eosinophils. Granules in the former cell type have typical scroll-like structures and granules in eosinophilic granulocytes possess crystalline bars, features not observed in the cells we identified in our specimens.

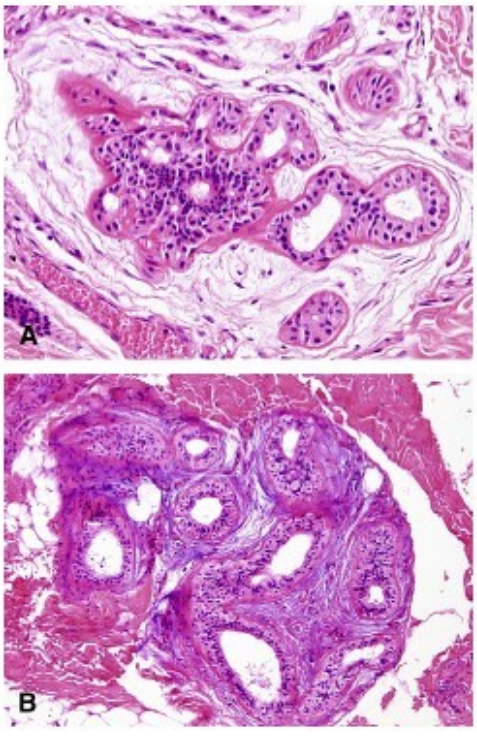

Fig 11. A, B, Eccrine glands with irregularly branching lumens in secretory parts and enlargement of outer layer cells that contained ample round cytoplasm and were round with well-delineated cellular membrane, thus imitating pagetoid spread. (Hematoxylin-eosin stain; original magnification: A, 3100; B, 3100.)

The surrounding connective tissue in endometriosis may exhibit various secondary changes, such as focal ossification, calcification, or myxoid change.[1] Among our cases, we identified two peculiar reactions of adipose and skeletal muscle tissue. The former displayed lipoblast-like cells having one or several intracytoplasmic vacuoles indenting the nuclei. Some areas had an appearance of a well-differentiated liposarcoma by featuring variably sized adipocytes sporting occasional lipoblast-like cells in a myxoid background. In fact, these lipoblast-like cells perfectly match the definition of authentic lipoblasts by exhibiting one or two large intracytoplasmic vacuoles indenting the nucleus, sometimes creating a signet-ring appearance. Although considered pathognomonic for liposarcoma, lipoblasts or lipoblast-like cells have been shown to occur in benign lesions with adipose differentiation. For example, they have recently been identified in nerve sheath tumors and have been interpreted as aberrant multidirectional differentiation.[36] Because in our cases these lipoblast-like cells were distributed in a haphazard fashion and were not confined to the advancing borders of the lesion, we refer to them as ''lipoblast-like'' rather than as ''pseudolipoblasts.''

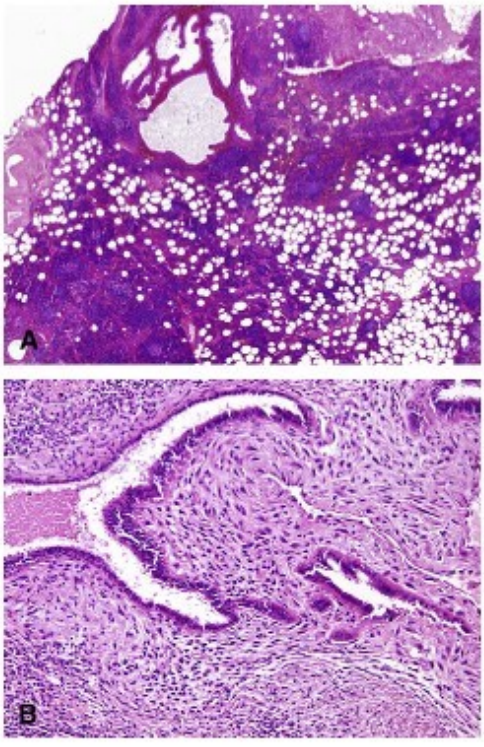

Fig 12. A and B, Lymph node involvement by endometriosis. Leaf-like gland with adjacent cellular stroma bears some resemblance to incipient mullerian adenofibroma¨ (B). (Hematoxylin-eosin stain; original magnification: A, 340; B, 3100.)

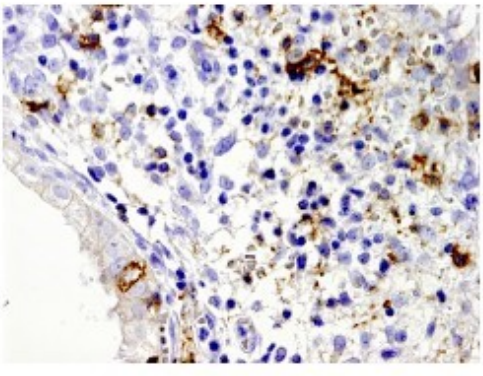

Fig 13. CD56 staining. Cells, some with intracytoplasmic granules, can be seen in epithelial lining as well as in stroma. (Original magnification: 3200.)

Additionally, in 7 cases we identified peculiar intranuclear inclusion in adipocytes in the form of so-called Lochkern (German; Loch = hole and Kern = nucleus), Kerbenkern (German; notched nucleus), and Ringkern (German; ring nucleus). First identified by Unna[37] as early as 1895, these inclusions have recently regained attention.[38] Originally believed to be present in adipocytic tumors, they may cause confusion, sometimes rendering a lipoblast-like appearance, but now these intranuclear inclusions are

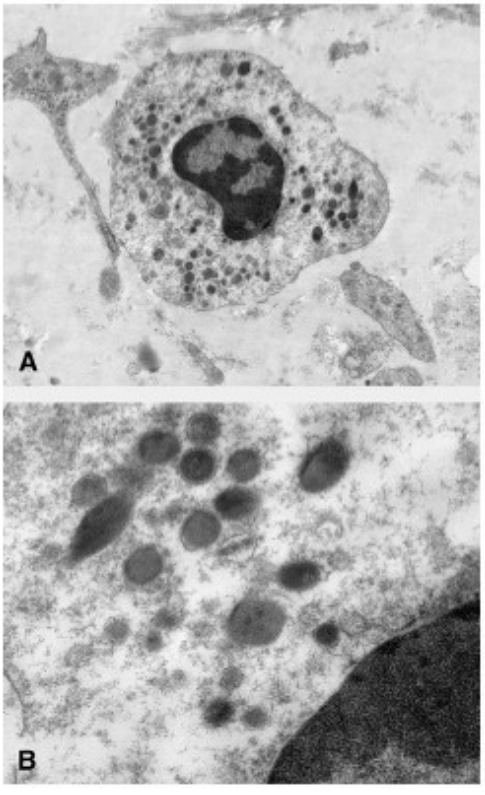

Fig 14. A and B, Large granular lymphocytes. Note round cytoplasmic granules, some with delimiting membrane. (Electron microscopic imaging.)

thought to be normally present within the subcutaneous fat and may be present in any lesion that contains adipocytes.[38]

The reports on cutaneous and subcutaneous endometriosis contain very few references on skeletal muscle involvement by endometriotic tissue.[39,40] Among 17 scar-related cases reported by Chatterjee,[40] there was only a single case with skeletal muscle involvement. The incidence is probably influenced by the surgical approach. Considering the propensity of endometriosis to recur/persist, in all the institutions where our patients were treated, a generous surgical excision was performed, including deeper tissues. The atypically appearing multinucleated myocytes we observed likely represent a degenerative change that is not specific for endometriosis. This type of change can be found in other tumors reaching skeletal muscle. Its recognition is, however, important because in some cases myocytes were located in close proximity to endometriotic glands, replacing the endometriotic stroma and thus simulating a sarcoma arising within endometriotic foci, a rare but known event in endometriosis.[41]

Rarely, a carcinoma may arise within endometriosis in various locations. Some cases have been reported in the skin and included endometrioid

carcinoma and clear cell adenocarcinoma.[42-44] The diagnosis in such cases is usually not difficult if one is familiar with the appearances of gynecological tumors and is aware of such a possibility. In our opinion, a potential pitfall in this situation may be overdiagnosis of hyperplasia, especially with the complex variation with atypia. In our series, we detected only one case of complex hyperplasia without atypia, but, clearly, cases of complex hyperplasia with atypia must exist in cutaneous endometriosis, as these have been described in other locations. Identification of atypical mitotic figures in the endometriotic glandular epithelium may be another source of frustration. Our 4 cases in which this feature was detected did not architecturally qualify as malignant; however, the significance of this finding is unknown. Endometriosis, although considered by many a nonneoplastic benign process, has been shown to be clonal in some locations, particularly in the ovary.[45] Moreover, somatic DNA alterations involving chromosome 17 in general and the p53 locus in particular, have been found in endometriotic lesions, especially in severe/late stage endometriosis.[46] Furthermore, it is known that endometriosis itself may induce a malignant tumor in the female genital tract. Histopathologic and epidemiologic studies have consistently demonstrated a strong link between endometriosis and endometriosis-associated ovarian cancers, in particular, the endometrioid, mucinous, and clear cell subtypes. Recent molecular studies provided evidence for common molecular genetic alterations in both endometriosis-associated ovarian carcinomas and corresponding endometriosis.[47]

Another possible diagnostic pitfall identified in our series may be the perivascular location of endometriotic foci. This may simulate perivascular or intravascular invasion, a phenomenon rarely occurring in this condition.[1,8] Close inspection of the vessels, however, revealed that they were remarkably similar to the spiral arteries of the human endometrium. Cut transversely, the vessels exhibited thick walls of several concentric layers of smooth muscle cells and had small round lumens. We failed to identify these vessels on longitudinal sections performed to see whether they really have the spiral shape. We suggest that these vessels indeed represent endometrial spiral arteries, but the cutaneous location may not allow them to develop the typical spiral configuration. In normal endometrium, spiral arteries respond to varying hormone levels by proliferation and by intermittent contraction during the luteal phase of the menstrual cycle. In the early proliferative phase they are thin-walled and straight, but as the proliferative phase proceeds, they become coiled and their walls increase in thickness. If implantation fails to occur, declining steroid levels induce their constriction, which results in ischemic necrosis of the functionalis and its subsequent sloughing during the menstruation.[48]

Analogous to previous reports, we observed myxoid changes around eccrine and apocrine glands. In addition, in 4 cases eccrine glands displayed a peculiar change characterized by irregularly branching lumens in the parts and enlargement of the outer layer cells that contained ample round cytoplasm, were round with well-delineated cellular membrane, thus imitating a pagetoid spread. Nonneoplastic alterations of eccrine units usually develop in response to tumors, mostly keratoacanthomas and basal cell carcinomas, but may be observed in inflammatory conditions as well. They may adopt various appearances, including proliferation of peripheral or luminal cells of duct, pseudocarcinomatous eccrine glandular hyperplasia, cystic dilation, formation of solid components. Mehregan[49] delimited 4 mains patterns, and our cases with branching lesions may be similar to one of them, although pagetoid-like change in eccrine glands may be a new finding.

Involvement of superficial lymph nodes by endometriosis, as was the case with one of our patients, is rare. This finding theoretically should be differentiated from lymph node inclusions of mullerian-type¨ epithelium that most commonly involve pelvic lymph nodes belonging to the secondary mullerian¨ system.

In conclusion, our study expands the spectrum of histopathologic changes encountered in cutaneous endometriosis, including so-called atypical features described in endometriosis affecting other anatomic sites. Cutaneous endometriotic lesions are apt to show a broad spectrum of metaplastic changes involving the epithelial component as well as the stromal component. In terms of the former, all types of mullerian differentiation can be found. Cutaneous¨ endometriosis may induce a peculiar reaction in the surrounding tissue, namely in fat and skeletal muscles that altogether may result in the appearances simulating a malignancy. Further studies would be needed to substantiate newly described findings in cutaneous endometriosis, such as LGL (by immunoelectron microscopy) and spiral vessels of endometrioid type.

We are grateful to our colleagues pathologists and gynecologists who kindly provided us with case material and clinical data: Romuald Curik, MD (Ostrava, Czech Republic [CR]), Karel Dedic, MD, PhD (Hradec Kralove,CR) Pavel Fajt, MD (Marianske Lazne, CR), Marek Greda,MD (Klatovy, CR), Vladimır Fruhauf, MD (Pilsen, CR),

Jozef Horak, MD (Mlada Boleslav, CR), Zdenek Kestranek, MD (Pilsen), Zdenek Knaizl, MD (Stod, CR), Jozef Reindl, MD (Pilsen), Miroslav Sulc, MD (Chomutov, CR), Vladislav Treska, MD, PhD (Pilsen), Jan Vileta, MD (Rokycany, CR), Lubomır Voltr, MD (Jicinn, CR), Miroslav Zahalka, MD (Pelhrimov, CR), Dalibor Zeman, MD (Ricany, CR).

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3.2 Borderline papilárny serózny tumor fimbrií tuby s peritonálnymi implantmi

(Borderline papillary serous tumor of the fimbriated end of the fallopian tube with peritoneal implants)

Borderline papilárny serózny tumor vajcovodu bol prvýkrát popísaný v roku 1996 Zhengom. Popis bol založený na histologickej podobnosti s identickým tumorom ovária. Jedná sa o raritnú jednotku. V roku 2011 v čase tejto publikácie bolo popísaných iba 8 prípadov, z toho 3 priamo lokalizované vo fimbriách. Klinicky sa u pacientky, ktorú sme identifikovali, prejavovala bolesť v dolnom podbrušku, dyspareunia a dyzúria. Ultrazvukové vyšetrenie identifikovalo adnexálnu masu. Po laparoskopicky vykonanej adnexektómii sa ukázalo, že sa jedná o svetložltobiely lobulizovaný jemne zrnitý tumor cca 7x5x4cm nachádzajúci sa v oblasti fimbrií vajcovodu, bez priameho spojenia s inými okolitými orgánmi. Ďalej boli nájdené neinvazívne implanty v oboch ováriách a na viscerálnom peritoneu. Imunohistochemicky bola zaznamenaná pozitívna expresia Ki67 v menej ako 5% jadier a pozitívna expresia p53 iba fokálne v oblastiach s prejavmi atypie. Tento nádor bol prvý serózny borderline tumor vajcovodu, u ktorého sa zisťovala prítomnosť mutácií genu KRAS, BRAF a p53. Do tej doby bola známa iba jediná analýza ploidity iného identifikovaného tumoru s potvrdením diploidného statusu. Analýza aktivačnej mutácie V600 E génu BRAF a aktivačných mutácií génu KRAS prebehla s použitím strip esej kitu a mutačná analýza kódujúcich exonóv génu p 53 pomocou PCR. PCR analýza bola vykonaná pomocou primerov dizajnovaných pomocou softvéru Primer 3. V nami popisovanom prípade mutácia žiadneho z génov v rozsahu vyšetrenia nebola identifikovaná. Prognóza tohoto nádoru je neistá. Najdlhšie zdokumentované prežívanie pacientky bolo 6 rokov. Aj keď vyššie spomínané mutácie génu KRAS a BRAF sa v seróznych borderline tumoroch nájdu u 50% a 30%, v našom prípade zaznamenané neboli. Anamnéza pacientky bola zaujímavá z dôvodu apendektómie a febrilnej epizódy v druhom puerpériu, čo podporuje hypotézu o význame chronického zápalu v etiopatogenéze borderline papilárneho serózneho tumoru. V čase publikácie bola otvorená aj otázka možnosti stagingu nádorov fimbrií ako stage 1 (F). Aktuálne je platný staging podľa 8. vydania AJCC z roku 2017. Tumor limitovaný na vajcovod s prítomnosťou nádoru na povrchu vajcovodu (pravdepodobne vrátene fimbrií – pozn. autora) je označený ako pT1c2 (stage group IC2). V prípade prítomnosti implantov v oblasti panvy ide o štádium pT2 (stage group II), so subklasifikáciou pT2a,b, stage group II A,B.

THE JOURNAL OF
Obstetrics and Gynaecology Research

doi:10.1111/j.1447-0756.2011.01556.x J. Obstet. Gynaecol. Res. Vol. 37, No. 11: 1702–1705, November 2011

Borderline papillary serous tumor of the fimbriated end of the fallopian tube with peritoneal implants

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Abstract

Diagnosis of Borderline papillary serous tumor of the fallopian tube was comprehensively established by Zheng in 1996 supported mostly by a histological similarity to its ovarian counterpart. It is a very rare entity with eight cases published so far and the ninth case described here as a 41-year-old woman presented with non-specific lower abdominal pain, dyspareunia and dysuria. Left adnexal mass was identified and she was operated on. It turned out the tumor was attached exclusively to the left tube, with no connection to any of the surrounding structures and with histology of borderline serous tumor with non-invasive implants in the left and right ovary and visceral peritoneum. Reviewing available data on genetics of these tumors there was diploid status in one examined tumor, and in our case no mutations of KRAS, BRAF and p53 genes were found. Histomorphology remains the mainstay of diagnosis and staging operation is the mainstay of patient management. Prognosis is uncertain with a 6-year survival documented in one case.
Key words: BRAF protein, fallopian tube, human, KRAS protein, neoplasm.

Introduction

The term Borderline papillary serous tumor of the fallopian tube (BPSTFT) was used for the first time by Zheng in 1996[1] and reviewed by Krasevic in 2005.[2] BPSTFT meets the histological criteria of epithelial stratification and nuclear atypia as defined in its ovarian counterpart.[1]

To our knowledge only eight such tumors have been sufficiently described.[1–7] Biological properties of BPSTFT including genetic characteristics are practically unknown due to its rarity.

Case Report

A 41-year-old patient presented with lower abdominal pain for the previous 6 months, dyspareunia and dysuria.

Personal history was remarkable for childhood appendectomy and abdominal hernioplasty in 2007. She was 2 para (1991, 1998, with second puerperium complicated by febrile episode) with a regular menstrual cycle since the age of 14 (28–30/7). Ultrasound and computed tomography scan revealed left adnexal tumor. Laparoscopic salpingo-oophorectomy was carried out.

During the course of the laparoscopy the surgeon visualized the tumor attached exclusively to the left tube fimbriae, protruding freely into the pouch of Douglas where visceral peritoneum was focally thickened, soft and of a white color (Fig. 1). A left salpingo-oophorectomy was performed. Perioperative diagnosis of low grade fallopian tube carcinoma led to a hysterectomy followed by a contralateral salpingo-oophorectomy accompanied by an infracolic omentectomy and peritonectomy of the pouch of Douglas.

Received: September 2 2010.
Accepted: December 17 2010.
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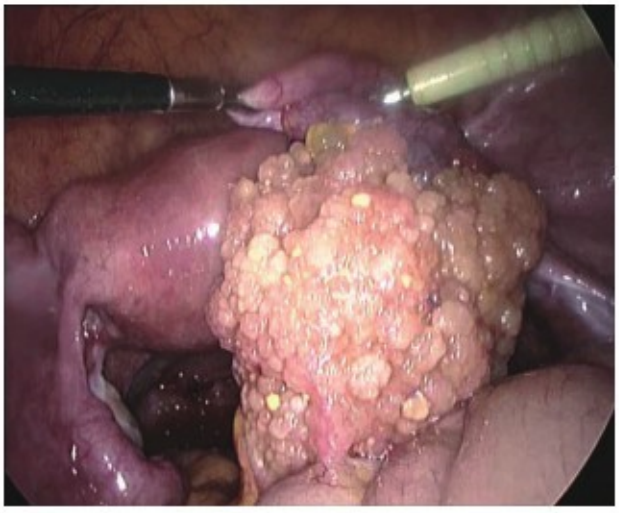

Figure 1 The tumor is pulled out from the pouch of Douglas. It occupies left tube fimbrial area. The left ovary is hidden behind the left tube above the tumor. Right adnexal structures are inconspicuous.

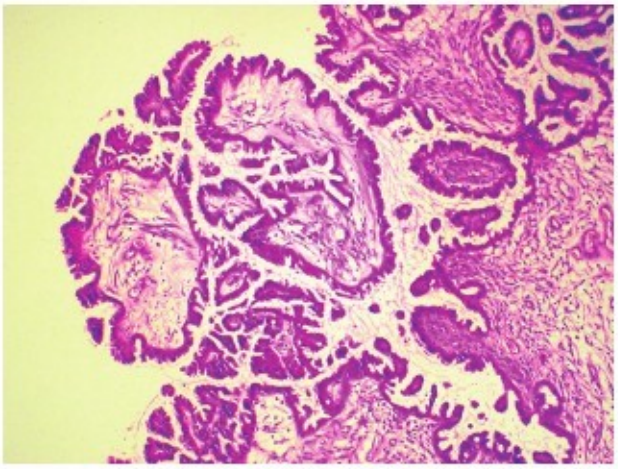

Figure 2 The histopathological appearance of the presented tubal borderline tumor with obvious mild stratification of the surface epithelium with multiple micropapillae formed as well (dark cells with red cytoplasm and blue nuclei) covering edematous papillae (nearly circular slightly indented structures). (Hematoxylin–eosin stain; magnification 100¥.)

The primary operative specimen consisted of an unremarkable left ovary and left tube with the fimbriated end replaced by tumor. It was a yellowish brown friable conical mass of cauliflower-like shape measuring 7 ¥ 5 ¥ 4 cm.

Microscopically, the lesion presented multiple papillary projections composed of fibrovascular cores covered by stratified cuboidal and columnar epithelium with focal 'hobnail' features and mild nuclear atypia (present in less than 1% of examined tissue), consistent with borderline morphology ascribed to ovarian borderline tumors (Fig. 2). Peritoneal lesions described above turned out to be serous desmoplastic non-invasive implants. The cortex of the left and right ovary contained similar microscopic lesions up to 3 mm in diameter.

The patient was well 6 months after the surgery, with no physical or ultrasonographic signs of tumor recurrence.

Sections from surgical specimen were fixed in 10% buffered formalin, embedded in paraffin and stained withhematoxylin–eosin.Immunohistochemical studies were performed on representative sections of the tumor examined using the routine avidin–biotin–peroxidase complex technique along with the appropriate positive and negative controls. We used Ki-67 (Clone MIB-1, mouse monoclonal, 1:1000, Dako, Denmark), and p53 (Clone DO-7, mouse monoclonal, 1:50, Dako, Denmark) antibodies. Immunohistochemistry revealed mild to moderate positive nuclear expression of antigen p53 limited to the areas of mild atypia. These were also characterized by positive expression of MIB1 antigen in less than 5% of epithelial cells. The rest of the examined tumor tissue was p53 negative and MIB1 index was down to 1%.

Genetic studies were also performed on paraffin-embedded representative tumor sections. KRAS, BRAF and p53 genes were analyzed for mutations claimed to be typical for ovarian borderline tumor.[8,9] DNA for molecular genetic investigation was extracted from formalin-fixed, paraffin-embedded tissues. Several 5-mm thick sections were placed on the slides. A hematoxylin–eosin stained slide was examined for determination of the area of tumor tissue. Then tumor tissue from an unstained slide was scraped and DNA was isolated by the NucleoSpin Tissue Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. In several cases, where the quality or amount of extracted DNA was low, concentration was enhanced with Microcon 100 (Millipore, Billerica, MA, USA) according to the manufacturer's protocol.

Analysis of the activating mutation V600E of the BRAF gene and also activating mutations of the KRAS gene was performed using StripAssay PGX KRAS BRAF Kit (ViennaLab, Vienna, Austria) according to the manufacturer's protocol.

Mutational analysis of coding exons of the TP53 was performed using polymerase chain reaction (PCR) and direct sequencing.

PCR was carried out using primers designed using Primer3 software (Rozen and Skaletsky, 2000) (http://frodo.wi.mit.edu/primer3)[10] with regard to the presence of known single nucleotide polymorphism. The reaction conditions were as follows: 12,5 mL of Hot-Start Taq PCR Master Mix (QIAgen, Hilden, Germany), 10 pmol of each primer, 100 ng of template DNA and distilled water up to 25 mL. The amplification program for all exons except 2, 9 and 10 of the TP53 gene consisted of denaturation at 95°C for 14 min and then 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1.5 min. The program was finished by 72°C incubation for 7 min. Annealing temperature for exons 2, 9 and 10 of the TP53 was 55°C. Successfully amplified PCR products were purified with magnetic particles Agencourt AMPure (Agencourt Bioscience Corporation, A Beckman Coulter Company, Beverly, MA, USA), products were then bi-directionally sequenced using the Big Dye Terminator Sequencing kit (PE/Applied Biosystems, Foster City, CA, USA) and purified with magnetic particles Agencourt CleanSEQ (Agencourt Bioscience Corporation) all according to the manufacturer's protocol and run on an automated sequencer ABI Prism 3130xl (Applied Biosystems) at a constant voltage of 13.2 kV for 11 min.

No mutation of KRAS, BRAF nor p53 gene was detected.

Discussion

Diagnosis of BPSTFT was comprehensively established by Zheng in 1996[1] supported mostly by a histological similarity to its ovarian counterpart, which is well described. Recently a body of information on genetic characteristics of ovarian borderline tumors has emerged detecting allelic loss of chromosome 17, 18q, 20q and gain of chromosome 12p13~q23,[11] further allelic alterations of chromosomes 1p, 5q, 8p, 18q, 22q, Xp and mutations of KRAS and BRAF genes. The last two being the most frequent, found in approximately 50 and 30% of cases, respectively.[8,9]

In our case fimbrial borderline tumor tissue did not harbor mutation of KRAS and BRAF genes, nor of the p53 gene. Combining this information with previous DNA ploidy determined in the case report by Krasevic et al.[2] with the result of normal diploid status of the examined tumor, it can be stated that tumor histology and location are still of the highest diagnostic importance.

Staging of fimbrial fallopian tube tumors is doubtful. Alvarado-Cabrero proposed a new category, Stage I (F) to be used.[4] The presence of structures herein described as implants raises and revives the question of correct classification and definition of the terms 'implant' versus 'metastasis' and 'border line tumor with implants' versus 'low grade serous carcinoma with metastases'.

Limited clinical experience with fimbrial tubal borderline papillary serous tumors still poses a great challenge in designing the optimal treatment of each particular patient. The prognosis is uncertain with documented survival up to 6 years.[1]

In conclusion this is the ninth report of BPSTFT, fourth with fimbriated end location, encompassing desmoplastic non-invasive implants of visceral peritoneum and both ovaries. Initially the patient presented with complaint of lower abdominal pain existing for 6 months.

Medical history of appendectomy and second puerperium complicated by a febrile episode lends some support to the theory of inflammation as an important factor in pathogenesis of ovarian and also fallopian tube tumors.[12]

Ahysterectomyandbilateralsalpingo-oophorectomy accompanied by infracolic omentectomy and peritonectomy of the Douglas pouch was performed. Tumor tissue did not harbor KRAS or BRAF mutation in spite of typical borderline histomorphology equaling that of ovarian borderline tumor. No mutation of the p53 gene was detected either. No other therapeutic modality was applied. The patient was free of the disease 6 months after surgery.

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3.3 HPV typizácia ťažkej dysplázie (CIN III) v konizátoch krčka maternice u 38 HPV-vakcinovaných žien

(HPV typing of high-grade dysplasia (CIN III) in cone biopsies of 38 HPV-vaccinated women)

V našom laboratóriu sme od zavedenia možnosti HPV vakcinácie boli sporadicky konfrontovaní s prípadmi ťažkej dysplázie HSIL (CIN 3) u žien s riadne ukončenou HPV vakcináciou v minulosti. Fenoménom DAV (dysplasia after vaccination) sa doteraz nikto systematicky nezaoberal. Na základe skúseností z nášeho pracoviska sme porovnávali HPV typy prítomné v konizátoch s HSIL u HPV vakcinovaných žien oproti publikovanému spektru HPV typov prítomných v cervikálnych HSIL léziách českej populácie. Hľadali sme faktory asociované s vývojom cervikálnej dysplázie po HPV vakcinácii. Krížové vyhľadávanie v databáze nášeho pracoviska z rokov 2006 - 2012 identifikovalo 38 pacientiek, u ktorých po riadne ukončenej anti-HPV vakcinácii bola histologicky potvrdená lézia HSIL v konizáte krčka maternice. Z reprezentatívneho parafínového bloku sme u týchto pacientiek následne vykonali HPV typizáciu 3 rozličnými metódami a pomocou HPV DNA ISH. Štatistické hodnotenie prebehlo pomocou Fisher exact testu pre porovnávanie prevalencie jednotlivých HPV typov programom STATA/1C10.1. Zistili sme že 1/3 žien bola v čase vakcinácie staršia ako doporučovaný vekový limit 26 rokov pre aplikáciu vakcíny. U 7 z týchto pacientiek bola použitá kvadrivalentná vakcína a u 5 bivalentná vakcína. Celkovo 37 (97%) prípadov vzniklo u žien starších ako 17 rokov v čase ukončenia vakcinácie. Všetky konizácie boli vykonané do 3 rokov od ukončenia vakcinácie. Väčšina z nich (28 prípadov) do 2 rokov. 7 krát (18%) boli pacientky konizované do roka od ukončenia vakcinácie. Je pozoruhodné, že 78% prípadov sa vyznačovalo tým, že pacientky mali v anamnéze aspoň 1 abnormálny nález skríningovej gynekologickej cytológie. U 6 pacientiek, ktoré mali negatívnu anamnézu skríningovej cytológie sme 5 krát cytologický nález revidovali na abnormálny. HPV typizácia bola úspešná u 33 pacientiek. Z toho v 2/3 prípadov (67%; 22/33) sme identifikovali HPV typ 16. Porovnanie spektra HPV typov nepreukázalo rozdiely v prevalencii jednotlivých typov. Ekologický shift HPV typov v populácii vakcinovaných pacientiek s dyspláziou sme nepozorovali. Závery štúdie svedčia pre potrebu pokračujúceho cervikálneho skríningu aj u HPV vakcinovaných žien. Ukazuje sa potreba kompetentnej osvety a kompetentného poradenstva ohľadne HPV vakcinácie u žien starších ako 18 rokov.

Virchows Arch
DOI 10.1007/s00428-013-1511-4

ORIGINAL ARTICLE

HPV typing of high-grade dysplasia (CIN III) in cone biopsies of 38 HPV-vaccinated women

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Received: 30 September 2013 / Revised: 31 October 2013 / Accepted: 7 November 2013

Abstract HPV-vaccinated women develop CIN III very rarely. We have identified a study group of 38 such patients and showed that a specific HPV genotype prevalence in those cases equals the prevalence of HPV genotypes in CIN III present in the general Czech population. In all cases, CIN III was diagnosed within 3 years after having completed the HPV vaccination. We conclude that dysplasia was present before the vaccination in those women. A history of abnormal pre-vaccination PAP smear result (present in 78 % of women in the study group) and age of over 17 by the time of vaccination completion (97 % of women in the study group) are identified as probable factors increasing the risk of CIN III development after HPV vaccination.

Keywords Cervix . Dysplasia . CIN III . HPV . Vaccination

Introduction

The Czech HPV vaccination program was launched in the month of December in the year 2006. Quadrivalent and bivalent vaccines were available for young women at the recommended age of 15 up to the age of 26. They have also been available for older women on the bases of consultation for individual cases. Vaccination has not been covered by any insurance program. Nevertheless, there is one exception since April 2012, and that is the newly approved voluntary vaccination of girls at the age of 13 with full insurance coverage. In our laboratory, we have been sporadically confronted with cases of CIN III in previously HPV-vaccinated women since 2009. This phenomenon had not been systematically studied before. Based on our institution's experience, we compared HPV types present in cone biopsies with CIN III in HPV-vaccinated women and HPV type spectrum in cone biopsies presenting CIN III in women of the general Czech population. We searched for factors associated with the development of cervical dysplasia after HPV vaccination.

Materials and methods

We took advantage of the busy surgical pathology and gynecologic cytology practice of our laboratory, which covers the whole area of Czech Republic. We process some 3,500 cone biopsies and more than 700,000 PAP smears per year. We cross searched our database for patients who underwent cone biopsy for CIN III after they had been vaccinated for HPV between 2006 and 2012. In November 2012, our laboratory database contained 24,709 HPV-vaccinated women (Table 1). From those, we identified 38 women who developed CIN III (high-grade dysplasia) subsequent to having completed vaccination. Histology slides were reviewed by one of the authors (O.O.). Subsequently, HPV type identification was performed using three different PCR methods and in situ hybridization assay (ISH). For HPV studies, genomic DNA was isolated from formalin-fixed, paraffin-embedded tissue using QIAsymphony SP. Special precautions were taken to prevent HPV DNA microcontamination. Briefly, five 5-μm-thick sections were cut from the blocks. A new microtome blade was

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Published online: 26 November 2013

Table 1 Age distribution at the time HPV vaccination was finished in women with PAP smear reading performed by Bioptická laboratoř s.r.o, between 2006 and 2012 (n =24,709)

Age when vaccination was finished	Quadrivalent vaccine applied (%)	Bivalent vaccine applied (%)
15	8.46	9.77
16	12.66	11.77
17	11.89	9.73
18	9.37	6.92
19	6.94	4.71
20	6.32	4.72
21	6.25	4.76
22	5.84	4.99
23	5.65	5.32
24	5.30	6.26
25	5.05	6.12
26	4.44	4.81
Older than 26	11.84	20.12

used each time a new case was sectioned. DNA was extracted by the QIAsymphony DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quality of isolated DNA was checked by PCR that amplificates a set of control genes [1]. In total, 33 patients were found to be eligible for DNA study. The HPV DNA detection was performed using a set of several PCRs with different primers (Table 2) to cover a wide detection range of predominantly high- and low-risk HPV types. For all samples, the primer's systems targeting both the L1 and E1 regions were used: CPSGB, GP5+/GP6+, as previously described [2]. To avoid false-negative findings (because of the loss of the L1 or E1 region due to a process of HPV integration into the host genome), PCR targeting HPV oncogenes E6 and E7 of the six most prevalent HR-HPV types, namely types 16, 18, 31, 33, 35, and 45, was performed [3]. Furthermore, the RHA kit HPV SPF10-LiPA25, version 1 (Bio-medical Products, Rijswijk, The Netherlands) was run in order to reveal possible multiple HPV type infections.

Table 2 Primers used for HPV detection in cone biopsies of HPV-vaccinated women presenting with CIN III

HPV	Type 16	TCA AAA GCC ACT GTG TCC TGA CGT GTT CTT GAT GAT CTG CAA
	Type 18	CCG AGC ACG ACA GGA ACG ACT TCG TTT TCT TCC TCT GAG TCG CTT
	Type 31	CTA CAG TAA GCA TTG TGC TAT GC ACG TAA TGG AGA GGT TGC AAT AAC CC
	Type 33	AAC GCC ATG AGA GGA CAC AAG ACA CAT AAA CGA ACT GTG GTG
	Type 35	CCC GAG GCA ACT GAC CTA TA GGG GCA CAC TAT TCC AAA TG
	CPSGB	ATATGTCTGAG CCTCCWAARTT ATGTTAATWSAGCCWCCAAAATT
	GP5+	TTA TCA WAT GCC CAY TGT ACC AT TTTGTTACTGTGGTAGATACTAC
		GAAAAATAAACTGTAAATCATATTC

All PCR were run on the cycler GeneAmp PCR System 9700 (PE/Applied Biosystem, Forster City, CA, USA). Amplicons were analyzed in 2 % agarose gel with ethidiumbromide. Positive PCR samples were genotyped by hybridization to type-specific probes or sequenced and compared to BLAST databases. Positive and negative controls were included in every single run. ISH for HPV DNA detection (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66) was performed in each case according to the manufacturer's protocol using the BenchMark automated slide staining system (Ventana Medical System) and the INFORM HPV III Family 16 Probe (B) set. All available pre-vaccination PAP smear results were evaluated. There were six cases without PAP smear results. Of the patients with available PAP smears, six of these had negative results. Slides from patients with negative pre-vaccination PAP smear history were reviewed.

Statistical analysis

We aimed to compare prevalence of different HPV types associated with CIN III in HPV-vaccinated patients (our study group), with findings of the Czech population HPV incidence study [4] which investigated HPV types detected in the general Czech population cone biopsies harboring CIN II and CIN III. We provide type-specific HPV prevalence as a proportion of patients tested positive for the particular HPV type among all patients successfully tested. When women were infected with multiple HPV types, they were counted positive regarding all involved types. We estimated relative risks (RR) of a type-specific HPV infection in the high-grade dysplasia specimens of vaccinated women in comparison to general population specimens with CIN III and CIN II+CIN III (Table 3). To test for the significance of association between type-specific HPV prevalence and vaccination, we used Fisher's exact test. All statistical analyses were performed using STATA/IC 10.1 (Stata Corp, College Station, USA).

Results

We have identified 38 women conizated for CIN III subsequent to HPV vaccination. Of those 12 (32 %) were older than the upper recommended age limit of 26 years for HPV vaccination in Czech Republic (Fig. 1). A quadrivalent vaccine was used in seven of those patients and a bivalent vaccine in five patients. Overall, 37 (97 %) of the above-mentioned cases developed in women of more than 17 years of age at the time

Table 3 Comparison of type-specific HPV prevalence in CIN III specimens of the study population (HPV-vaccinated women) and cone biopsies with CIN II and CIN III of the general Czech population

HPV type	Population CIN2 (N =111) Positive (%)	Population CIN3 (N =200) Positive (%)	Population CIN2+3 (N =311) Positive (%)	Study CIN3 (N =33) Positive (%)	Study CIN3 vs. pop. CIN3		Study CIN3 vs. pop. CIN2+3	
					RR	P value	RR	P value
16	46 (41.4)	135 (67.5)	181 (58.2)	22 (66.7)	0.99	1.00	1.15	0.46
18	5 (4.5)	11 (5.5)	16 (5.1)	0 (0.0)	0.00	0.37	0.00	0.38
26	1 (0.9)	0 (0.0)	1 (0.3)	0 (0.0)			0.00	1.00
31	9 (8.1)	29 (14.5)	38 (12.2)	2 (6.1)			0.50	0.40
33	13 (11.7)	28 (14.0)	41 (13.2)	2 (6.1)	0.42	0.27	0.46	0.40
35	3 (2.7)	2 (1.0)	5 (1.6)	2 (6.1)	0.43	0.27	3.77	0.14
39	2 (1.8)	0 (0.0)	2 (0.6)	0 (0.0)	6.06	0.10	0.00	1.00
45	7 (6.3)	6 (3.0)	13 (4.2)	1 (3.0)			0.72	1.00
51	4 (3.6)	3 (1.5)	7 (2.3)	1 (3.0)			1.35	0.56
52	6 (5.4)	4 (2.0)	10 (3.2)	1 (3.0)			0.94	1.00
53	1 (0.9)	0 (0.0)	1 (0.3)	0 (0.0)	1.01	1.00	0.00	1.00
56	4 (3.6)	6 (3.0)	10 (3.2)	0 (0.0)	2.02	0.46	0.00	0.61
58	3 (2.7)	11 (5.5)	14 (4.5)	2 (6.1)	1.52	0.54	1.35	0.66
59	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)				
66	2 (1.8)	3 (1.5)	5 (1.6)	0 (0.0)				
68	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.00	0.60		
82	0 (0.0)	4 (2.0)	4 (1.3)	1 (3.0)	1.10	1.00		
6	2 (1.8)	1 (0.5)	3 (1.0)	1 (3.0)				
11	0 (0.0)	1 (0.5)	1 (0.3)	0 (0.0)				
					0.00	1.00	0.00	1.00
					1.52	0.54	2.36	0.40
					6.06	0.26	3.14	0.33
					0.00	1.00	0.00	1.00
40	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)				
42	0 (0.0)	3 (1.5)	3 (1.0)	0 (0.0)	0.00	1.00	0.00	1.00
43	1 (0.9)	0 (0.0)	1 (0.3)	0 (0.0)			0.00	1.00
54	1 (0.9)	1 (0.5)	2 (0.6)	1 (3.0)			4.71	0.26
70	1 (0.9)	1 (0.5)	2 (0.6)	0 (0.0)	6.06	0.26	0.00	1.00
81	1 (0.9)	1 (0.5)	2 (0.6)	0 (0.0)	0.00	1.00	0.00	1.00
89	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.00	1.00		

No statistically significant difference (at a significance level of 0.05) in type-specific HPV prevalence between the study and population groups was found

RR relative risk for type-specific HPV infection (study group vs. comparison population group)

they completed the vaccination. All conizations were performed within 3 years after vaccination had been completed, most of them (28 cases) though within the first 2 years. There were seven (18 %) cases of conization in the same year as vaccination was completed. Interestingly 78 % (25/32) of cases were associated with at least one abnormal PAP smear

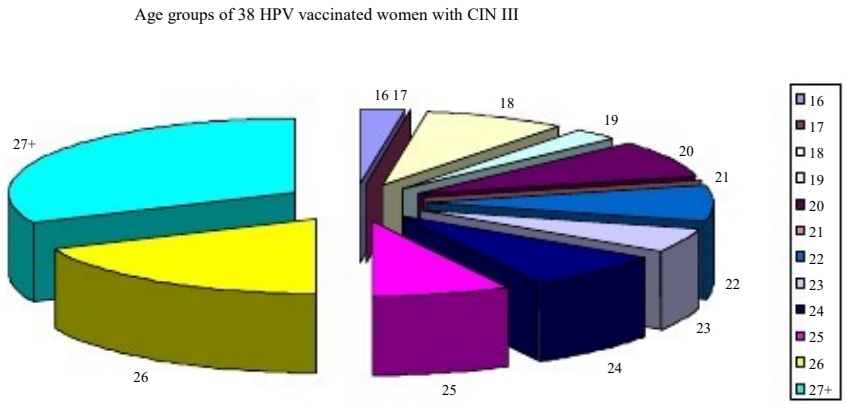

Fig. 1 Distribution of age at the time HPV vaccination was finished in women who were subsequently diagnosed with CIN III (n =38)

prior to vaccination. Moreover, five out of six patients, who originally had a negative PAP smear history, were reclassified to have an abnormal PAP smear history. The reclassification was due to false negativity of the PAP smear results of these patients (Table 4). HPV typing was successful in 33 patients. In two thirds of cases (67 %; 22/33) HPV 16 was identified. Eight other high-risk and two low-risk HPV types were identified (Table 3). There was no statistically significant difference in the prevalence of any particular HPV type between the study group and the population control group of patients (Table 3). Ecological HPV type shift in the population of vaccinated women was not observed.

Discussion

It can be stated that conization for CIN III subsequent to HPV vaccination is a rare event. Its significance remains to be fully determined by further long-term studies. Until then, a new role of pathology is emerging in assisting gynecologists to elucidate the unfortunate and generally unexpected circumstances which may possibly undermine patient–doctor relationship. Providing information on cone biopsy tissue HPV type and the review of preceding PAP smears may prove useful for gynecologists. Likewise, cytopathologists and pathologists familiar with this possibility may take more prompt decisions examining PAP smears and cone biopsies, opting for HPV typization when correlating the findings with available clinical information. The phenomenon of high-grade dysplasia in HPV-vaccinated women has already been noticed in the literature. Szarewski et al. [5] identified 8 HPV-vaccinated patients with CIN III associated with HPV types 16 (six cases) and 18 (two cases) at the study endpoint. The methodology of that investigation was based on liquid-based cytology (LBC). Authors do not comment on this phenomenon any further. Interestingly, we have identified two cases of CIN III associated with low-risk HPV type infection, namely HPV 6 and 54. Association of low-risk HPV types with CIN III and invasive cervical carcinoma is not well understood so far. The mechanism of carcinogenesis is thought to be different from high-risk HPV types. According to Geraets et al. [6], HPV types belonging to alpha species other than alpha species 5, 6, 7, 9, and 11 (including HPV types 6 and 54) were found in only 0.22 % of cervical cancers. Low incidence makes it difficult to study these cases systematically. Moreover, a laboratory phenomenon of preferential PCR amplification could explain some findings of solitary low-risk HPV type identification in case of coinfection with a low dose of high-risk HPV type [7]. We are aware of our study bias. The institutional study design misses a number of cases of interest due to the limited number of screening PAP smears processed in our laboratory (approximately 35 % of national screening program volume). Although we evaluate approximately 3,500 cone biopsies per year, the unknown total number of conizations performed nationwide prevents us to calculate the precise number of cases of interest on national level. Establishment of the specialized national registry or upgrade of the existing national PAP smear registry could be a solution. Furthermore, the age distribution of HPV-vaccinated women in Czech Republic was suboptimal compared to Czech national vaccination guidelines (Table.1). This may lead to a higher number of cases of high-grade cervical dysplasia after HPV vaccination (DAV) in Czech Republic. In the light of our small group findings, most of the European national vaccination programs with a recommended vaccination limit up to 18 years of age should be highly effective in the prevention of the above-described DAV phenomenon. Those programs also promote the vaccination of women older than 18 after consultation with a gynecologist on individual basis [8]. As majority of CIN III cases have been identified in women over 17 years of age, our study supports the fact that HPV vaccination's preventive effect for CIN III in the age group over 17 years is diminished. It appears that a personal history of an abnormal PAP smear (including ASCUS!) scales up the probability of CIN III development after HPV vaccination. Due to the short interval between vaccination and conization (typically less than 2 years), it can be reasonably assumed that dysplasia was present in all patients before the vaccination. A recent study by Castle [9] analyzing LBC results and subsequent HPV detection and typing in 617 patients supports this view. They show that “most CIN III cases diagnosed within the two-year time frame were prevalent cases”, meaning that cytologic abnormalities in those patients had been present before HPV vaccination. The above-described short time frame has to be taken into consideration in our study as well. Due to the above-mentioned reasons, with consideration of the methodological limitations of our study, we suppose that DAV is not a representation of immunological vaccination failure. Our opinion is that it is a consequence of improper pre-vaccination decision-making process, including false negativity of PAP smear reading and clinical counseling. We conclude that cervical high-grade dysplasia was present in all 38 HPV-vaccinated women before vaccination. Type-specific

Table 4 Results of meticulous review of 10 PAP smears, prior to HPV vaccination, in six patients that had originally been signed out as negative (NILM)

Case no.	Review results		
1	ASC-H	LSIL+infl.	ASCUS
2	ASCUS	ASCUS	
3	NILM		
4	LSIL	LSIL	
5	LSIL		
6	ASCUS		

HPV prevalence in these cases was not different from CIN III cases occurring in the general Czech population. Ecological HPV type shift in the population of HPV-vaccinated women was not observed. Higher age and personal history of abnormal PAP smear may be associated with DAV. This advocates for the need of continuing cervical screening in HPV-vaccinated women and the need for competent HPV vaccination counseling in women of 18 years of age and over.

Conflict of interest We declare that we have no conflict of interest.

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3.4 Genetické testovanie leiomyómov u žien mladších ako 30 rokov môže byť efektívnym skríningom pre syndróm hereditárnej leiomyomatózy a renálneho karcinómu (HLRCC syndróm)

(Genetic testing of leiomyoma tissue in women younger than 30 years old might provide an effective screening approach for the hereditary leiomyomatosis and renal cell cancer syndrome (HLRCC))

Zárodočná heterozygotná mutácia génu pre fumarát hydratázu (FH) bola nedávno identifikovaná ako príčina vrodenej dispozície k leiomyómom maternice a kože a niekoľkým typom renálnych karcinómov – syndróm hereditárnej leiomyomatózy a karcinómov z renálnych buniek (HLRCC) syndróm. Tento genetický defekt bol pôvodne popísaný u 25 fínskych probandov. Asociácia s renálnym karcinómom nie je dobre definovaná. Penetrancia tohto symptómu je nízka a nie je presne stanovená. Dlhodobé sledovanie pacientov s identifikovaným HLRCC syndrómom je jediným preventívnym opatrením. Identifikácia týchto pacientov v populácii je komplikovaná a nie je vyriešená. Navrhli sme špecifický cielený skríningový protokol pre vyhľadávanie symptomatickych pacientiek. V tejto štúdii sme overovali jeho efektivitu. V rámci českej nemocnice univerzitného typu sme retrospektívne za obdobie troch rokov 2010-2012 identifikovali 140 pacientiek, ktoré boli operované pre symptomatický leiomyóm vo veku menej ako 40 rokov. Pilotnú skupinu predstavovalo 14 pacientiek (10%) operovaných pre symptomatický leiomyóm vo veku menej ako 30 rokov (s priemerným vekom 28 rokov, v rozsahu 23-30 rokov). Revidovali sme histologické preparáty každého prípadu a hľadali sme histomorfologické rysy, ktoré sú považované za špecifické pre HLRCC asociovaný leiomyóm (veľké eozinofilné jadierka s perinukleolárnym haló na pozadí celulárneho alebo epiteloidného leiomyómu,eozinofilné globuly, fibrilárna cytoplazma, tenké rohaté cievy.) Reprezentatívny parafínový bloček bol použitý pre imunohistochemické a molekulovo genetické vyšetrenie. V pozitívnych prípadoch sme po informovanom súhlase pacientky odobrali periférnu krv pre potvrdenie germinálnej mutácie. Imunohistochemicky sme vyšetrovali expresiu antigénu 2SC. Bola použitá nedávno popísaná nekomerčná protilátka 2SC v riedení 1 ku 2000 a pre hodnotenie expresie fumarát hydratázy bola použitá komerčná protilátka. Hodnotenie imunohistochemickej expresie antigénov vykonal jeden z autorov bez znalosti klinických údajov a výsledkov molekulovo genetických vyšetrení. Mutačná analýza génu fumarát hydratázy bola vykonaná s použitím in-house primerov, z reprezentatívneho parafínového bločku a následne v pozitívnych prípadoch aj z periférnej krvi. Bola vykonaná mutačná analýza všetkých kódujúcich exónov FH génu Sangerovým sekvenovaním. PCR bola vykonaná s použitím in-house primerov. PCR produkty sa overovali na 2% agarovom gély elektroforézou. Analýza pozitívnych vzoriek bola opakovaná. Stratu heterozygozity lokusu 1q43 sme zisťovali za použitia sady 6 STR markerov v okolí FH génu. Sekvencie primerov boli získané z databázy National centre for

biotechnology informations probe. PCR produkty boli analyzované na agarózovom gély. Vzorku sme považovali za pozitívnu pre LOH ak bol konečný pomer peakov pre alely FH génu v nádorovom a nenádorovom tkanive viac ako 1,5 alebo menej ako 0,66. Identifikovali sme dve pacientky s germinálnou mutáciou FH génu. Prvá identifikovaná pacientka mala v čase diagnózy 27 rokov a syndróm sa prezentoval desiatkami leiomyómov, ktoré výrazne malformovali maternicu. Pacientka bola zaradená do dlhodobého monitorovacieho programu a genetické testovanie bolo doporučené všetkým členom rodiny. U druhej pacientky bolo podľa ultrazvukového vyšetrenia v maternici 6-7 leiomyómov od priemeru 11 mm do 55 mm. Napriek zmenám maternice spôsobených myómami pacientka vo veku 32 rokov porodila zdravé dieťa. Nádor obličky ani u nej prítomný nebol. Bola zaradená do dispenzárnej starostlivosti s ultrazvukom obličiek 1x ročne a členom rodiny bolo navrhnuté genetické testovanie. Záver: Vzhľadom na raritnosť syndrómu a náklady spojené s jeho diagnostikou navrhujeme cielený molekulovo genetický skríning u žien so symptomatickými leiomyómami vo veku menej ako 30 rokov. Podkladom je fakt, že tieto leiomyómy vo veľkej väčšine prípadov predchádzajú možnému vzniku renálnych karcinómov, ktoré možno pri dispenzárnej starostlivosti včas identifikovať. Histomorfologické znaky HLRCC leiomyómu boli prítomné iba u jednej z dvoch identifikovaných pacientiek. Imunohistochemická analýza podľa našich skúseností rovnako dokázala identifikovať iba jednu pacientku a tak zlatým diagnostickým štandardom zostáva molekulovo genetická analýza mutácie FH génu. Podľa našich skúseností prítomnosť mnohopočetných leiomyómov u ženy pod 30 rokov, ktoré sú symptomatické a vedú minimálne k myomektómii, alebo identifikácia leiomyómu so simultánnou prítomnosťou všetkých histologických znakov HLRCC asociovaného leiomyómu by mala viesť k podozreniu na možnosť, že sa u danej pacientky jedná o HLRCC syndróm. Zavedenie akéhokoľvek skríningového protokolu pre HLRCC syndróm do praxe patológov ostáva otvorenou otázkou.

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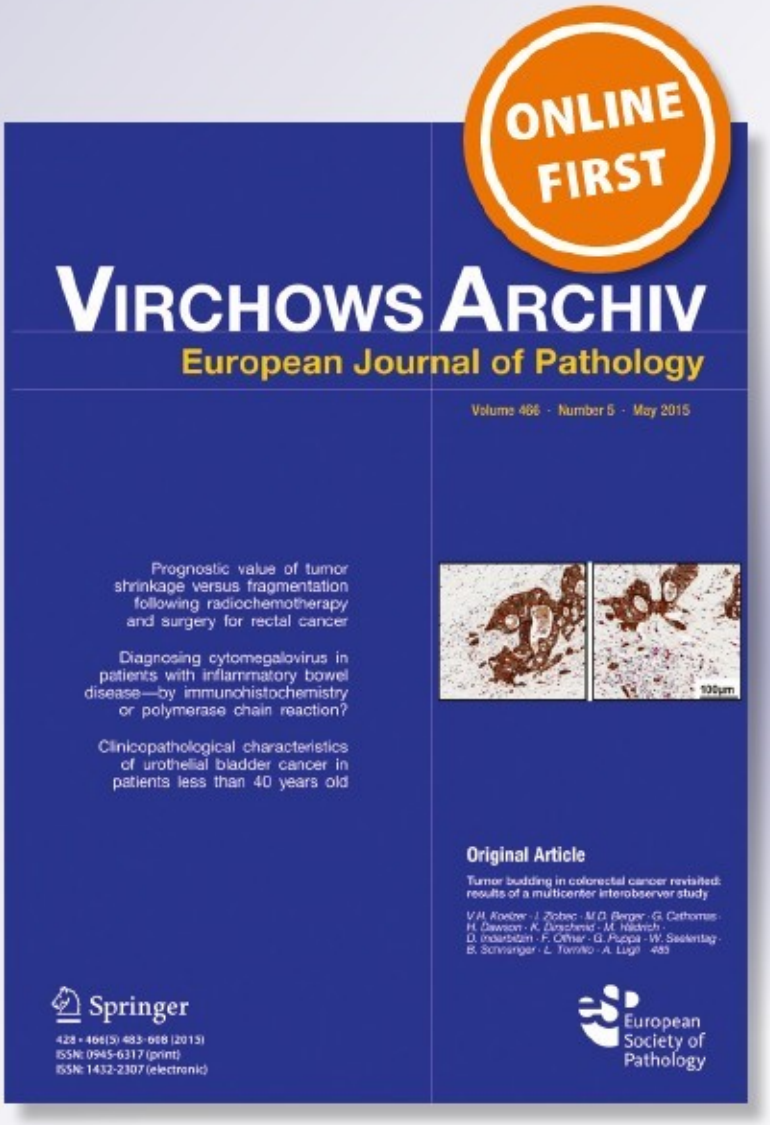
Virchows Archiv
The European Journal of Pathology

ISSN 0945-6317

Virchows Arch
DOI 10.1007/s00428-015-1783-y



Virchows Arch
DOI 10.1007/s00428-015-1783-y

ORIGINAL ARTICLE

Genetic testing of leiomyoma tissue in women younger than 30 years old might provide an effective screening approach for the hereditary leiomyomatosis and renal cell cancer syndrome (HLRCC)

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Received: 11 January 2015 / Revised: 8 April 2015 / Accepted: 4 May 2015

Abstract We have studied the viability of targeted molecular screening for the identification of female patients with hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome. Affected patients harbor a germ-line heterozygous mutation of the fumarate hydratase (FH) gene. Clinically, some patients present with aggressive renal cell carcinoma. Concerning women, in almost all cases, this is preceded by symptomatic uterine leiomyoma. We aimed to identify women operated on for symptomatic leiomyoma by the age of 30. Archived paffin-embedded leiomyoma tissue was tested for the FH gene mutation in 14 cases. Two patients with multiple leiomyomas and with the confirmed germ-line mutations c.1433_1434dupAAA, p.(Lys477dup) and c.953A>T, p.(His318Leu) were identified and enrolled in a surveillance program. Statistically significant correlation between the presence of multiple uterine leiomyomas (more than seven in our experience) and the FH gene mutation was found. The immunohistochemical expression pattern, of simultaneous FH absence and S-(2-succino)cysteine (2SC) positivity, correlated with the results of the molecular genetic study in only one case. The histomorphologically simultaneous detection of enlarged nucleoli with a clear halo of leiomyocyte nuclei, their fibrillary cytoplasm, the presence of eosinophilic globules, and staghorn vessels proved to be only a partially sensitive indicator of HLRCC-associated leiomyoma and fully correlated with immunohistochemistry and molecular genetic study only in one case. Molecular genetic testing is presently the only reliable diagnostic tool able to identify HLRCC patients. The sensitivity and specificity of the presence of multiple leiomyomas in women with the FH gene mutation who are younger than 30 years old should be confirmed in larger scale studies. The applied targeted molecular screening protocol proved to be effective, resulting in identification of two positive patients out of fourteen tested individuals.

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Published online: 19 May 2015

Keywords HLRCC . Fumarate hydratase . FH Leiomyoma . Renal cell carcinoma

Introduction

The germ-line heterozygous mutation of the fumarate hydratase (fumarase, FH) gene has recently been identified as the cause of a hereditary predisposition to uterine and cutaneous leiomyomas and several types of renal cell carcinoma (RCC)—hereditary leiomyomatosis and renal cell cancer syndrome (HLRCC) [1, 2]. This genetic defect was originally described in 25 Finnish probands. The associated RCC

present several, as yet not well established, histomorphologic features. Their most upsetting biological feature is the late stage at the time of diagnosis conferring adverse prognosis for the patient [3]. That is why newly identified probands are offered long-term follow-up with regular ultrasound kidney examinations twice a year, aiming to identify possible carcinoma in an early stage of evolution. We postulate that non-symptomatic carriers of the biologically significant heterozygous FH mutation would benefit from early identification and inclusion into the above-described surveillance protocol. Due to the rarity of the syndrome, cost-effective focused identification of these patients in the general population remains an open question. In this study, we examine the feasibility of a specific targeted screening protocol for symptomatic female probands. We took advantage of the fact that uterine leiomyomas belong to the HLRCC phenotype. It was shown that the FH gene mutation may induce uterine leiomyoma formation earlier in life, compared to the general population with an average age at hysterectomy (due to symptomatic leiomyoma) at 30 years [3]. If leiomyoma tissue contains the germ-line FH gene mutation, it might be readily identified using paraffin-embedded (FFPE) tissue.

Materials and methods

The study was approved by the local ethics committee. The size of the studied population was limited by age at the time of operation for leiomyoma. In the setting of the Czech University hospital in a 3-year period (2010–2012), we have retrospectively identified 140 women operated on for uterine leiomyoma at an age younger than 40 years. Our pilot study group consisted of 14 (10 %) women operated on for leiomyoma at an age younger than 30 years (mean age was 28 years, range 23–30). Histologic slides were reviewed in each case, searching for histomorphologic features described as being specific for HLRCC-associated leiomyomas, including leiomyocytes with large eosinophilic nucleoli and a perinucleolar halo in a background of cellular or epithelioid leiomyoma [2], presence of eosinophilic globules, fibrillary cytoplasm, and staghorn vessels [4]. Representative FFPE tissue blocks were submitted for genetic and immunohistochemical studies—one for each case. In positive cases, a peripheral blood sample was obtained with informed consent of the patient to confirm the presence of a germ-line mutation.

Mutation analysis of FH gene

DNA from FFPE tissue was extracted using a QIAsymphony DNA Mini Kit (Qiagen, Hilden, Germany) on an automated extraction system (QIAsymphony SP, Qiagen) according to the manufacturer's supplementary protocol for FFPE samples (Purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution). Peripheral blood was isolated using the QIAsymphony DNA DSP Mini Kit. Concentration and purity of isolated DNA was measured using NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). DNA integrity was examined by amplification of control genes in a multiplex PCR [5]. Mutation analysis of the whole coding sequence of the FH gene was performed using Sanger sequencing. PCR was carried out using primers shown in Table 1. The reaction conditions were as follows: 12.5 μl of HotStar Taq PCR Master Mix (Qiagen, Hilden, Germany), 10 pmol of each primer (Table 1), 100 ng of template DNA and distilled water up to 25 μl. The amplification program consisted of denaturation at 95 °C for 15 min and then 40 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1.5 min for all amplicons. The program terminated with 72 °C incubation for 7 min. The PCR products were checked on 2 % agarose gel electrophoresis. Successfully amplified PCR products were purified with the AMPure magnetic particles (Agencourt Bioscience Corporation, A Beckman Coulter Company, Beverly, MA), both side sequenced using the Big Dye Terminator Sequencing Kit (Applied Biosystems,

Table 1 Primers used for mutation analysis of FH gene

Primer name	sequence 5' -3'	product size (bp)
FH-e1-F	gcggaacggtttctgaca	263
FH-e1-R	caggagggctgaaggtcact	
FH-e2-F	gatgcgattacttttgatcctg	235
FH-e2-R	ccaaaatagccaacatttcca	
FH-e3-F	gccaaaaataataaacttccatgc	
FH-e3-R	agtatggcatgggtctgagg	230
FH-e4-F	ggcataatcagcattattatttcctt	
FH-e4-R	aaaaacagcaaagctcacatactg	262
FH-e5a-F	tttgtttttgttgcctctgattt	
FH-e5a-R	ggattttgcatcaagagcatc	
FH-e5b-F	cttttcccacagcaatgcac	169
FH-e5b-R	catttgtaccaagctctaaattgaa	
FH-e6a-F	ctttgctcatcataagatttgaagt	218
FH-e6a-R	caacagcagtgcctccag	
FH-e6b-F	tcaggaatttagtggttatgttcaa	
FH-e6b-R	cagaccacgtataatgagaaatgaa	262
FH-e7a-F	ttgctaatggtagaaaaatgtttagtt	
FH-e7a-R	cccaaaaatcgaatatcatttgc	224
FH-e7b-F	ctcatgacgctctggttgag	
FH-e7b-R	caagttttagctccaacatttactagc	
FH-e8-F	tttctttattctcctgattatttgcat	200
FH-e8-R	ccaagataataagcctttggtca	
FH-e9-F	ctctctctctctctctctcactcac	197
FH-e9-R	tggtttagctttttaattttgcatt	
FH-e10-F	aacccatatgtcgtctttttattttt	249
FH-e10-R	tttttaagaaatgggagtctgttttt	
		244
		245

Primer name: FH fumarate hydratase, e exon with number, F forward, R reverse

Foster City, CA) and purified with the CleanSEQ magnetic particles (Agencourt) all according to the manufacturer's protocols. Subsequently, the sequencing products were run on an automated sequencer ABI Prism 3130xl (Applied Biosystems, Foster City, CA) at a constant voltage of 13.2 kV for 20 min. The analysis of positive samples was repeated.

Loss of heterozygosity analysis of 1q43 locus

The sample of non-tumoral tissue of the endometrium (from another biopsy of patient no. 2) and myometrium in the case no.4 was available to be compared with the leiomyoma sample. Analysis of loss of heterozygosity was performed. A set of six polymorphic short tandem repeat (STR) markers (D1S517-F, D1S2785-F, D1S180-F, AFM214, D1S547-F, and D1S2842-F), surrounding the FH gene, was used to detect loss of heterozygosity (LOH) [1]. Primer sequences were obtained from the National Center for Biotechnology Information's database Probe [6]. Markers were amplified using a fluorescence-labeled primer at the same PCR conditions as described above. PCR products were examined by agarose gel electrophoresis, diluted if necessary, mixed with GeneScan-500LIZ size standard (Applied Biosystems), and run on ABI Prism 3130xl analyzer at a constant voltage of 15 kV for 20 min resulting in two peaks representing two alleles, if informative markers were found in non-tumor tissue. Shorter allele height was divided with longer allele height, and the final ratio of normal and leiomyoma samples was calculated. The sample was considered LOH positive if the final ratio was >1.5 or <0.66.

Immunohistochemistry

Immunohistochemistry (IHC) for fumarate hydratase and 2SC was performed on the same representative FFPE tissue block as molecular genetic testing. For fumarate hydratase, a commercially available mouse monoclonal antibody was used at a dilution of 1 in 2000 (clone J-13, cat no sc-100743, Santa Cruz Biotechnology, USA). For 2SC, a previously described rabbit polyclonal antibody was employed at a dilution of 1 in 2000 [7, 8]. To our knowledge, this antibody is not currently commercially available. IHC was performed using an automated staining platform—the Leica BOND-III autostainer (Leica Biosystems, Mount Waverley, Victoria, Australia). For fumarate hydratase, heat-induced epitope retrieval (HIER) was performed for 30 min at 97 °C in the manufacturer's alkaline retrieval solution ER2 (VBS part no: AR9640). For 2SC, HIER was performed for 30 min at 97 °C in the manufacturer's acidic retrieval solution ER1 (VBS part no.AR9961). Slides were scored by a single observer (AG) who was blind to all clinical and molecular data. For fumarate hydratase, the absence of staining in all neoplastic cells (arbitrarily defined as greater than 95 % of neoplastic cells) was defined as negative and considered an abnormal pattern of staining, suggestive of fumarate hydratase abnormality. Positive staining in any neoplastic cells (arbitrarily defined as more than 5 % of neoplastic cells) was considered positive and indicated a normal pattern of staining. If tumor cells were negative but non-neoplastic cells of the normal histomorphologically discernible uterine wall and/or endometrium present in the slide (which were considered internal positive controls) were also negative, staining was considered indeterminate and repeated. For 2SC, the presence of positive staining (which was diffuse and both nuclear and cytoplasmic) in all neoplastic cells (defined as greater than 95 % of cells) was considered positive and represented an abnormal pattern of staining suggestive of fumarate hydratase abnormality. All other patterns of 2SC staining were considered negative (normal pattern of staining).

Results

Two patients with mutation of the FH gene were identified. In the medical history of case no. 2, multiple hysteroscopic resections of submucous leiomyomas between the age of 23 and 26 and laparotomy with subserous uterine leiomyoma enucleation at the age of 24 years are remarkable. The patient was 27 when the HLRCC syndrome was diagnosed. She was engaged in IVF consultation, and her uterine cavity was distorted and very small. Eventually, the decision was taken to perform laparoscopically assisted vaginal hysterectomy. The specimen contained more than 20 subserous leiomyomas of 5 mm diameter and dozens of intramural leiomyomas ranging from 3 to 20 mm. Ultrasound examination of the kidneys was performed, and no tumor was found. The patient enrolled in a kidney ultrasound surveillance program. Genetic testing was offered to the family members who postponed their decision. Personal history of the patient no. 4 was remarkable for laparoscopic myomectomy of the subserous and intramural leiomyoma measuring 55 and 45 mm, respectively, at the age of 29 years. Subsequently, she developed four other uterine myomas measuring up to 11 mm. Nonetheless, the patient gave birth to a healthy child at the age of 32. An ultrasound examination of the kidneys was performed and no tumor was found. The patient enrolled in a kidney ultrasound surveillance program. Genetic testing was offered to the family members. Immunohistochemically, convincing loss of FH expression in combination with positive expression of 2SC (FH-, 2SC+) was identified only in one mutation positive patient (Table 2, case no. 4). The other mutated case (no. 2) showed normal IHC status (FH+, 2SC−). The simultaneous presence of all four histomorphologic features of leiomyoma in the FH gene-mutated patient reported as specific recently [4] was

Table 2 Histomorphologic, immunohistochemical, and clinical features studied in 14 young women leiomyomas (a - cases tested positive for FH gene mutation)

Case no.	Cytoplasmic fibrillarity	Staghorn vessels	Eosinophilic globules	Nucleoli	FH expression	2SC expression	Leiomyoma number/type	Para/gravida status
1	−	−	−	+	+	−	1/SM	0/1
2a	−	−	−	+	+	−	Multiple - X	0/0
3	+	−	+	+	+	−	1/S	1/1
4a	+	+	+	+	−	+	Multiple - 7	0/0
5	−	−	−	+	+	−	1/SM	0/0
6	−	−	−	−	+	−	1/S	1/1
7	+	+	−	−	+	−	1/S	0/0
8	−	−	−	−	+	−	1/IM	0/0
9	−	−	−	+	+	−	1/SM	0/0
10	+	−	−	+	+	−	1/IM	0/0
11	−	−	−	+	+	−	1/IM	1/4
12	+	+	−	+	+	−	1/S	0/0
13	−	−	−	+	+	−	1/S	0/0
14	+	+	−	+	+	−	1/IM	1/1

confirmed only in one positive case—no.4 (Fig. 2). In summary, enlarged nucleoli, focally even with perinucleolar halo, were identified in 11 cases. It is of note that each of those tumors presented at least focal morphology of cellular leiomyoma. Eosinophilic globules were present in two cases, focally increased vasculature with staghorn vessels in four cases, and "fibrillary" cytoplasm of leiomyocytes in six cases (Table 2).

In case no.2, a germ-line mutation c.1433_1434dupAAA, p.(Lys477dup) was detected in one subserous leiomyoma (Fig. 1a). It is a known mutation with confirmed pathogenic significance (reference SNP: rs367543046) reported as the most frequent variant (~30 %) in fumarate hydratase deficiency [9]. It was also detected in the DNA from the patient's peripheral blood, and therefore, we concluded that it was a germ-line mutation. LOH analysis for the FH gene was negative in two tested samples. In case no. 4, a missense mutation c.953A > T, p.(His318Leu) was found in the FFPE sample (Fig. 1b). A pathogenic role of this mutation was described by Deschauer et al. [10] under different codon number (H275L) due to now outdated version of FH reference sequence (NM_000143.1). Analysis of peripheral blood confirmed status of this mutation as germ-line. LOH analysis of case no. 4 was positive. No mutations were found in the other 12 patients.

Discussion

Patients with HLRCC carry one wild-type and one mutated allele of the FH gene. During their lifetime, inactivation of the wild-type allele by somatic mutation results in the development of a tumor. In one third of families with HLRCC syndrome, renal cell carcinoma is diagnosed later in life [11]. It was found in eight women out of 46 identified by Toro et al. in the North American study [3] with median age at detection of renal tumor being 44 years. The mean age at diagnosis of renal tumor was 39 years in a study from Singapore [11]. Screening for HLRCC syndrome is justifiable in view of the fact that HLRCC-associated renal cell carcinomas present aggressive behavior irrespective of the histological type and a majority of patients present with an advanced stage tumor. Renal carcinomas associated with this syndrome reportedly have a peculiar morphology characterized by voluminous eosinophilic cells arranged in a papillary, tubulopapillary, or cribriform pattern with a prominent eosinophilic nucleolus and a clear halo [12]. The rarity of the syndrome and absence of an effective screening protocol hamper the identification of additional patients and better understanding of the disease. Several screening approaches have been proposed. Sanz–Ortega suggested morphological screening of leiomyomas followed by genetic testing for germ-line mutation [2]. Bardella proposed the use of a 2SC antibody for immunohistochemical screening of suspicious RCC cases for further FH gene mutation testing [8]. According to Reyes, combination of some specified histomorphological features of uterine leiomyomas combined with positive 2SC immunohistochemistry might help identify patients with possible HLRCC syndrome [4].We decided to perform targeted molecular genetic screening of leiomyomas of women younger than 30 years old, in view of the early onset of symptomatic uterine leiomyomas in HLRCC patients [3]. In this pilot study, we identified two symptomatic carriers out of 14 tested individuals. The probands presented with multiple symptomatic

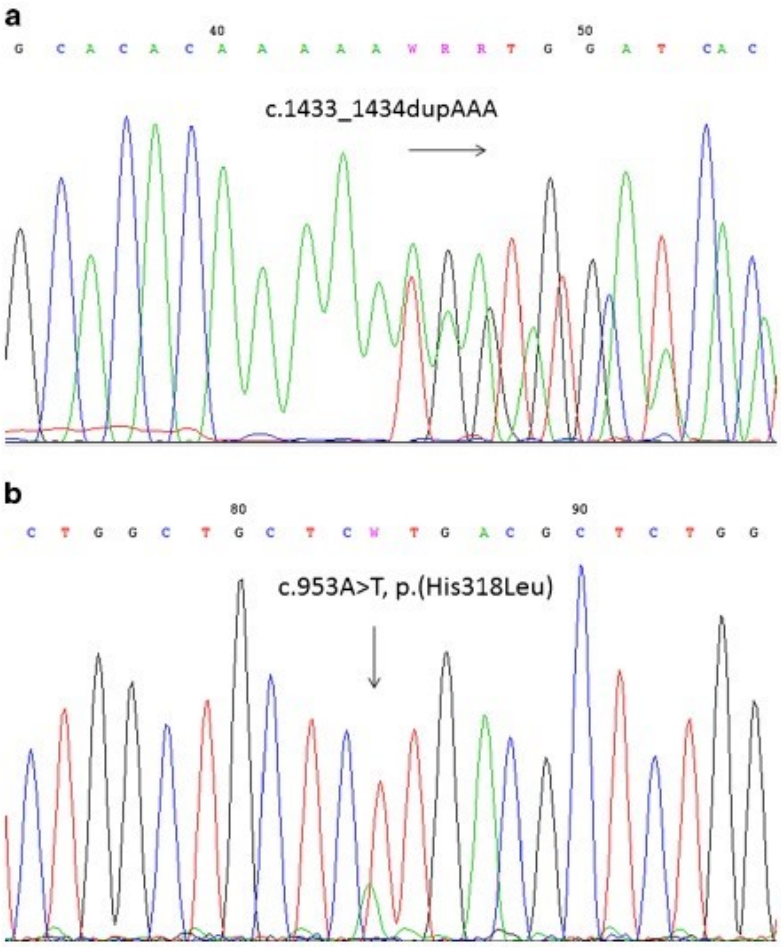

Fig. 1 Mutations in FH gene detected by sequencing: a case no. 2 with insertion mutation in exon 10 and b case no. 4 with missense mutation in exon 7

leiomyomas by the age of 27 and 29 years old (case no. 2 and no. 4, respectively). The correlation between the presence of multiple leiomyomas and the FH gene mutation was statistically significant with a 95 % confidence interval (Fisher's exact test, two tailed p=0.0110). No renal carcinomas were identified in those patients. We found that histomorphologic features [2, 4] correlated with molecular genetic status only in one of the two positive cases. Eosinophilic mildly enlarged inclusion-like nucleoli with a perinucleolar halo in leiomyocytes were found only focally in leiomyomas of the patient no. 2 and also in case no. 4. Nucleoli were large but none attained the size of a typical nuclear inclusion associated, for example, with a CMV infection. Large nucleoli, focally even with perinucleolar halo, were also found in leiomyomas of nine other patients in the studied group without FH gene mutation (Table 2). Moreover, large nucleoli are a regular finding in epithelioid leiomyoma, leiomyoma with hormonal-related changes (apoplectic leiomyoma and hemorrhagic cellular leiomyoma), leiomyoma with bizarre nuclei, and even low-grade leiomyosarcoma. Identification of perinucleolar halo necessitates a determined search.

Evaluation of the "cleanness" of perinucleolar halo is subject to the pathologist's personal judgment. Taken together, our data do not provide convincing evidence that the presence of large eosinophilic nucleoli with perinucleolar halo in

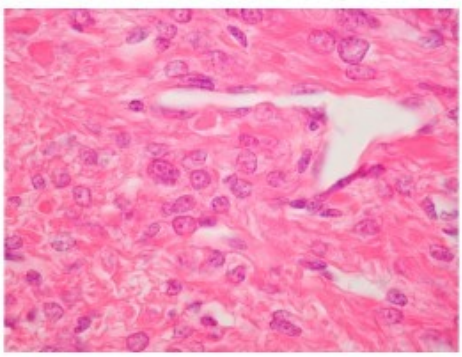

Fig. 2 Histomorphologic features of leiomyoma in patient with FH gene mutation (case no. 4): multiple enlarged eosinophilic nucleoli with a perinucleolar halo, fibrillary cytoplasm in leiomyocytes (better appreciated on the right side of the picture), eosinophilic globules (present in the upper left), staghorn vessel (hematoxylin-eosin, ×1000)

leiomyocytes alone constitutes a reliable pathognomonic feature of HLRCC-associated leiomyoma. The other above-described histologic features occur in case no.4 but also in leiomyomas without FH gene mutation (Table 2). Interestingly, simultaneous presence of all histologic features was noted only in leiomyoma with FH gene mutation (Fig. 2). A correlation between the presence of all four histomorphologic features and the FH gene mutation was not statistically significant at 95 % confidence interval (two tailed p=0.0714, Fisher's exact test) in our small cohort. To date, there has been limited experience with screening IHC for either FH and 2SC to identify neoplasms associated with HLRCC, and to our knowledge, there have been no published reports of FH and 2SC staining in uterine leiomyomas. Llamos-Velasco et al. [13] reported abnormal (negative) FH staining in 10 of 13 cutaneous smooth muscle tumors from patients with confirmed or suspected HLRCC. Although these authors did not assess 2SC IHC, they concluded that negative staining for FH in cutaneous smooth muscle tumors had a sensitivity of 83.3 % and a specificity of 75 % for HLRCC. After the initial report of Bardella et al. [8], suggesting 2SC as a biomarker for HLRCC in renal neoplasms, Chen et al. [14] investigated 2SC IHC in a large series of renal carcinomas and found positive (abnormal) staining in 9 of 9 (100 %) of HLRCC-associated renal carcinomas and negative (normal) staining in 184/184 (100 %) clear cell carcinomas, 93/97 (96 %) high-grade unclassified carcinomas, and 35/45 (78 %) type 2 papillary renal carcinomas. In our study, IHC for both FH and 2SC was successful in identification of one of two FH-mutated leiomyomas (case no.4). The second mutated case (no. 2) showed a normal IHC profile. As only one genetic hit was detected in this case (LOH was negative), one might speculate that a non-mutated allele was present and functional, resulting in a normal IHC staining pattern. On the other hand, the presence of an unknown second hit cannot be excluded. Therefore, at the current stage of knowledge, screening approaches based on IHC for either FH or 2SC should be considered investigational and require further study. We postulate that direct mutational analysis via sequencing will provide a more reliable approach. Mutations in the FH gene are almost evenly distributed throughout the 10 exons of the gene [15] and mostly are missense (81 %) or other types of point mutations with only occasional large whole gene deletions. Therefore, Sanger sequencing of the whole coding sequence of the gene will detect more than 97 % of all known mutations. Our primers (Table 1) are designed to be used on partially degraded DNA from FFPE samples (average amplicon length of 230 base pairs) making them suitable for both leiomyoma and RCC samples. The sensitivity of Sanger sequencing is more than sufficient to detect germ-line mutations in the FFPE samples. LOH analysis used for detecting allelic loss compared to normal tissue is evenly sensitive however requires normal tissue sample. With the current cost of Sanger sequencing, sequencing of the FH gene is likely to be cost effective. Using next-generation sequencing might improve the mutation detection rate, as larger areas can be effectively searched for mutations (e.g., non-coding regions of the gene) and furthermore copy number changes can be detected allowing recognition of large deletions. Finally, it could bring down the prize of the analysis when large numbers of samples are tested.

In conclusion, we present a targeted molecular screening protocol for HLRCC syndrome in women based upon genetic testing of tissue of symptomatic early onset leiomyomas obtained through hysterectomy or myomectomy in women younger than 30 years, using a specifically designed primer set. This approach identified two patients with HLRCC syndrome. Further narrowing of the tested population by selective inclusion only of the patients with multiple leiomyomas might be considered. We show that the presence of multiple leiomyomas in women younger than 30 years is the clinical feature with high specificity for HLRCC syndrome. Recently described combination of histomorphological features (enlarged nucleoli with a perinucleolar halo, fibrillary cytoplasm of leiomyocytes, eosinophilic globules, and staghorn vessels) and the immunohistochemical pattern of lost FH expression and 2SC positivity are of limited sensitivity for HLRCC syndrome-associated leiomyomas. Currently, molecular genetic testing is the only reliable diagnostic procedure able to identify patients with the HLRCC syndrome. According to our findings, the presence of multiple leiomyomas in a woman under 30 years of age or identification of leiomyoma with simultaneous presence of the above-mentioned histologic criteria should alert pathologist to consider molecular genetic testing for the FH gene mutation. Implementation of any HLRCC syndrome screening protocol in surgical pathology practice remains to be determined.

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3.5 Leiomyómy s deficitom fumarát hydratázy vznikajú v rámci syndrómu i sporadicky

(Fumarate Hydratase–deficient Uterine Leiomyomas Occur in Both the Syndromic and Sporadic Settings)

Syndróm HLRCC známy jako Reedov syndróm je autozómovo dominantne dedičný nádorový syndróm charakterizovaný inaktivačnou mutáciu génu pre fumarát hydratázu (FH), ktorý je lokalizovaný na chromozóme 1 (1q42.3 – q43). Pacienti s HLRCC syndrómom sú predisponovaní k vzniku kožných leiomyómov, leiomyómov maternice a najzávažnejšie je to, že u niektorých z nich dochádza k vzniku zvláštneho agresívneho typu renálneho karcinómu. Vyhľadávanie pacientov s HLRCC syndrómom je problematické vzhľadom k nízkej incidencii a penetrancii tohoto syndrómu. Pri spornej senzitivite histologických znakov popísaných u HLRCC asociovaného leiomyómu by imunohistochemické (IHC) vyšetrovanie leiomyómov mohlo byť vhodným doplnkom diagnostického algorytmu. Hodnotenie významu FH IHC je predmetom tejto štúdie. Bola použitá metóda tissue array (TMA). Z celkového množstva 1052 vyšetrovaných leiomyómov bolo 25 so stratou expresie FH. Následné vyšetrenie z celého klasického parafínového rezu absenciu expresie FH potvrdilo v 12 prípadoch. Z toho vyplýva, že 1 % leiomyómov sú FH deficientné leiomyómy. Prítomnosť mnohopočetných leiomyómov bola v čase diagnózy potvrdená u 4 pacientiek. Sangerovým sekvenovaním bola potvrdená mutácia FH génu u 6 pacientiek. Zistili sme, že IHC identifikácia FH deficientných leiomyómov môže byť dôležitou pomôckou v diagnostike HLRCC syndrómu, ale jej význam nemožno preceňovať, pretože má obmedzenú senzitivitu i špecificitu. Napríklad u 11 leiomyómov od 5 pacientiek s potvrdeným HLRCC syndrómom bola strata expresie FH zaznamenaná u 10 leiomyómov. Okrem toho 1 tumor pacientky s geneticky potvrdenou mutáciou FH génu pozitívne exprimoval FH imunohistochemicky. Limitom tejto štúdie bolo, že Sangerovo sekvenovanie a MPS (masívne paralelné sekvenovanie) prebehlo z DNA extrahovanej z archivovaných parafínových blokov a nevykonalo sa MLPA (multiplex ligation-dependent probe amplification), takže sme nemohli identifikovať rozsiahle delécie, ktoré nie sú u HLRCC syndrómu zriedkavé. Incidencia FH deficientných leiomyómov je podľa našich zistení cca 1% . Žiadna z pacientiek nemala v tejto štúdii zárodočnú mutácia FH génu, ale jednalo sa iba o somatickú mutáciu. Z toho vyplýva, že incidencia HLRCC syndrómu v bežnej populácii je naozaj nízka. Z praktického hľadiska sa teda zdá, že IHC detekcia FH deficinetného leiomyomu rozhodne neznamená, že sa jedná o HLRCC asociovaný leiomyóm. Doporučenie genetického poradenstva je namieste až po potvrdení zárodočnej mutácie metódami molekulovej genetiky, alebo pri zistení ďalších príznakov HLRCC syndrómu, ako je osobná alebo rodinná anamnéza kožných leiomyómov, renálneho karcinómu, alebo leiomyómov maternice so vznikom v mladom veku. Použitie histologických znakov pre HLRCC asociovaný leiomyóm je

limitované. Senzitivitu a špecificitu IHC vyšetrovania by mohlo zvýšiť paralelné hodnotenie overexpresie antigénu S-(2-succino)- cysteín (2SC). Táto je však zatiaľ komerčne nedostupná. Záver: Asi 1% neselektovaných leiomyómov maternice prezentuje stratu expresie FH. Väčšina je asociovaná so somatickou mutáciou FH génu a morfologicky môže vykazovať niektoré znaky zodpovedajúce HLRCC asociovanému leiomyómu. Genetické testovanie týchto pacietniek na HLRCC syndróm pri absencii ďalších klinických faktorov asociovaných s HLRCC syndrómom, ako je rodinná anamnéza, kožné leiomyómy, renálny karcinóm a symptomatické leiomyómy v mladom veku nie je doporučované. Veríme, že prospektívne zisťovanie deficitu FH v leiomyómoch má význam pre následnú klinickú úvahu o možnej asociácii s HLRCC syndrómom.

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Fumarate Hydratase–deficient Uterine Leiomyomas Occur in Both the Syndromic and Sporadic Settings

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Abstract: Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) syndrome secondary to germline fumarate hydratase (FH) mutation presents with cutaneous and uterine leiomyomas, and a distinctive aggressive renal carcinoma. Identification of HLRCC patients presenting first with uterine leiomyomas may allow early intervention for renal carcinoma. We reviewed the morphology and immunohistochemical (IHC) findings in patients with uterine leiomyomas and confirmed or presumed HLRCC. IHC was also performed on a tissue microarray of unselected uterine leiomyomas and leiomyosarcomas. FH-deficient leiomyomas underwent Sanger and massively parallel sequencing on formalin-fixed paraffin-embedded tissue. All 5 patients with HLRCC had at least 1 FH-deficient leiomyoma: defined as completely negative FH staining with positive internal controls. One percent (12/1152) of unselected uterine leiomyomas but 0 of 88 leiomyosarcomas were FH deficient. FH-deficient leiomyoma patients were younger (42.7 vs. 48.8 y, P = 0.024) and commonly demonstrated a distinctive hemangiopericytomatous vasculature. Other features reported to be associated with FH-deficient leiomyomas (hypercellularity, nuclear atypia, inclusion-like nucleoli, stromal edema) were inconstantly present. Somatic FH mutations were identified in 6 of 10 informative unselected FH-deficient leiomyomas. None of these mutations were found in the germline. We conclude that, while the great majority of patients with HLRCC will have FH-deficient leiomyomas, 1% of all uterine leiomyomas are FH deficient usually due to somatic inactivation. Although IHC screening for FH may have a role in confirming patients at high risk for hereditary disease before genetic testing, prospective identification of FH-deficient leiomyomas is of limited clinical benefit in screening unselected patients because of the relatively high incidence of somatic mutations.

Key Words: leiomyoma, HLRCC, fumarate hydratase, fumarate hydratase–deficient leiomyoma

(Am J Surg Pathol 2016;40:599–607)

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Conflicts of Interest and Sources of Funding: Supported by the Cancer Institute NSW through the Sydney Vital Translational Research Centre program (A.J.G.) and the Royal College of Pathologists of Australasia through the medical student pathology scholarship program (W.J.H.). The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

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Hereditary leiomyomatosis and renal cell cancer HLRCC) syndrome, also known as Reed syndrome,[1] is a rare autosomal dominant hereditary tumor syndrome associated with inactivating germline mutations of the fumarate hydratase (FH) gene located at

chromosome 1q42.3-q43.[2–4] Patients with HLRCC are predisposed to the development of cutaneous and uterine leiomyomas and, more seriously, a unique type of aggressive renal cell carcinoma.[3,5]

The majority of female individuals with HLRCC will develop symptomatic uterine leiomyomas and require surgery at a young age, usually before the development of renal carcinoma.[6–8] In 1 study, 98% (46/47) of female HLRCC patients with cutaneous leiomyomas also developed uterine leiomyoma, and these were sufficiently symptomatic to warrant surgery in 91% (42/46).[6] Of particular note, 57% of these patients required a hysterectomy at or before the age of 30 years (mean, 30 y), which was significantly earlier than the development of renal carcinoma at a median age of 44 years.[6] The presentation of patients with leiomyomas at a significantly younger age than renal carcinoma presents a clear opportunity for early diagnosis and intervention if these patients could be prospectively identified.

Unlike the syndrome of HLRCC, uterine leiomyomas are extremely common. Perhaps as many as 20% to 50% of women will develop uterine leiomyomas by 30 years, and up to 80% of females may have uterine leiomyomas by 50 years.[9] Although the majority of those affected will not undergo surgery, uterine leiomyomas are still among the most common visceral tumors received in the diagnostic surgical pathology laboratory. Therefore, any screening test to identify patients with HLRCC presenting first with uterine leiomyomas must be highly specific.

It has previously been suggested that uterine leiomyomas arising in the setting of germline FH mutation may show distinctive morphologic features including prominent nucleoli with perinucleolar clearing, hypercellularity, symplastic-type nuclear atypia, a hemangiopericytomatous vascular pattern, cytoplasmic globules, and stromal edema.[7,10,11] However, the significance and specificity of these features has recently been questioned,[8] and it seems that morphology alone will be inadequate to identify HLRCC-associated uterine leiomyomas prospectively.[10]

Several groups have demonstrated that positive immunohistochemical (IHC) staining for S-(2-succino)-cysteine (2SC), a metabolite that accumulates when FH is inactivated, is highly sensitive for the identification of HLRCC-associated renal carcinomas[12,13]; however, it lacks specificity.[14,15] This lack of specificity is a significant problem in a screening test for a rare entity. Although there is some evidence that 2SC IHC may be useful to identify HLRCC-associated uterine leiomyomas,[10] others have found it less useful.[8] Perhaps most importantly, to our knowledge, IHC for 2SC is not commercially available and, therefore, cannot be deployed in the routine surgical pathology laboratory.

IHC stain for FH is commercially available. In early investigations loss of staining for FH in conjunction with morphology has been suggested to be useful in the diagnosis of uterine leiomyomas,[16] cutaneous leiomyomas,[17] and renal carcinomas[14,15] occurring in the setting of HLRCC. Our experience in renal carcinoma is that loss of staining for FH is less sensitive than positive staining for 2SC but highly specific for identifying loss of FH expression.[14,15] Because of its commercial availability and high specificity, IHC screening for FH has the potential to identify HLRCC patients presenting with uterine leiomyomas.

We therefore sought to investigate the utility of FH IHC in the diagnosis of uterine leiomyomas associated with HLRCC, first by reporting the patterns of FH staining in uterine leiomyoma patients with known HLRCC and then assessing the results of FH IHC in unselected patients with uterine leiomyomas and leiomyosarcomas. We describe the pathologic features, FH mutation status, and clinical significance of the group of uterine leiomyomas, which show negative staining for FH, a class of tumor we term FH-deficient leiomyomas.

METHODS

The consultation files of one of the authors (A.J.G.) were searched for all patients with confirmed or presumed HLRCC who had also undergone surgical resection for uterine leiomyomas and had material available in formalin-fixed paraffin-embedded (FFPE) blocks. The database of the Department of Anatomical Pathology, Royal North Shore Hospital, Sydney, Australia was searched for all patients with uterine leiomyoma who underwent surgical resection during calendar years 2009 to 2013. A search was also made for all patients with leiomyosarcomas (including both uterine and extrauterine) who underwent surgery or biopsy from June 1998 to 2013 at the same institution. Material from patients with neoplastic tissue remaining in FFPE blocks was then used to construct tissue microarrays (TMAs) containing two 1 mm cores. In patients with multiple tumors the largest tumor was selected for annotation and TMA construction.

IHC for FH was performed on FFPE sections using previously described methods.[8] Briefly, a commercially available anti-FH mouse monoclonal antibody was used at a dilution of 1 in 2000 (cloneJ-13, cat no sc-100743; Santa Cruz Biotechnology), using an automated staining platform—the Leica Bond III Autostainer (Leica Biosystems, Mount Waverley, Vic., Australia) with heat-induced epitope retrieval for 30 minutes at 971C in the manufacturer's alkaline retrieval solution ER2 (VBS part no: AR9640).

FH IHC was scored by a single observer (A.J.G.). Absent staining in all neoplastic cells in the presence of a positive internal control in non-neoplastic cells such as endothelial cells was interpreted as true negative staining. If tumor cells were negative but there was no internal positive control, staining was considered indeterminate and repeated on whole sections or different blocks. All other patterns of staining including focally positive staining were considered positive, provided the staining was cytoplasmic and granular (ie, mitochondrial).

For patients with known HLRCC, IHC was performed on whole sections, and the observer was not blinded to the underlying diagnosis. For all other patients with leiomyoma and/or leiomyosarcomas, the observer was blinded to all clinical and pathologic features at the time of interpreting IHC. For these unselected cases IHC was initially performed on TMA sections and then repeated on whole sections if staining was negative or indeterminate.

All patients with confirmed HLRCC were offered FH germline testing as part of their clinical care. Genetic testing was performed using massively parallel sequencing (MPS) for small nucleotide variants with Sanger confirmation and, multiplex ligation-dependent probe amplification (MLPA) for detection of large-scale deletions.

All patients without a clinically confirmed diagnosis of HLRCC but with a uterine leiomyoma that demonstrated negative staining for FH underwent mutation testing on DNA extracted from macrodissected neoplastic and non-neoplastic FFPE tissue. Mutation testing was performed by both Sanger sequencing and MPS. For Sanger sequencing, previously described custom primer sets were used.[8] For MPS a MiSeq Platform and TruSeq Custom Amplicon Assay (Illumina, CA) was used. If a mutation was identified by MPS but not found on Sanger sequencing, repeat targeted Sanger sequencing of the exon of interest was performed before the mutation was considered confirmed. Loss of heterozygosity (LOH) studies were performed using a previously described set of 6 polymorphic short tandem repeat markers (D1S517, D1S2785, D1S180, AFM214xe11, D1S547, and D1S2842), surrounding the FH gene.[8] This study was approved by the North Sydney Local Health District medical ethics review board.

RESULTS

The details of 5 patients with a clinical diagnosis of HLRCC who previously or subsequently underwent resection of uterine leiomyomas are presented in Table 1. Briefly, although some had previously undergone myomectomies with tissue unavailable for review, the material available for testing was from surgery performed at a mean age of 35 years (range, 25 to 41 y). The mean tumor size was 65 mm (range, 30 to 115 mm). Ten of 11 uterine leiomyomas from these patients demonstrated negative IHC staining for FH. One uterine leiomyoma from a patient who had a point mutation (c.689A > C, p.Lys230Arg) demonstrated patchy staining, which, although weaker than usual, was interpreted as positive. Four patients demonstrated some of the morphologic features previously reported to be associated with HLRCC-related uterine leiomyomas (symplastic-type nuclear atypia, hemangiopericytomatous vascular pattern, hypercellularity); however, these features were absent in all the leiomyomas from 1 patient.

The database search identified 1176 patients with uterine leiomyomas who underwent surgical resection during the period 2009 to 2013. Of these patients, 1152 had sufficient material in TMA sections for IHC to be interpreted. Tumors from 25 patients demonstrated either negative staining for FH in the presence of a positive internal control or indeterminate staining when interpreted on TMA sections. When IHC was repeated on whole sections from these 25 patients it was definitively interpretable in all cases. Tumors from 13 patients demonstrated positive staining, and 12 were confirmed to be genuinely negative in the presence of an internal positive

TABLE 1. Clinical, Morphologic, IHC, and Molecular Features of Uterine Leiomyomas in Patients With HLRCC

Patient	Age	Surgery	Germline FH Mutation	No. Leiomyomas	FH IHC	Size of Largest Leiomyoma (mm)	Morphology
1	35	Myomectomy	Confirmed c.689A > C p.Lys230Arg	1	Neg	40	Usual leiomyomas
2	37 38	Hysterectomy Hysterectomy	Presumed*	Multiple Multiple	Neg in 2, Pos in 1 Neg in 2	100	Symplastic nuclear atypia, hypercellularity, hemangiopericytomatous vascular pattern
3	36	Myomectomy	Confirmed large-scale deletion on MLPA	1	Neg	40	Symplastic nuclear atypia, hypercellularity, hemangiopericytomatous vascular pattern
4	25	Myomectomy	Confirmed c.301 C>T p.Arg101X	1	Neg	115	Symplastic atypia, hypercellularity
5	41	Hysterectomy	Presumedw	Multiple	Neg in 3	30	Mild nuclear atypia, hypercellularity, hemangiopericytomatous vascular pattern

*Patient 2 was subsequently diagnosed with a typical HLRCC-type renal carcinoma at age 44. She is currently undergoing formal genetic testing and is presumed to have HLRCC.

wBoth patient 5 and her sister had large symptomatic leiomyomas requiring myomectomy in their 30s. They declined genetic testing but are presumed to have HLRCC.

TABLE 2. Morphologic and Demographic Features of Patients With FH-deficient Leiomyomas Identified by Screening IHC in a Cohort of 1152 Consecutive Patients

Patient	Age (y)	Size (mm)	Hypercellularity	Nuclear Atypia	Nucleoli	Hyaline Globules	Staghorn Vessels	Slit-like Vessels	Alveolar Edema
1	39	60	+	+	À	À	+	+	+
	30	Unknown	À	À	À	+	+	+	+
2	50	25	+	À	À	+	+	+	À
3	38	52	+	+	À	+	+	+	À
4	46	55	À	+	+	+	+	+	+
5	48	50	À	+	À	+	+	+	+
6	30	130	+	À	À	+	+	+	+
7	35	95	+	À	À	+	+	+	+
8	53	55	À	+	À	+	+	+	À
9	43	10	+	À	+	À	+	+	À
10	43	35	À	À	+	À	+	+	À
11	50	80	À	À	À	+	+	+	+
12	37	6	À	À	À	À	+	+	+
Total (n [%])			6/13 (46)	5/13 (38)	3/13 (23)	9/13 (69)	13/13 (100)	13/13 (100)	8/13 (62)
Average	42.7	54.4							

The details of the FH-deficient leiomyoma presenting 9 years earlier in patient 1 are also included.

control. That is, 12 of 1152 (1%) unselected uterine leiomyomas demonstrated genuine negative IHC staining for FH and were therefore classified as FH-deficient leiomyomas (Table 2).

Of the 12 patients from the unselected cohort with FH-deficient tumors, 4 had multiple leiomyomas resected at the time of index surgery. All of these additional tumors demonstrated positive staining for FH. One patient had previously undergone myomectomy 9 years earlier (patient 1 in Table 2). This previously resected leiomyoma demonstrated negative staining for FH. No other patients with FH-deficient leiomyomas recurred, and all patients were alive and disease free at the last known follow-up.

The pattern of FH IHC staining in FH-deficient leiomyomas is illustrated in Figure 1. Briefly, all non-neoplastic tissues (including adjacent non-neoplastic smooth muscle and endothelial cells within the leiomyomas) demonstrated positive staining in a mitochondrial pattern (ie, granular and cytoplasmic) (Fig. 1B), whereas the neoplastic cells were either negative or exhibited only a weak cytoplasmic blush of nonspecific staining (Fig. 1D).

The clinical and demographic features of the FH-deficient leiomyomas from the unselected cohort are presented in Table 2. Compared with other unselected uterine leiomyoma patients, patients with FH-deficient leiomyomas underwent surgery at a significantly younger mean age of 42.7 years versus 48.8 years (odds ratio, 0.919 [0.854-0.989]; P = 0.024). Although the average tumor size was slightly larger in FH-deficient leiomyomas, the difference was not significant (54.4 vs. 51.6 mm, P = 0.82).

The morphology of the 13 FH-deficient leiomyomas from 12 patients thus identified (comprising the 12 tumors from the truly unselected cohort plus the tumor resected 9 years earlier from patient 1) were reviewed in a specific search for the features previously associated with HLRCC-related uterine leiomyomas—that is, hypercellularity, symplastic nuclear atypia, prominent inclusion-like nucleoli, hyaline globules, a hemangiopericytomatous vasculature, a slit-like vasculature, and alveolar edema. The findings are presented in Table 2 and illustrated in Figures 2 and 3. Briefly, all cases demonstrated a hemangiopericytomatous/staghorn vasculature at least focally. In some cases this was a focal finding, evident only after a careful search, whereas in other cases this was widespread. Only 3 cases (23%) demonstrated prominent eosinophilic inclusion-like nucleoli. Five cases (38%) demonstrated symplastic-type nuclear atypia, which in 1 case was diffuse and in 4 cases was focal. Six cases (46%) demonstrated notable hypercellularity. Nine cases (69%) contained eosinophilic cytoplasmic globules, although in some cases this finding was very subtle and evident only after a dedicated search. Alveolar edema, defined as prominent stromal edema, which, when mixed with spindled smooth muscle cells, imparted an alveolar architecture, was evident in 8 cases (62%) but was often very focal. There was no evidence of coagulative or hyaline necrosis in any of the 13 FH-deficient leiomyomas, and all cases had a mitotic count of <5 per 10 high-power fields.

A total of 116 tumors from 88 patients who had received a diagnosis of leiomyosarcoma between 1998 and 2013 were available for IHC. Forty-four tumors from 35 patients were uterine leiomyosarcomas, 27 tumors from 22 patients were retroperitoneal, and the remainder arose in the soft tissue, subcutaneous connective tissue, or the gastrointestinal tract. All these leiomyosarcomas demonstrated positive staining for FH. However, we noted that 1 smooth muscle tumor of uncertain malignant potential (STUMP) was inadvertently included in the TMA of leiomyosarcomas because the initial reporting pathologist had favored a diagnosis of leiomyosarcomas, before retracting this diagnosis in favor of STUMP after consultation with a subspecialist gynecologic pathologist. This tumor, arising in a 31-year-old woman, demonstrated hypercellularity and areas of symplastic-type

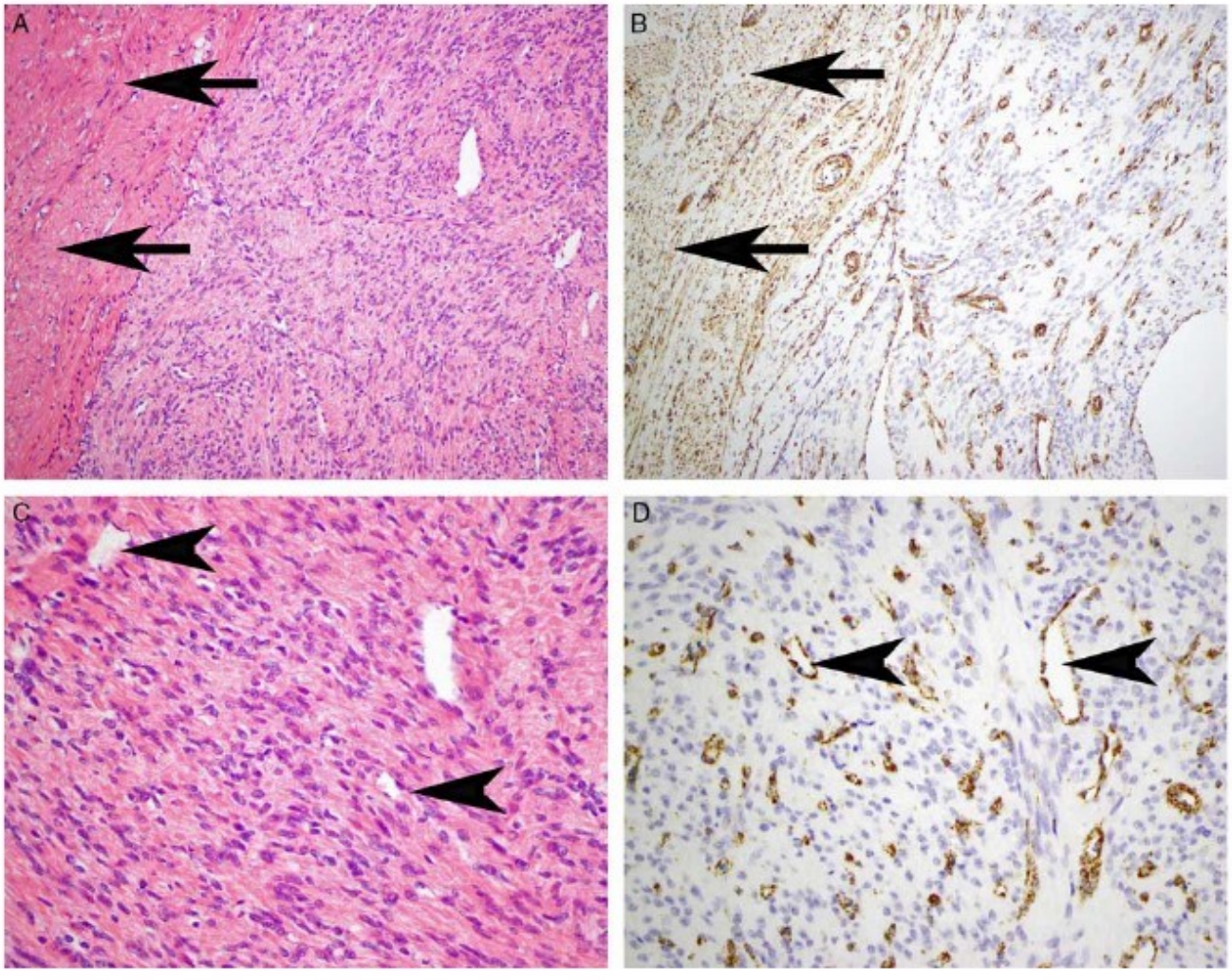

FIGURE 1. Serial hematoxylin and eosin–stained (A, C) and FH IHC-stained (B, D) sections. The non-neoplastic uterine smooth muscle (arrows) and endothelial cells within the main tumor mass (arrowheads) demonstrate positive staining for FH. This staining, which is distinctly mitochondrial (granular and cytoplasmic), serves as an internal positive control and contrasts with the leiomyoma, which is completely negative. The positive staining of the endothelial cells also serves to highlight the hemangio-pericytomatous and slit-like vascular pattern.

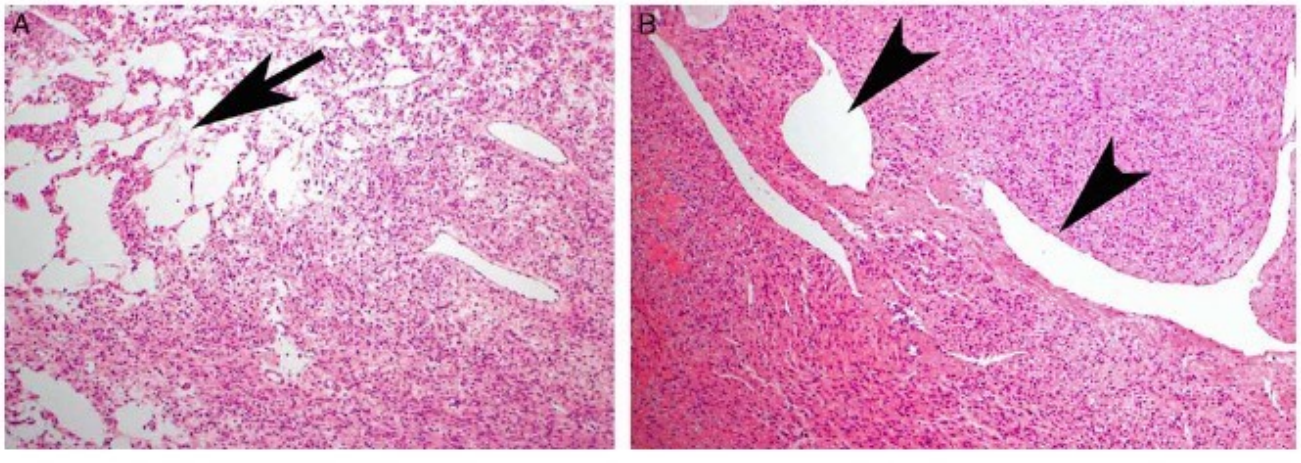

FIGURE 2. A, "Alveolar edema" (arrow) . B, Hemangiopericytomatous vascular pattern (arrowheads).

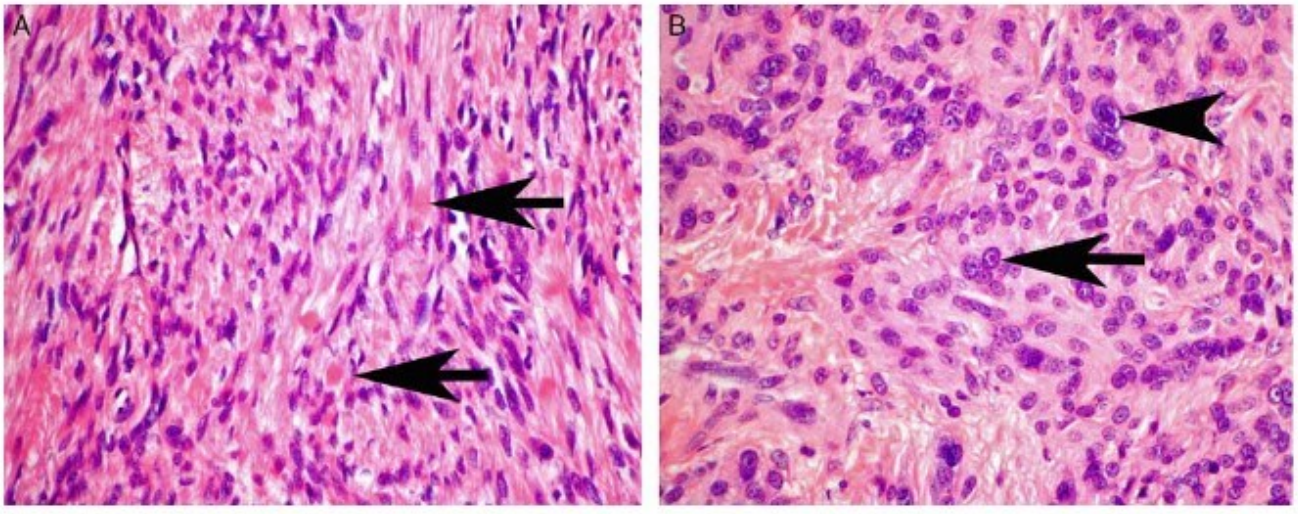

FIGURE 3. A, Eosinophilic cytoplasmic globules (arrows) were a subtle feature but commonly identified when sought. B, A few cases demonstrated either prominent inclusion-like nucleoli (arrow) or symplastic-type nuclear atypia (arrowhead).

nuclear atypia but lacked coagulative necrosis or mitotic activity and is illustrated in Figure 4. IHC for FH was negative in both TMA and whole sections from this tumor. The patient was alive and disease free 8 years after surgery, confirming that the tumor was biologically benign but also illustrating that FH-deficient leiomyomas may mimic leiomyosarcomas.

The results of molecular testing performed on DNA extracted from FFPE tissue from the 14 apparently sporadic FH-deficient leiomyomas, comprising 12 patients from the unselected cohort (1 with 2 FH-deficient leiomyomas presenting 9 y apart) and this additional STUMP patient, are presented in Table 3. Briefly, complete coverage by Sanger sequencing was achieved in 10 of 14 FH-deficient leiomyomas. Of these, somatic FH mutations were identified in 6 tumors from 6 separate patients (43% of all FH-deficient leiomyomas in the cohort, and 60% of those with complete coverage). Five of these mutations were also identified by MPS interpreted blinded to the results of Sanger sequencing, whereas 1 mutation was not identified by MPS due to incomplete coverage at the exon of interest but was confirmed on repeat Sanger sequencing. No confirmed somatic mutations were found in 4 FH-deficient leiomyomas with complete coverage by Sanger sequencing and partial coverage by MPS. LOH was identified in all 5 informative tumors with FH mutation. LOH at the FH locus was also identified in 1 FH-deficient leiomyoma in which no mutation was identified. LOH studies in the remaining tumors were not informative. Four FH-deficient leiomyomas had inadequate coverage by both Sanger sequencing and MPS to exclude mutation.

Sanger sequencing was successful in non-neoplastic tissue from 5 of 6 patients with FH-deficient leiomyomas harboring FH mutation. All were wild type for FH. No other mutations were identified by MPS on non-neoplastic tissue from any of the 13 patients; however, coverage was poor and insufficient to definitively exclude germline mutations in the 7 patients with no mutation identified in their tumors.

No FH mutations were identified in all 4 FH IHC-positive leiomyomas comprising second or third leio-

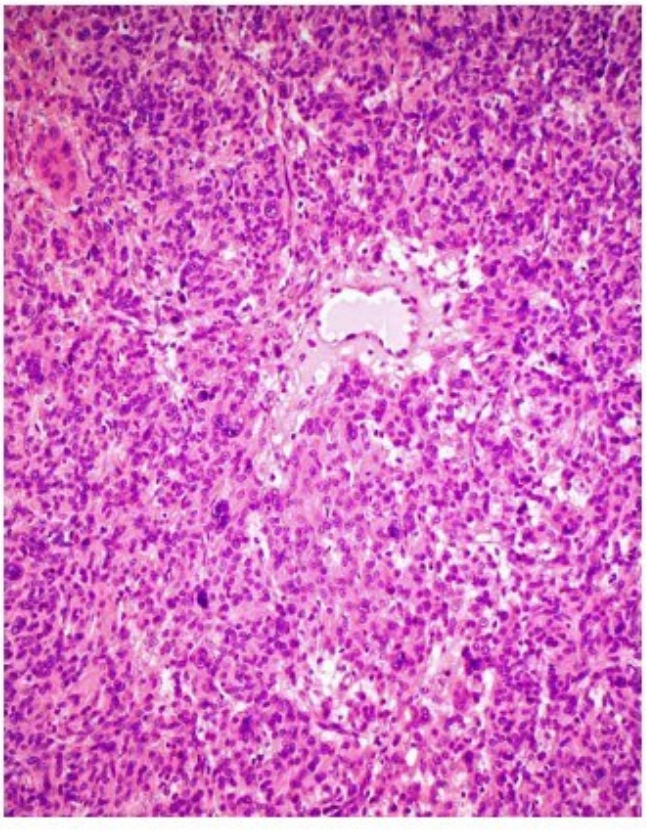

FIGURE 4. The diagnosis of leiomyosarcoma was originally considered in this FH-deficient leiomyoma. However, the tumor was reclassified as STUMP upon review. It is hypercellular and shows nuclear atypia but lacks significant mitotic activity or coagulative necrosis.

TABLE 3. Summary of FH Status in Leiomyomas

Patient	Germline FH Status (Sanger)	Tumor	FH IHC	Tumor FH Status (Sanger)	LOH
1	WT*	1	Neg	WT*	NA
		2	Neg	c.1040C > A (p.Ser347Tyr)w	Yes
2	WT*	1	Neg	c.1390+1G > A (splice site)w	Yes
		2	Pos	WT*	NA
3	WT*	1	Neg	WT*	Yes
4	NP*	1	Neg	NA*	NA
5	NPz	1	Neg	WTy	NA
		2	Pos	WT*	
6	NA*	1	Neg	c.712G > A (p.Asp238Asn)8	NA
7	WT*	1	Neg	c.830C > T (p.Thr277Ile)w	Yes
8	NPz	1	Neg	WTz	NA
		2	Pos	WT*	
		3	Pos	WT*	
9	WT*	1	Neg	c.583A > G (p.Met195Val)w	
10	NP*	1	Neg	NA#	Yes
11	WT*	1	Neg	c.1217A > T (p.Asn406Ile)w	NA
		2	Pos	NA*	Yes
12	NP*	1	Neg	NA*	
13**	NP*	2	Neg	NA*	NA
					NA

Results of molecular testing in 14 FH-deficient leiomyomas and 5 non–FH-deficient (second and third) leiomyomas from 13 patients.

*MPS performed, not suggestive of mutations; however, complete coverage was not achieved.

wConfirmed by MPS.

z MPS performed—wild-type sequence observed in all exons, with the exception of exon 1, which was uninterpretable due to insufficient coverage.

y c.838G > A (p.Gly280Ser) detected by MPS (47% variant call, depth of coverage 34); however, this mutation was not identified by repeated targeted Sanger sequencing and is therefore considered WT.

8 Not confirmed by MPS due to insufficient coverage.

z c.290G > A (p.Gly97Asp) detected by MPS (24% variant call, depth of coverage 102); however, this mutation was not identified by repeated targeted Sanger sequencing and is therefore considered WT.

c.1367T > C (p.Val456Ala) detected by MPS (41% variant call, depth of coverage 27); however, this mutation was not identified by repeated targeted Sanger sequencing and is therefore considered WT.

**Uterine leiomyoma initially diagnosed as leiomyosarcoma.

NA indicates results inconclusive due to insufficient coverage (Sanger or MPS) or noninformative (LOH); NP, not performed as no mutation identified in tumor; WT, wild-type.

myomas from patients with FH-deficient leiomyomas (patients 2, 5, and 8), which were successfully tested.

DISCUSSION

FH is the enzyme immediately following succinate dehydrogenase in the Krebs cycle.[18] IHC for succinate dehydrogenase B, which is performed and interpreted in an identical way to FH, is used diagnostically as a marker of integrity of the succinate dehydrogenase complex. The standard nomenclature for tumors that show negative staining for succinate dehydrogenase B is succinate dehydrogenase deficient.[18] Succinate dehydrogenase–deficient tumors include distinct subtypes of pheochromocytoma/paraganglioma, gastrointestinal stromal tumor, renal carcinoma, and pituitary adenoma—summarized in Gill.[18] Using similar nomenclature, we therefore believe that FH-deficient leiomyoma is the most appropriate nomenclature for a leiomyoma, which shows IHC-negative staining for FH in the presence of an internal positive control. We prefer this terminology to HLRCC-associated leiomyoma, not only because of the obvious parallels with succinate dehydrogenase deficiency in terms of interpretation of the stain but also because it is clear from this study that, although most patients with HLRCC (ie, germline FH mutation) will develop FH-deficient leiomyoma, the great majority of FH-deficient leiomyomas seem to occur sporadically and are not associated with HLRCC.

Our study demonstrates that the identification of FH-deficient uterine leiomyomas may be an important clue to the diagnosis of HLRCC, but its significance should not be overinterpreted as it is not completely sensitive and it is far from specific. For example, in 11 leiomyomas from 5 confirmed HLRCC patients, only 10 tumors (91%) demonstrated negative IHC staining for FH. One tumor from a patient with a clearly pathogenic germline FH point mutation c.689A > C (the patient subsequently went on to develop typical HLRCC-related renal cell carcinoma) demonstrated positive staining for FH. Therefore, negative staining for FH will clearly not identify all patients with HLRCC presenting with leiomyoma.

Although FH-deficient leiomyomas account for only 12 of 1152 (1%) of all unselected uterine leiomyomas, uterine leiomyomas are among the most common visceral tumors submitted for pathologic examination. That is, because of the sheer volume of uterine leiomyomas resected, FH-deficient leiomyomas will be encountered frequently in most diagnostic surgical pathology practices. In this respect is it noteworthy that no germline FH mutations were identified in 12 patients with FH-deficient leiomyomas from an unselected cohort encountered over a 5-year period. We caution that Sanger

sequencing and MPS were performed on DNA extracted from archived FFPE tissue, and this approach, being subject to uneven coverage, could not be expected to identify all FH mutations. Furthermore, we did not perform MLPA to look for large-scale deletions, which are not uncommon in HLRCC and in fact account for 1 of our patients from the confirmed germline mutation cohort (patient 3, Table 1). Therefore, it is possible that not all germline mutations would be identified by our approach. Nevertheless, the fact that FH-deficient leiomyomas are relatively common (1% of all uterine leiomyomas) but HLRCC is rare, and no germline mutations or subsequent clinical evidence of HLRCC were identified in our unselected patients, suggests that germline mutation testing for FH mutation is a low-yield test in the absence of other features to suggest syndromic disease even in patients presenting with FH-deficient leiomyomas.

Our findings are in keeping with previous studies, which primarily used molecular testing to screen for FH mutations in leiomyomas. For example, Barker et al[19,20] detected no FH mutations in 129 unselected uterine leiomyomas but did identify LOH at 1q43 in 7 tumors, and Lehtonen et al[21] found 2 somatic mutations in 153 uterine leiomyomas from 46 patients and also confirmed that no patients had germline mutation. Taken together, 2 of 282 (0.7%) unselected uterine leiomyomas from these molecular studies harbored somatic FH mutation, and neither of these were associated with germline mutation. Although our study was not intended or designed to assess the sensitivity of FH IHC in identifying FH mutations in uterine leiomyomas, given that we found an FH-deficient leiomyoma incidence of 1% it is likely that FH IHC identifies most but not all FH mutations.

From a practical point of view we would recommend that if an FH-deficient uterine leiomyoma is diagnosed, the possibility of HLRCC should be considered clinically. However, genetic counseling and formal mutation testing may not be indicated in the absence of suspicious features identified after a detailed family history and physical examination—such as a personal or family history of cutaneous leiomyomas, renal carcinoma, or uterine leiomyomas with onset at a young age.

Compared with other uterine leiomyomas, FH-deficient leiomyomas were resected at a significantly younger age (mean, 42.7 vs. 48.8 y, P = 0.024). Although a hemangiopericytomatous vascular pattern was a relatively constant feature, other morphologic clues to the diagnosis of FH-deficient leiomyomas (such as hypercellularity, prominent nucleoli, symplastic nuclear atypia, and hyaline globules) were subtle, inconstant, or infrequent. Therefore, although we emphasize that FH-deficient leiomyomas are overrepresented among symplastic, atypical, or hypercellular leiomyomas, and these features can certainly be a clue to the diagnosis, we are in agreement with Alsolami et al[10] and Martinek et al[8] who suggested that these morphologic features lack sufficient objectivity or robustness to be useful to definitively confirm or exclude a diagnosis of FH-deficient leiomyoma in routine clinical practice.

Our experience and that of others, in renal carcinoma is that not all HLRCC-associated tumors will be FH IHC negative and that some definite HLRCC-associated renal carcinomas will show positive staining for FH and could potentially be identified by positive staining for 2SC.[14,15] The potential to identify HLRCC-associated uterine leiomyomas by positive staining for 2SC has been demonstrated by others,[8,10,11] and we know that 1 of our uterine leiomyomas arising in a patient with a confirmed germline FH mutation but with positive IHC staining for FH was demonstrated to show positive staining for 2SC (data not shown). Perhaps in the future both positive staining for 2SC and negative staining for FH may be used to diagnose FH-deficient leiomyomas, and our experience has been that the combination shows potential in renal carcinoma albeit limited by the lack of sensitivity of FH and lack of specificity of 2SC.[14] However, at the time of writing, IHC for 2SC is not commercially available and therefore not practical for routine clinical use. Because of this lack of availability we were unable to test the sensitivity and specificity of 2SC on the entire cohort for this study.

Although it is clear that FH-deficient leiomyomas commonly show cytologic atypia, the relationship between FH deficiency and true leiomyosarcomas (ie, biological evidence of malignancy) is unclear and muddied by previous reports of cases of uterine leiomyosarcoma diagnosed on the basis of histology, often without expert pathologic review, which do not provide evidence of the biological behavior of these tumors.[3,22,23] In fact we are only aware of 1 case of uterine leiomyosarcoma arising in the setting of FH mutation in which biological evidence of malignant behavior is reported, and this patient was still alive 12 years after presentation.[23] For this reason we believe that overinterpretation of the cytologic atypia and hypercellularity, which may occur in FH-deficient leiomyoma, may lead to "overdiagnosis" of leiomyosarcomas. This is well illustrated in our case of STUMP, which was initially considered a leiomyosarcoma but reclassified as symplastic leiomyoma on review. We note that none of the FH-deficient leiomyomas in our cohort behaved in a malignant manner, and we found no cases with negative FH IHC staining among 116 genuine leiomyosarcomas from 88 patients. This is similar to Reyes et al[11] who demonstrated that none of 29 leiomyosarcomas showed positive staining for 2SC. Furthermore, it has previously been demonstrated that FH mutations are not a common driver of uterine leiomyosarcoma.[24] Therefore, in the absence of more definitive evidence to the contrary, we believe FH mutations occur rarely (if at all) in association with biologically malignant smooth muscle tumors—that is, true leiomyosarcomas. However, we emphasize that FH-deficient leiomyomas may morphologically mimic leiomyosarcomas because of their hypercellularity and tendency to symplastic nuclear atypia. In this respect, loss of staining for FH may be considered a reassuring finding in a mildly atypical uterine smooth muscle tumor, and we would be hesitant to make a diagnosis of uterine leiomyosarcoma in the setting of FH deficiency unless there is unequivocal evidence of malignancy.

In conclusion, the great majority of uterine leiomyomas arising in the setting of HLRCC will show negative staining for FH, and therefore in the appropriate clinical context FH-deficient leiomyomas can be an important clue to the diagnosis of HLRCC. There are some morphologic clues to assist in the diagnosis of FH-deficient leiomyomas including hypercellularity, prominent nucleoli, symplastic-type nuclear atypia, cytoplasmic eosinophilic globules, and a staghorn vasculature. However, these features are inconstant and may be subtle. Morphology therefore cannot be used to replace IHC or molecular analysis in the routine clinical setting. Not all uterine leiomyomas arising in the setting of HLRCC will show negative staining for FH and positive staining for FH does not completely exclude the diagnosis of HLRCC. Loss of staining for FH occurs rarely, if at all, in true uterine leiomyosarcomas.

Most importantly, at least 1% of all unselected uterine leiomyomas show negative staining for FH. Although these leiomyomas are commonly associated with somatic FH mutation and show similar morphology to cases associated with germline FH mutation, these patients very rarely have demonstrable germline FH mutation. We therefore would not recommend genetic testing for HLRCC in these patients in the absence of other clinical risk factors such as a personal or family history of cutaneous leiomyomas, renal carcinoma, or symptomatic leiomyomas at a very young age. We do, however, believe there is merit in prospectively identifying these patients so that the possibility of syndromic disease can be considered clinically.

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3.6 Bizarná dysplázia krčka maternice

(Bizarre cell dysplasia of the cervix)

Väčšina dysplastických lézií dlaždicového epitelu krčka maternice je ľahko klasifikovateľná v súlade s WHO 2014 ako LSIL, alebo HSIL. Malá časť z nich je však diagnosticky problematická hlavne z dôvodu, že sa jedná o metaplastický fenotyp alebo nízky dysplastický epitel, ktorý sa ťažko hodnotí. Okrem toho existuje ďalšia morfologická varianta s obsahom mnohojadrových a bizarných buniek. Bizarná dysplázia nikdy nebola ciele a konzistentne popísaná. Čiastočný popis sa objavuje v roku 2007 rámci Parkovho popisu dysplázie nízkeho stupňa. Cieľom tejto práce je cielený histologický a imunohistochemický popis bizarnej dysplázie a hľadanie možnej asociácie s infekciou HPV alebo inými non-HPV DNA vírusmi. Štúdia je založená na revízii a hodnotení 29 konizátov obsahujúcich dysplastický dlaždicový epitel s bizarnými bunkami. Tieto prípady boli získané ako náhodné prípady z rutinnej biopsie do roku 2011 a systematické retrospektívne prehľadávanie konizátov z roku 2013 až 2015 prinieslo ďalších 24 prípadov. U každej pacientky sme získavali demografické údaje, revidovali sme preparáty skríningovej cytológie a v tkanive z vybraného reprezentatívneho parafínového bloku sme doplnili imunohistochemické vyšetrenie expresie antigénu p16 a Ki67 a boli použité metódy molekulovej genetiky (PCR, HPV DNA *in situ* hybridizácia). Bizarnú dyspláziu sme definovali ako podtyp ťažkej dysplázie (HSIL) charakterizovaný prítomnosťou individuálnych bizarných buniek nerovnomerne rozložených v celej šírke dysplastického epitelu. Bizarné bunky sú charakterizované veľkosťou a tvarom podobným superficiálnym dlaždicovým bunkám dlaždicového epitelu v normálnom ektocervixe, ďalej anizonukleózou a mnohojadrovosťou s prekrývaním sa hyperchrómnych alebo aj ojedinele hypochrómnych jadier, čo vedie k nukleomegálii s bizarným tvarom jadier, ktorý niekedy pripomína atramentové machule. Z 231 konizátov krčka maternice obsahujúcich ťažkú dyspláziu (HSIL) bizarná dysplázia bola prítomná v 29 prípadoch s priemerným vekom pacientiek 33,6 rokov. Bizarná dysplázia bola spojená s konvenčnou ťažkou dyspláziou v 18 prípadoch a z blandným typom ťažkej dysplázie (tak ako bol nedávno popísaný skupinani autorov Park a Kitahara, ktorý ju označil ako „deceiving dysplasia“) v 10 prípadoch. V jednom prípade sme bizarnú dyspláziu zastihli i s extenziou do endocervikálnych krýpt. Vo všetkých prípadoch bola imunohistochemicky prítomná bloková pozitivita expresie antigénu p16. In situ hybridizačný signál bol hodnotiteľný v 12 prípadoch takto: Integrovaný typ bodkovitého signálu bol zaznamenaný v bizarných bunkách iba v 3 prípadoch, keď sa tieto bunky nachádzali v dolnej tretine dysplastického epitelu. Difúzny typ signálu epizomálneho typu bol zaznamenaný v bizarných bunkách pokiaľ sa nachádzali v horných dvoch tretinách dysplastického epitelu. Podľa našich zistení je bizarná dysplázia významne častejšie asociovaná s prítomnosťou HPV typu 16, v porovnaní s českou populáciou podľa výsledkov štúdie pražských autorov obsahujúcej skupinu 311 pacientiek. Rovnaký výsledok sme získali i v porovnaní s kontrolnou skupinou 49 HSIL lézií nevýberových konizátov vyšetrených

na našom pracovisku. Asociácia s non-HPV DNA vírusmi bola zaznamenaná iba v jedinom prípade, kedy sme zaznamenali koinfekciu vírusom HHV 6. Jednalo sa o imunokompromitovanú pacientku dlhodobo liečenú kortikosteroidmi pre Crohnovu chorobu. Vzťah s inými non-HPV vírusmi sme nezaznamenali. Bizarná dysplázia je identifikovateľná približne v 13% konizátov s ťažkou dyspláziou. Nie je rozdiel v priemernom veku pacientiek v porovnaní s konvenčnou ťažkou dyspláziou. Tento typ dysplázie má potenciál endocervikálnej extenzie. Správna identifikácia bizarnej dysplázie by mohla prispieť k spresňovaniu diagnostiky cervikálnych dysplázií, najmä v malých vzorkách a takisto by mohla podporiť vznik longitudinálnych štúdií, ktoré by ďalej presnejšie určili biologické vlastnosti bizarnej dysplázie a to najmä jej riziko progresie v porovnaní s konvenčnou ťažkou dyspláziou. Na základe unikátnych histologických a cytologických charakteristík a asociácie s infekciou HPV 16 si myslíme, že bizarná dysplázia reprezentuje špecifickú variantu HSIL.

THE JOURNAL OF
Obstetrics and Gynaecology Research

doi:10.1111/jog.13196 J. Obstet. Gynaecol. Res. Vol. 43, No. 2: 345–351, February 2017

Bizarre cell dysplasia of the cervix

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Abstract

Aim: The aim of this study was the characterization of a new subtype of high-grade cervical squamous intraepithelial lesion (HSIL) with enlarged cells containing bizarre nuclei: so-called bizarre cell dysplasia (BCD).
Methods: A total of 29 cervical cone biopsy samples of this type of dysplasia were studied. Multi-target polymerase chain reaction and in situ hybridization human papillomavirus (HPV) detection was performed in all cases. BCD was defined as a subtype of HSIL characterized by the presence of large dysplastic cells with abnormal, large pleomorphic nuclei or multinucleation causing nucleomegaly. This results in bizarre nuclear shapes. Bizarre cells are scattered throughout the whole thickness of the dysplastic squamous epithelium.
Results: The BCD lesions arise within the conventional/classic high grade or "bland" type squamous dysplasia HSIL. Statistically they were significantly associated with HVP type 16. A significant association with other studied viruses (Herpes simplex virus [HSV]1, HSV2, Varicella zoster virus, Epstein–Barr virus, cytomegalovirus, human herpesvirus 6, and human polyomaviruses BK and JC) was not confirmed.
Conclusions: BCD involves cytologically characteristic morphologic changes that are recognizable, but which may pose some risk of misdiagnosis as low-grade squamous intraepithelial lesion due to the enlargement of dysplastic cells and multinucleation. Based on the unique histological, cytological and biological features of BCD including strong association with HPV 16 infection, we believe that this is a specific, and so far unrecognized variant of HSIL.
Key words: cervical dysplasia, DNA virus, high-grade squamous intraepithelial lesion, human papillomavirus.

Introduction

Most of the cervical squamous intraepithelial lesions are readily classified according to the existing histologic criteria as either cervical intraepithelial neoplasia (CIN) I, II, III or more conveniently as low-grade or high-grade squamous intraepithelial lesion (LSIL or HSIL) according to the World Health Organization 2014 classification.[1] The small number of cases is problematic (mostly because of the metaplastic phenotype).[2–4] Still other types of rare lesions present multinucleation and bizarre nuclei. Bizarre cell dysplasia (BCD) has never been concisely described before. It was partly described in the setting of low-grade dysplasia by Park et al. in 2007.[4] The aim of this study was therefore to better characterize BCD lesions histologically and immunohistochemically, and to investigate the association with human papillomavirus (HPV) infection and possible non-HPV DNA virus coinfection (i.e. Herpes simplex virus [HSV]1, HSV2, Varicella zoster virus [VZV], Epstein–Barr virus [EBV], also called human herpesvirus [HHV]4, cytomegalovirus [CMV], HHV6 and human polyomaviruses BK and

Received: February 17 2016.
Accepted: September 7 2016.
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JC), which is known to cause multinucleation in various histopathologically defined lesions.

Methods

Study design and case selection

We identified 29 cases of BCD featuring bizarre nuclei within squamous dysplastic epithelium. These consisted of five incidentally identified routine cervical cone biopsy cases since 2011, and 24 prospectively identified cases between September 2013 and June 2015 in the setting of cone biopsy harboring HSIL performed and diagnosed at Šikl's Department of Pathology and Bioptická Laboratory by O.O., and reviewed by two co-authors (M.M., R.F.). In each case, demographic data and pap smear history were obtained. A representative formalin-fixed and paraffin-embedded (FFPE) block was provided for immunohistochemistry (IHC), in situ hybridization (ISH) and molecular genetic studies.

Immunohistochemistry of p16 antigen

The IHC was performed using a Ventana Benchmark XT automated stainer (Ventana Medical System, Tucson, AZ, USA). Visualization was carried out using diaminobenzidine tetrahydrochloride or Fast Red as the chromogen. Monoclonal antibody p16 (p16 Protein INK 4a CINtec V–kit, clone E6H4, Ventana) and Ki 67 (CONFIRM anti-Ki–67 (30–9), clone Confirm 30–9, Ventana) was used with the appropriate positive control for classic HSIL. In accordance with lower anogenital squamous terminology (LAST) recommendations,[5] the lesions were scored as positive when intense diffuse nuclear or simultaneous nuclear and cytoplasmic staining reaching at least one-third of the width of the

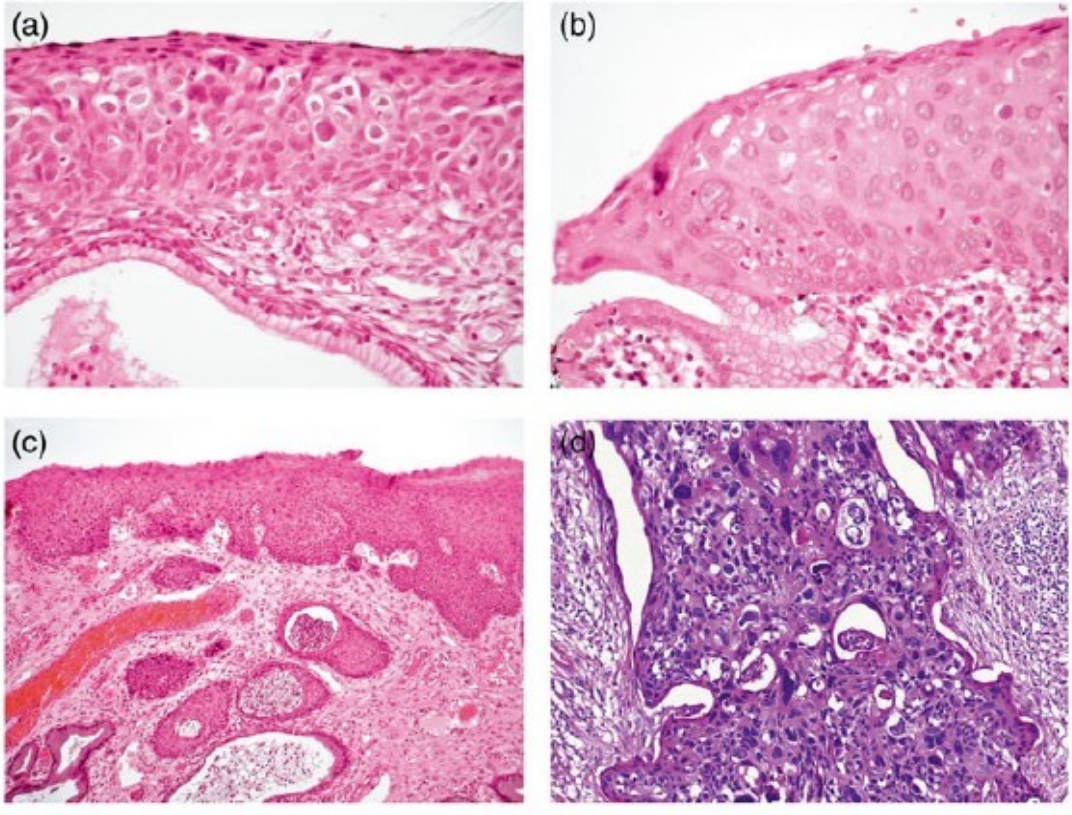

Figure 1 (a) Bizarre cell dysplasia (BCD) is characterized by the presence of large dysplastic cells with dysmorphic nuclei of bizarre shapes including the confluence of multiple nuclei (HE, 400x). (b) BCD of bland type resembles immature metaplastic epithelium at first sight. Observed significant variation of the size of the nuclei, occasional multinucleation and sporadic bizarre hyperchromatic nuclei qualifies this type of epithelium as high-grade squamous intraepithelial lesion. Molecular genetics indicates an association with human papillomavirus (HPV) type 16 (HE, 400x). (c) BCD is capable of glandular extension. Scattered bizarre nuclei can be found in the superficial dysplastic epithelium as well as in the endocervical crypts (HE, 400x). (d) Cells with bizarre nuclei may be present in the invasive component of squamous cell carcinoma of the cervix (HE, 400x).

dysplastic squamous cervical epithelium (so called "block-type positivity") was observed. Focal or scattered cytoplasmic and/or nuclear staining of the epithelium was scored as negative.

Molecular genetics

In situ hybridization

In order to determine the site-specific infection pattern, ISH of HPV DNA was done using the commercial kit INFORM HPV III Family 16 Probe on Ventana Benchmark XT automated stainer (Ventana Medical System). Results were interpreted according to manufacturer recommendations.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in all cases in the course of routine examination with special anti-cross-contamination precautions. HPV DNA detection was performed using three different in-house PCR methods with primers GP5+/6+, CPSGB, and type-specific primers for HPV16, 18, 31, 33, 35, 45.[6–9] In the case of possible multi-type HPV infection, the commercial system RHA (reverse hybridization assay) kit HPV SPF10, version 1 (Bio-medical Products, Rijswijk, The Netherlands) was used. DNA detection of HSV1, HSV2, VZV, EBV, CMV, and HHV6 was performed using real-time PCR with specific primers and hydrolysis probes.[10]

Results

We defined BCD as a subtype of HSIL characterized by the presence of individual bizarre cells irregularly scattered throughout the thickness of the dysplastic epithelium (Fig. 1a). Bizarre cells are characterized by (i) similar shape and size to superficial squamous cells of a squamous epithelium in a normal ectocervix; and (ii) anisonucleosis and multinucleation with overlapping hyperchromatic or hypochromatic dysplastic nuclei, causing nucleomegaly with bizarre nuclear shapes resembling an ink spot. BCD was identified in 29 of 231 cone biopsies diagnosed as HSIL (CIN III). Mean patient

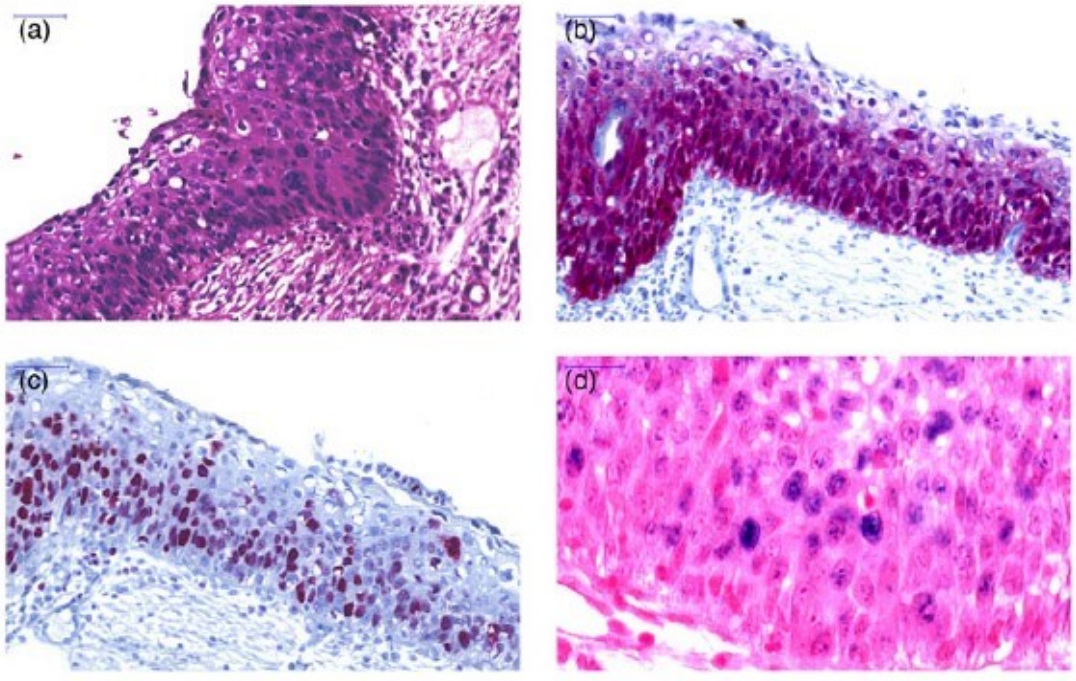

Figure 2 (a) Patient 12. Bizarre cells with overlapping nuclei in the lower third of dysplastic epithelium in the center, as well as in the upper third, where they intermingle with koilocytes (HE). (b) Block-type positivity of p16 antigen of at least two-thirds of the thickness of dysplastic epithelium (immunohistochemistry, Fast Red). (c) Nuclear positive expression of Ki67 antigen in the lower third of dysplastic epithelium, sporadically reaching the middle third including some of the bizarre cells on the right (immunohistochemistry, Fast Red). (d) Dotted in situ hybridization (ISH) signal of integrated type represented by scattered nuclei of dysplastic epithelium. Diffuse ISH signal of episomal type is seen in sporadic nuclei of the intermediate and superficial layer of the dysplastic epithelium. In the background non-specific dotted staining of some nucleoli and partial weak diffuse staining of some nuclei can be seen (ISH). Scale bars: (a) 50μm; (b) 50μm; (c) 50μm; (d) 20μm.

age was 33.6 years. It was associated with classic HSIL (18/29 cases) or with a bland-type of high-grade dysplasia (10/29 cases; Fig. 1b) earlier partly described by Park et al.[4] and by Kitahara et al. as "deceiving dysplasia".[3] One case was associated with both classic and bland-type dysplasia. In some cases BCD was also identified in dysplastic epithelium extending into the endocervical crypts (Fig. 1c). Sporadically bizarre cells may also be present in the infiltrative portion of cervical squamous cell carcinoma (Fig. 1d). Positive p16 expression was confirmed on IHC in all BCD cases (Figs 2–5). Interestingly, when bland-type dysplasia was present the area of p16 positive expression reached one-half to two-thirds that of the dysplastic epithelium. There was also no ISH signal in 10 of 22 ISH-analyzable BCD cases. Bizarre cells produced rare scattered individual weak dotted signals of integrated type only in three cases when bizarre cells were located in the lower or intermediate third of the epithelium. Diffuse medium–strong signal pattern of episomal type was usually found in cells present in the upper two-thirds of the dysplastic epithelium, rarely in the lower third (Table 1). BCD is significantly more often associated with HPV 16.

This is statistically significant (P = 0.029 at significance level 0.05, Fisher exact test) compared with the HPV type distribution in HSIL (n = 311) in the general Czech population,[11] as well as with the present control group of 49 consecutive HSIL lesions (mean patient age, 31.3 years) in cone biopsies examined at Bioptická laboratory (P = 0.049 at significance level 0.05, Fisher exact test). Association with HHV6 was found in one case (no. 24) in an immunocompromised patient on long-term corticosteroid treatment since 2014 due to Crohn's disease. We did not note an association with any other non-HPV DNA viruses studied (Table 2).

Discussion

In this study we have designated a distinctive cervical squamous intraepithelial lesion as BCD. It is a histologically identifiable entity in approximately 13% of cone biopsies with HSIL characterized by the presence of individual bizarre cells. There is no difference in mean age of BCD patients and those with the usual HSIL lesion. BCD has the potential for endocervical crypt

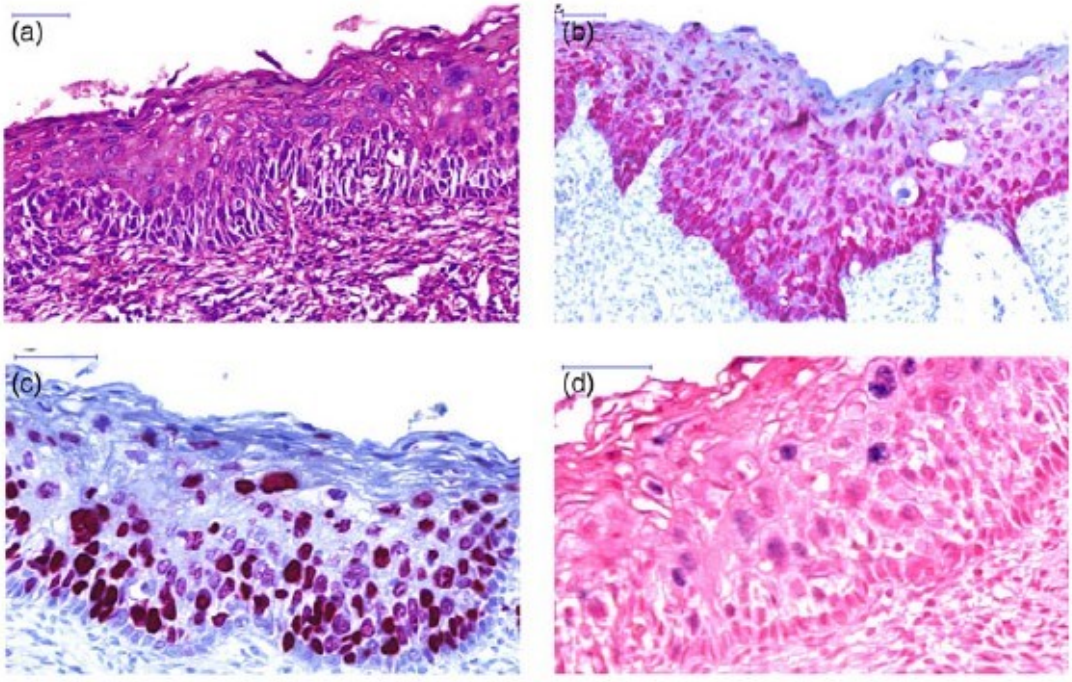

Figure 3 (a) Patient 18. Bizarre cells are present in the middle third of dysplastic epithelium on the right (HE). (b) Block-type positivity of p16 antigen of at least two-thirds of the thickness of dysplastic epithelium including bizarre cells (immunohistochemistry, Fast Red). (c) Significant positive expression of Ki67 antigen. Bizarre cells are present in the upper two-thirds of dysplastic epithelium on the left and in the middle (immunohistochemistry, Fast Red). (d) Diffuse in situ hybridization (ISH) signal of episomal type in sporadic nuclei of the intermediate and superficial layer of dysplastic epithelium (ISH). Scale bars: (a) 50μm; (b) 50μm; (c) 50μm; (d) 50μm.

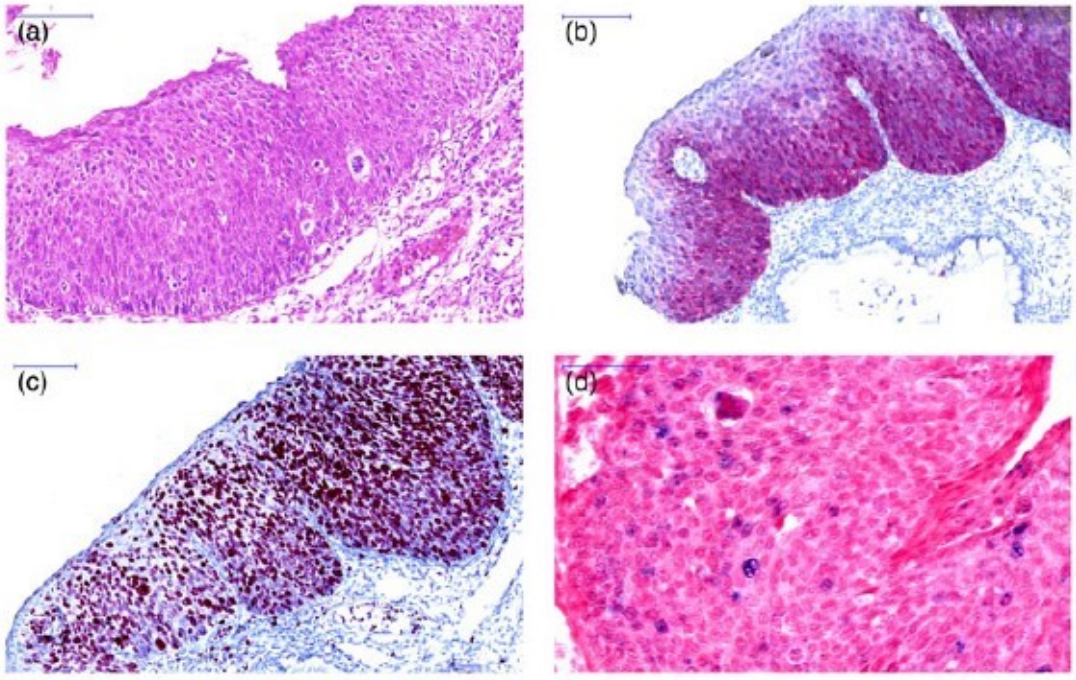

Figure 4 (a) Patient 21. Sporadic bizarre cells are scattered throughout the dysplastic epithelium (HE). (b) Block-type positivity of p16 antigen of at least two-thirds of the thickness of dysplastic epithelium. The bizarre cell in the middle has only weak cytoplasmic positivity (immunohistochemistry, Fast Red). (c) Strong positive expression of Ki67 antigen in dysplastic epithelium (immunohistochemistry, Fast Red). (d) Dotted in situ hybridization (ISH) signal of integrated type in the nuclei of the lower two-thirds of dysplastic epithelium. Diffuse ISH signal of episomal type is seen in sporadic nuclei of all three layers of the dysplastic epithelium (ISH). Scale bars: (a) 100μm; (b) 100μm; (c) 100μm; (d) 50μm.

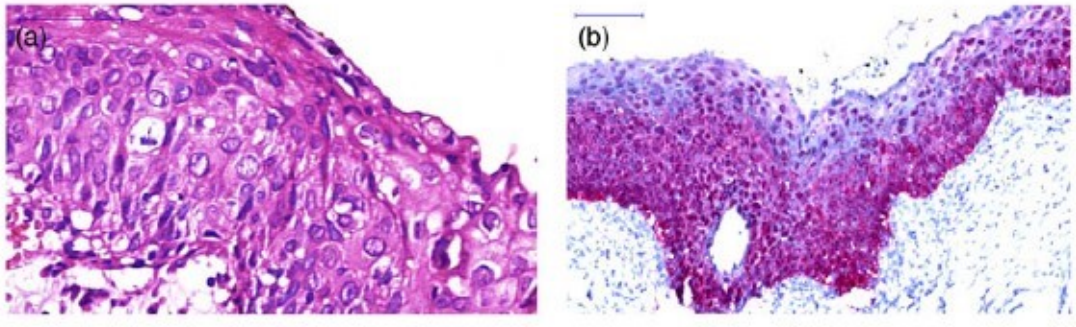

Figure 5 (a) Patient 22. Dysplastic epithelium with sporadic bizarre cell (HE). (b) Block-type positivity of p16 antigen of at least two-thirds of the thickness of dysplastic epithelium (immunohistochemistry, Fast Red). (c) Flat dysplastic epithelium with strong nuclear positivity of Ki67 antigen including sporadic bizarre cells (immunohistochemistry, Fast Red). (d) Sporadic dotted in situ hybridization (ISH) signal of integrated type in some nuclei of the lower third of the epithelium and sporadic diffuse episomal type signal in the middle third, partly obscured by non-specific background staining. Unequivocal bizarre cells are not seen in this part of the dysplastic epithelium (ISH). Scale bars: (a) 50μm; (b) 50μm; (c) 50μm; (d) 50μm.

extension and invasive carcinomatous growth. It could be the source of diagnostic difficulties in punch biopsy of the cervix and in pap smears (Table 3). We agree with others that it may be misdiagnosed as LSIL both cytologically[12] and even histologically.[4] Fortunately, BCD is in our experience histologically associated with "classic" HSIL in 62% of cases (18/29). This is of great convenience when a pathologist is in doubt when dealing with this type of dysplasia. Small sample biopsies are more problematic in the case of "bland type of squamous dysplasia". In those instances, familiarity with the BCD phenotype is of great help because

Table 1 ISH signal pattern in 12/22 analyzable BCD samples

Type of hybridization signal	n	Intraepithelial location	ID no.
Episomal	8	Superficial	1,4,5,8,13,18,21, 24
Episomal	3	Intermediate	14,18,20
Episomal	2	Basal	8,12
Integrated	2	Intermediate	20, 24
Integrated	1	Basal	6

There was no ISH signal in 10/22 analyzable BCD samples. Unanalyzable samples: ID nos 3, 9, 10, 11, 25, 27, 29. BCD, bizarre cell dysplasia; ISH, in situ hybridization.

Table 2 BCD in cervical cone biopsy: Patient characteristics (n = 29)

Patient ID no.	Age (years)	Dysplasia type	HPV type	Non-HPV virus
1	28	Classic	16	Negative
2	46	Classic	16	Negative
3	41	Bland	31	Negative
4	21	Classic	16	Negative
5	25	Bland	16	Negative
6	60	Classic	16	Negative
7	34	Bland	16	Negative
8	31	Bland + classic Classic	16	Negative
9	26	Classic	16	Negative
10	47	Classic	16	Negative
11	39	Bland	16	Negative
12	31	Bland	16, 45	Negative
13	29	Classic	16	Negative
14	39	Bland	16	Negative
15	29	Classic	16	Negative
16	28	Bland	16	Negative
17	45	Bland	16	Negative
18	39	Classic	16	Negative
19	37	Classic	16	Negative
20	24	Classic	16	Negative
21	31	Classic	16	Negative
22	40	Bland	16	Negative
23	38	Classic	16	Negative
24	26	Classic	16	HHV6
25	20	Bland	58	Negative
26	19	Classic	56	Negative
27	39	Classic	56	Negative
28	41	Classic	26	Negative
29	22		31	Negative

BCD, bizarre cell dysplasia; HHV6, human herpesvirus 6; HPV, human papillomavirus; non-HPV viruses (polyomavirus BK, polyomavirus JC, herpesviruses 1-6).

Table 3 Cytologic diagnosis directly preceding BCD cervical cone biopsy

Pap smear result	n
NA	8
NILM	1
ASCUS	1
LSIL	4
ASC-H	2
HSIL	13

ASC-H, atypical squamous cells cannot exclude HSIL; ASCUS, atypical squamous cells of undetermined significance; BCD, bizarre cell dysplasia; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NA, not available; NILM, negative for intraepithelial lesion or malignancy.

identification of bizarre cells inside the lesion provides the clue to the correct diagnosis of HSIL. The issue of "bland-type dysplasia" has been discussed by Park et al.[4] and Kitahara et al.[3] They commented on "low-grade squamous intraepithelial lesions of the cervix with marked cytological atypia", and "deceiving dysplasia", respectively. On IHC BCD shows whole thickness p16 positivity when associated with the classic type of dysplasia, and one-half to two-thirds thickness positivity when associated with bland-type dysplasia. We believe that, in accordance with the LAST recomendation,[5] the aforementioned intensity and continuity of the staining – so called "block-type positivity"– allows us to consider this epithelium as dysplastic cervical squamous cells associated with high-risk HPV. Finally, molecular genetic studies showed that the high-risk HPV spectrum in BCD does differ from ordinary HSIL. HPV type 16 in BCD is more frequent. Moreover, a few cases were associated with possible high-risk HPV types (namely types 26, 67). Those cases could be missed in the setting of molecular gynecologic screening by all approved HPV tests. The eye-catching phenomenon of bizarre nuclei and multinucleation of dysplastic squamous cells cannot be attributed to non-HPV DNA viruses (i.e. HSV1, HSV2, VZV, EBV, CMV, HHV6, human polyomaviruses BK and JC).

In conclusion, BCD represents a so far unrecognized and potentially clinically significant subgroup of cervical intraepithelial lesions. Its proper identification would further improve the diagnostic accuracy of cervical punch biopsy. It would also facilitate longitudinal studies to further determine the biological properties of

BCD, namely the risk of progression compared with conventional HSIL lesions. Based on the unique histological and cytological features of BCD together with its strong association with HPV 16 infection, we believe that BCD represents a specific variant of HSIL.

Acknowledgments

We are indebted to Dr Leopold Rotter, Brno, Czech Republic for providing additional clinical information on the patient with HHV 6 coinfection. We also acknowledge the patients and the microphotography expertise of Ms Nina Šefčovičová in the preparation of this manuscript.

Disclosure

The authors declare no conflicts of interest.

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3.7 Význam bizarných buniek v skríningovej LBC cytológii: prospektívna štúdia 15 prípadov

(Significance of bizarre cells in cervical screening liquid-based cytology: A prospective study of 15 cases)

V LBC (liquid based cytology) preparátoch skríningovej gynekologickej cytológie sú niekedy prítomné objemné mnohojadrové bunky dlaždicového pôvodu, veľkosťou zodpovedajúce superficiálnej vrstve dlaždicového epitelu krčka maternice, so zväčšenými jadrami prezentujúce bizarné tvary jadier a napodobujúce atramentové machule. Podľa 3. edície Bethesda klasifikácie z roku 2014 tieto bunky patria do kategórie LSIL (ľahká dysplázia dlaždicového epitelu). Táto práca popisuje prospektívny zber prípadov, ktoré na úrovni LBC obsahujú bizarné bunky, s následou histologickou, alebo cytologickou koreláciou. Na základe revízie materálov sme sa pokúsili určiť význam cytologického nálezu bizarných buniek pre praktickú cytologickú diagnostiku a pre manažment pacientiek s týmto nálezom. V rámci gynekologického skríningu s využitím LBC a systému ThinPrep s asistovaným počítačovým vyhodnocovaním obrazu sme identifikovali 15 prípadov s prítomnosťou bizarných buniek. Súčasne sem vykonali detekciu mRNA alebo DNA HPV so zbytkového LBC materiálu metódou Aptima (Hologic), alebo Digene Hybrid Capture (Qiagen, Hildenberg, Germany). U všetkých 15 pacientiek sme potvrdili prítomnosť vysoko-rizikových typov HPV. V 10 z 15 prípadov bol nález hodnotený ako ASC-H so sekundárnou diagnózou LSIL. V 5 z 15 prípadov bol cytologický záver HSIL. U všetkých pacientiek sme doporučili histologické overenie cytologického nálezu. Následná histologická verifikácia v podobe punch biopsie, konizácie, alebo hysterektómie bola vykonaná u 13 prípadov v rozpätí 1 až 11 mesiacov od cytologického odberu. 10 krát sa diagnóza HSIL potvrdila a prítomnosť bizarných buniek bola zistená v 5 prípadoch. U ďalších 3 pacientiek sa jednalo o LSIL. Cytologické sledovanie gynekológ zvolil u 2 pacientiek. Záver: Identifikácia bizarných buniek v skríningovej LBC je relatívne jednoduchá a rýchla. Prispieva k presnosti a rýchlosti gynekologického cytologického skríningového vyšetrenia. Podľa našich skúseností cytologický nález bizarných buniek koreluje s histologickým nálezom HSIL v 77 % prípadov. Preto navrhujeme aby sa takého cytologické nálezy hodnotili ako ASC-H so sekundárnou diagnózou LSIL, na rozdiel od aktuálneho doporučenia hodnotiť takéto nálezy ako LSIL. BCD ako špecifický podtyp HSIL môže za istých okolností slúžiť ako vysvetlenie diskordantných prípadov cytologického nálezu LSIL a následnej histologickej diagnózy HSIL.

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Accepted: 21 September 2017

DOI: 10.1111/cyt.12494

ORIGINAL ARTICLE

WILEY

Significance of bizarre cells in cervical screening liquid-based cytology: A prospective study of 15 cases

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Objective: The aim of this study was to assess the significance of bizarre cells (cells of squamous origin with a superficial squamous cell-type cytoplasm and characterised by multinucleation that produces bizarre nuclear shapes) in liquid–based cytology (LBC) Papanicoaou (pap) smears with clinical and histological follow-up correlation.

Methods: Fifteen patients, all with LBC samples containing bizarre cells, were identified in routine ThinPrepâ LBC workload. HPV testing was performed in each case using residual LBC material. Cytological-histological correlations were reviewed.

Results: All 15 LBC samples contained bizarre cells and tested positive for high-risk HPV types. Ten of the 15 cases were identified as atypical squamous cells – cannot exclude an HSIL (ASC–H) with secondary diagnosis of low-grade squamous intraepithelial lesion (LSIL), while five cases were identified as high-grade squamous intraepithelial lesion (HSIL), and a subsequent biopsy was recommended. Additionally, 13⁄15 cases underwent cone biopsy or hysterectomy within 1-11 months, of which 10 showed histologically confirmed HSIL end-points. LSIL was present in three cases. Bizarre cells were identified in the HSIL epithelium of five cone biopsies.

Conclusions: Identification of bizarre cells in LBC is straightforward and may facilitate diagnosis. The cytology of bizarre cells is associated with HSIL in cone biopsies. We recommend assigning LBC samples containing bizarre cells as ASC-H with secondary diagnosis of LSIL.

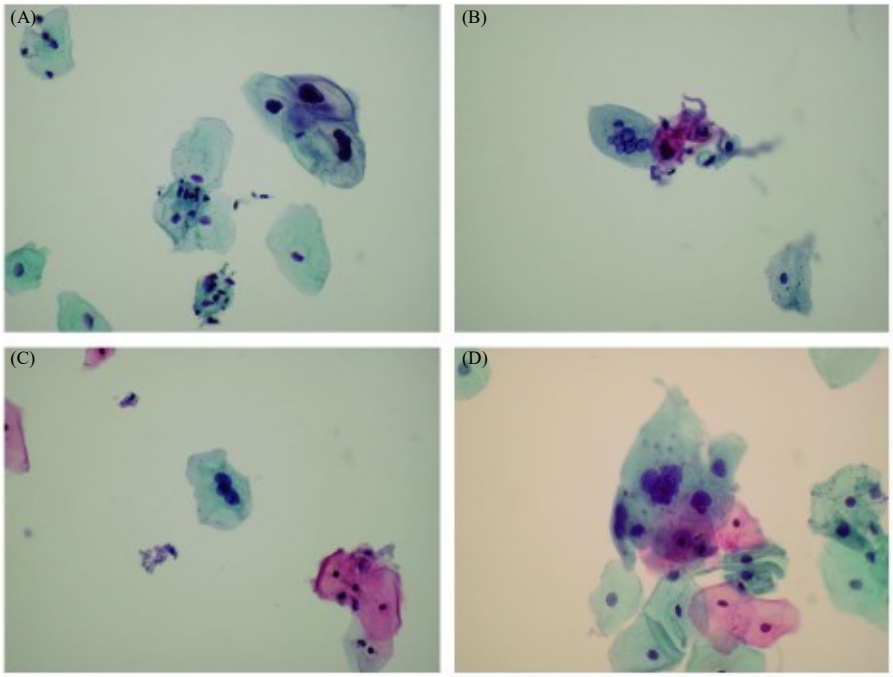
KEYWORDS

ASC-H, cervix, high-grade squamous intraepithelial lesion, human papillomavirus , liquid-based cytology, multinucleation

1 | INTRODUCTION

LBC Papanicolaou (pap) smears may sometimes contain noticeable cells of squamous origin with superficial squamous cell-type cytoplasm. These cells are characterised by significantly enlarged nuclei (more than three times the area of normal intermediate nuclei) showing binucleation or multinucleation and possibly presenting distorted/bizarre nuclear shapes, sometimes resembling an ink spot (Figure 1). These cells are categorised as low-grade squamous intraepithelial lesions (LSILs) according to the 2014 Bethesda classification. In general, the cytological appearance of bizarre cells is similar to that of the dominant LSIL population (Figure 2), with occasional scattered enlarged and mildly atypical metaplastic cells of parabasal cell-type, which are classified as atypical squamous cells – undetermined significance (ASCUS), although ASC–H or even HSIL categories cannot be excluded. We have recently described bizarre cell dysplasia (BCD) in the cervix[1] as an unrecognised variant of HSIL that is strongly associated with high-risk HPV type 16.

In this study, we have taken a step further to prospectively identify LBC pap smear cases containing bizarre cells as described earlier

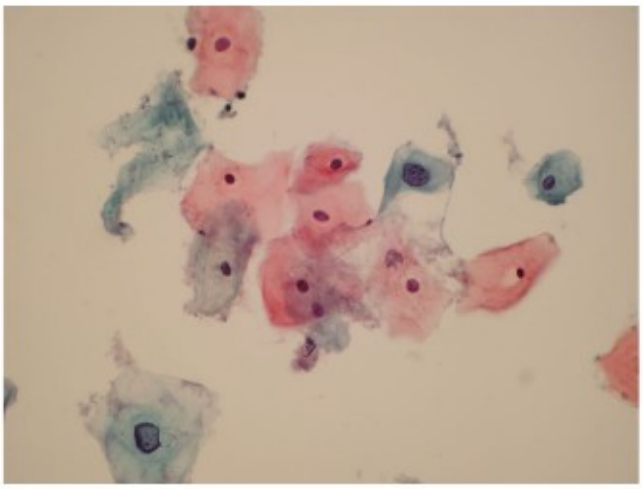


F I G U R E 1 Multinucleated cells of squamous origin and superficial level size – designated bizarre cells – were present in all 15 liquid-based cytology samples. Papanicolaou: (A) 4009, (B) 2009, (C) 2009, (D) 4009

and followed them up in order to assess the significance of such findings in LBC samples.

2 | MATERIALS AND METHODS

Using ThinPrep® Imager-assisted screening, we identified 15 patients with LBC pap smear slides showing bizarre cells from May 2014 to February 2015 in the routine workload of one of the authors (O.O.). Each case was identified as HSIL or ASC-H and LSIL with a note that biopsy verification of the lesion should be considered. Hybrid Capture (HC2) or an Aptima HPV test was performed in each case using the residual LBC material. All of the original LBC slides were reviewed by a panel of three experienced cytopathologists, and follow-up biopsies were reviewed by a panel of four gynaecopathologists. Follow-up cytology was only available for two patients. Punch biopsy, cone biopsy or hysterectomy was available for 13/15 patients.



F I G U R E 2 Typical low-grade squamous intraepithelial lesion cells in liquid-based cytology sample (Papanicolaou, 4009)

2.1 | Molecular genetic studies

2.1.1 | Hybrid capture

ThinPrep® (Hologic, Marlborough, MA, USA) LBC specimens were processed for Digene HC2 High-Risk HPV DNA testing (Qiagen, Hilden, Germany). Cellular pellets from 4 mL of LBC media and 400 lL of conversion buffer (Qiagen) were dissolved in 300 lL of STM (Qiagen) medium. Then, a standard hybrid capture was run on a semiautomatic Rapid Capture System-1 (Qiagen). Briefly, denatured samples were hybridised with High-Risk HPV RNA probes targeting types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68; subsequently formed DNA–RNA hybrids were captured by the primary antibody on the microplate. After incubation with a secondary antibody labelled with alkaline phosphatase and stringent washing, the specific signal was measured as chemiluminescence after reaction with substrate and read as relative light units on the luminometer (QIAGEN).

2.1.2 | Aptima

One millilitre of LBC medium was sampled for mRNA analysis using a liquid pap transport collection device kit (Hologic). The presence of mRNA of oncogenes E6/E7 of 14 HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) was analysed on an automatic

Panther device from Aptima HPV Array (Hologic). In brief, the molecular principle of an Aptima Array consists of three main steps, which occur in a single tube: target capture, target amplification by transcription-mediated amplification and detection of the amplification products by the hybridisation protection assay. The assay incorporates an internal control to monitor nucleic acid capture, amplification and detection, as well as operator or instrument error.

3 | RESULTS

During the study period, 1284 abnormal LBC pap smears were reviewed, of which 15 cases containing bizarre cells were identified. In five cases, the coexistence of bizarre cells and HSIL cells was noted. In the remaining 10 cases, no equivocal HSIL cytology was found. Only immature metaplastic cells were present, with uncertain levels of nuclear atypia and a marginal increase in the nucleo–cytoplasmic ratio. Identification of bizarre cells led to the straightforward ASC-H diagnosis and, in fact, determined the management of the patients. Of the 10 cases with no equivocal HSIL cytology, HSIL was diagnosed in five cone biopsies, and LSIL was diagnosed in three cases. Two patients were followed-up by LBC only with LSIL diagnosis. Altogether, 13 of 15 patients underwent cone biopsy or hysterectomy, with LBC follow-up without biopsy being implemented in two patients. The interval between the original LBC sampling and follow-up procedure was 1-11 months (mean 3 months). Cone biopsy was performed in 12 patients, which was preceded by punch biopsy in three patients. In one patient, hysterectomy and bilateral adnexectomy were performed. Of 13 cases with available follow-up histological examinations, 10 were positive for HSIL and three were LSIL positive (Table 1). Five of 13 cases with available follow-up histological examination showed bizarre cells within HSIL dysplastic epithelium (Figure 3). In two patients with cytology follow-up (no histological examination available), both were LSIL. Fourteen of 15 cases were positive for high-risk HPV genotypes; data were not available in one case.

4 | DISCUSSION

BCD in the cervix is an under-recognised cytological feature, which has just recently been concisely described by our group.[1] However, Park et al[2] were among the first to describe so-called LSIL with marked cytological atypia, which appears to be similar to BCD. In 2013, Washiya et al from Japan[3] assessed the significance of binucleated cells with compression and multinucleated squamous epithelial cells in Thinprep LBC samples. Despite taking a slightly different approach from that of Bethesda 2001[4] and before the publication of the LAST study[5] and the WHO 2014 classification of cervical dysplasia,[6] the Japanese group studying ASCUS cases found a strong association between the presence of binucleated and multinucleated squamous epithelial cells in ThinPrep[8] LBC specimens and (1) high-risk HPV positivity and (2) follow-up histological diagnosis of CIN 1-2.

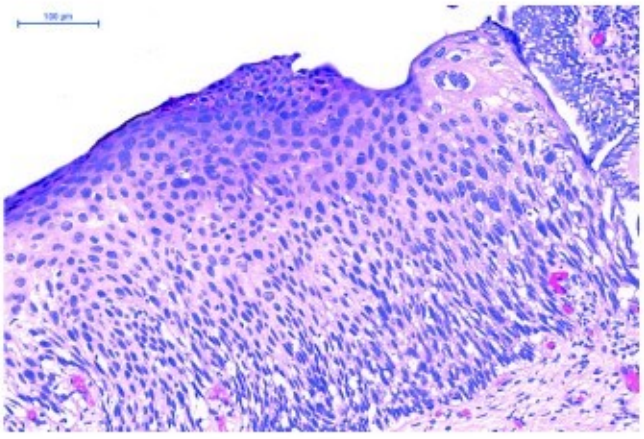
T A B L E 1 Correlation of the results of LBC, HPV testing and histology in 15 patients

Case no.	Original LBC	HPV testing	Follow-up	Diagnosis	Bizarre cells
1	HSIL	HC2+	Cone biopsy	HSIL	
2	HSIL	HC2+	Cone biopsy	HSIL	
3	HSIL	mRNA +	Cone biopsy	HSIL	
4	ASC-H	mRNA +	Cone biopsy	LSIL	
5	ASC-H	mRNA +	LBC	LSIL	
6	ASC-H	mRNA +	Cone biopsy	HSIL	+
7	HSIL	HC2+	Cone biopsy	HSIL	+
8	ASC-H	HC2+	LBC	LSIL	
9	ASC-H, LSIL	HC2+	HYE+AE	LSIL	
10	ASC-H, LSIL	HC2+, 16, 18, 45	Cone biopsy	LSIL	
11	LSIL	HC2+, 16, 18, 45	Cone biopsy	HSIL	
12	HSIL	mRNA +	Cone biopsy	HSIL	
13	ASC-H	mRNA +	Cone biopsy	HSIL	+
14	ASC-H, LSIL	mRNA+, 16, 18, 45	Cone biopsy	HSIL	+
15	ASC-H, LSIL	NA	Cone biopsy	HSIL	+

LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells – cannot exclude HSIL; HC2, Digene HC2 high-risk HPV DNA testing; mRNA, Aptima HPV array; LBC, liquid-based cytology; NA, not available; HYE +AE, simple hysterectomy and bilateral adnexectomy.

Most recently, Masand et al[7] have described low-grade-like HSIL (LLHSIL) histological lesions and their correlation with high-risk HPV genotypes. The authors concluded that LLHSIL morphologically mimics LSIL and that p16 immunostaining may be required for accurate diagnosis. An interesting finding in their study was that they reported LLHSIL to be commonly associated with non-16/18 HPV genotypes.

In this study, we assessed the significance of bizarre cells in LBC pap samples and found consistent findings. Bizarre cells are defined as



F I G U R E 3 Cone biopsy in five cases contained high-grade squamous intraepithelial lesion with bizarre cells as shown in the upper right part of the photo (haematoxylin-eosin, 2009)

cells of squamous cell origin with superficial squamous cell-type cytoplasm. They present bizarre nuclear shapes due to overlap of two or more enlarged nuclei (more than three times the area of normal intermediate nuclei), with irregular nuclear membrane and hyperchromasia. They sometimes present finely granular chromatin but rarely hypochromasia and no or inconspicuous nucleolus. Such cells fall into the LSIL category as defined by the 2014 Bethesda classification. We are fully aware of the fact that bizarre cells of the ectocervix, as described above, may defy straightforward cytological classification in terms of the 2014 Bethesda classification. However, this classification may assist in explaining potential cytological-histological discordant cases. This specific morphological cell type is not fully discussed in the third edition of the Bethesda classification. Nonetheless, a single picture resembling this type of cells is included (Figure 5.3 on page 139)[9] and properly described but ultimately designated LSIL. In routine LBC practice, the dominant feature of LSIL is easily and accurately identified, and then a search for individual parabasal cells with possible atypia follows. We believe that in some instances, it may well be possible that large individual bizarre cells are either overlooked or underreported. We believe that so-called bizarre cells are easy to recognise, and their identification strongly correlates with HSIL in follow-up biopsy (77% of cases in our study). Our laboratory gynaecological cytology service covers the entire country. Internal quality control data for 2016 contain nearly 800 000 gynaecological screening cases, which constitutes approximately 33% of the annual national volume. There were 3756 LBC LSIL cases, of which 376 included histological follow-up data. End-point HSIL diagnosis was rendered in 48% of cases. At the same time, there were 598 LBC ASC-H cases, of which 133 included histological follow-up data. End-point HSIL diagnosis was rendered in 71% of cases. Similarly, in the current study, the finding of bizarre cells was associated with histological end-point HSIL diagnosis in 77% (10/13) of cases. Moreover, our laboratory frequency of cytology cases with follow-up histological data increased from 10% for LSIL to 22% for ASC-H and 32% for HSIL. This shows a twofold increase in the chance for histological verification of the lesion between LSIL and ASC-H. Therefore, we suggest that LBC samples showing bizarre cells may be identified as ASC-H with secondary diagnosis of LSIL. We also suggest a comment on the nature of the slide in which LSIL cells dominate and are accompanied by scattered individual bizarre cells and that such cytological features may be associated with HSIL in follow-up biopsies. In our study, we showed that the cytological finding of bizarre cells in LBC specimens is histologically associated with a specific subtype of high-grade dysplasia, which comprises bizarre cells (found in 50% of studied HSIL cases). Diagnosing LBC samples containing bizarre cells as ASC-H leads to proper management of the patient, including colposcopic examination with possible cone biopsy. However, identifying such cases as LSIL would lead to some delay, as LSIL management consists of repeated pap smears according to most of the European and Northern American guidelines.[10–12] The current third edition of the Bethesda system places bizarre cells, as we described them above, implicitly into the LSIL category.[9] There are some authors who are in favour of use of the LSIL-H category[13–16]; however, such suggestions have not reached a consensus yet.[17] We suppose it is conceivable that, from a practical point of view, the term LSIL-H could be used with a certain degree of convenience in cases of BCD when no HSIL cells are found in a smear. It should be noted that the third edition of the Bethesda system allows such types of LBC specimen to be properly designated ASC-H with secondary diagnosis of LSIL, which may lead to a subsequent cone biopsy diagnosis of HSIL in most cases. Further work on a larger sample is being undertaken by our group to assess whether the findings of the current study can be reproduced on a larger scale with the aim of improving patient management and facilitating the diagnostic process in LBC samples containing bizarre cells.

5 | CONCLUSIONS

The presence of bizarre cells in LBC specimens is associated with squamous dysplasia in cone biopsies, particularly with HSIL in 77% of the cases. Further, the presence of bizarre cells in LBC specimens is associated with bizarre cell dysplasia in 50% of cone biopsies harbouring HSIL. We believe that it may be more appropriate to diagnose LBC samples containing bizarre cells as ASC-H with a secondary diagnosis of LSIL in routine practice as opposed to only LSIL. BCD, as a specific subtype of HSIL, could potentially serve, in a proper setting, as an explanation for discordant cases of cytological LSIL and histological HSIL end-point in cone biopsies.

ACKNOWLEDGMENTS

We are grateful to our colleague pathologists and clinicians who kindly supported this study by providing follow-up data and slides of their patients. Namely, we appreciate the assistance of Dr Bilina (Praha), Dr Bolen (Kraslice), Dr Bosakova (Ceske Budejovice), Dr Brozova (Jihlava), Dr Bresky (Jilemnice), Dr Gregor (Jicin), Dr Hawawrehova (Sokolov), Dr Klicman (Bor), Dr Langmajerova (Plzen), Dr Laznickova (Brno), Dr Majerova (Rokycany), Dr Mejchar (Plzen), and Dr Svec (Plzen).

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How to cite this article: Ondic O, Ferko R, Kicinova J, et al. Significance of bizarre cells in cervical screening liquid-based cytology: A prospective study of 15 cases. Cytopathology. 2017;00:1–5. https://doi.org/10.1111/cyt.12494

4 Záver

Skúsenosti z práce na jednotlivých projektoch ukazujú, že v dnešnej dobe je nevyhnutná úzka spolupráca patológa a molekulového genetika. Šírka ponuky metód molekulárnej genetiky vedie k subšpecializácii i v rámci tohoto oboru a možnosti ktoré sa patológom vďaka metódam molekulovej genetiky otvárajú sú nesmierne široké. To, či ich patológ dokáže efektívne využiť a výsledkom budú informácie s praktickým významom pre diagnostiku a liečbu pacientov, podľa môjho názoru v prvom rade závisí na uvedomení si hraníc oboru patologická anatómia a na ochote vstúpiť s rešpektom do dlhodobého dialógu s erudovanými genetikmi. V tejto atmosfére možno očakávať, že vzájomná diskusia prinesie identifikáciu správnej metódy a správnych pacientov a tkanív, ktoré touto metódou možno efektívne vyšetrovať. V rámci oboru patologická anatómia následne dochádza k výraznému prehlbovaniu a k získavaniu nových poznakov.

Výrazne stúpa význam a cena tkaniva, ktoré patológ spracováva. Enormne sa zvyšujú nároky na vzdelanie patológa. Objavuje sa výrazná tendencia požiadavky na zvyšovanie komunikačných schpností, pretože do ustáleného komunikačného okruhu, ktorý zahŕňa histologické a imunohistochemické laboratórium, klinika a pacienta vstupuje ďalší významný partner - molekulový genetik.

Vzhľadom na cenu metód molekulárnej genetiky stále existuje veľký priestor pre imunohistochémiu a hľadanie korelačných vzťahov medzi expresiou antigénu a prítomnosťou mutácie konkrétneho génu alebo inej hrubej chromozómovej aberácie. Takéto nálezy a koreláty môžu mať obrovský finančný efekt. Použitie imunohistochémie v špecifickej reflexnej aplikácii niekedy umožňuje selektovať vzorky, ktoré budú následne potvrdzované metódami molekulovej genetiky s výraznou úsporou finančných prostriedkov. Máme tak možnosť vo väčšom merítku poskytovať pacientom a ich ošetrujúcim lekárom diagnózy s presnejšími charakteristikami nádorov a prispievať k výberu kandidátov vhodných pre cielenú liečbu.

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6.4 Prezentácie na vedeckých konferenciách

102th Annual Meeting of the United States & Canadian Academy of Pathology, March 2 - 8, 2013, Baltimore, MD, USA
Poster: Ondrej O., Kašpírková J. Cervical Conisation for High Grade Dysplasia (CIN III) in 38 HPV Vaccinated Women.
104th Annual Meeting of the United States & Canadian Academy of Pathology,March 21-27,2015, Hynes Convention Centre, Boston, MA, USA
Poster: Kašpírková J., Ondič O., Michal M. Bizarre Cell dysplasia of the Cervix.
105th Annual Meeting of the United States & Canadian Academy of Pathology's , March 12-18, 2016 in Seattle, WA.
Poster: Kašpírková J, Gomolčáková B, Ondič O., Michal M. The Assessment of Potential Biomarkers of Disease Progression in High Grade Squamous Cervical Lesions. Promoter Methylation Analysis of Selected Tumour-Suppressor Genes and Viral/Microbial Co-Infection".
EAU 16th Central European Meeting, 7.-8.10.2016, Vienna, Austria
Poster: Pitra T, Pivovarcikova K, Vanecek T, Alaghehbandan R, Gomolcakova B, Ondic O,Peckova K, Rotterova P, Hora M, Michal M, Hes O. Comparative study of TERT gene mutation analysis on voided liquid-based urine cytology and paraffin-embedded tumorous tissue.
106th Annual Meeting of the United States & Canadian Academy of Pathology, 4.-10.3.2017 Henry B. Gonzalez Convention Center, San Antonio, TX, USA
Poster: Alaghehbandan R, Stehlik J, Trpkov K, Magi-Galluzzi C, Foix MP, Berney D, Sibony M, Suster S, Agaimy A, Montiel DP, Pivovarcikova K, Michalova K, Daum O, Ondic O, Rotterova P, Michal M, Hes O. Programmed Death-1 (PD-1) Receptor/PD-1 Ligand (PD-L1) Expression in Fumarate Hydratase-Deficient Renal Cell Carcinoma.
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Poster: Isikci OT, He H, Grossmann P, Alaghehbandan R, Ulamec M, Petersson F, Perez Montiel D, Michalova K, Pivovarcikova K, Ondic O, Saskova B, Rotterova P, Hora M, Michal M, Hes O. Low-Grade Spindle

Cell Proliferation in Clear Cell Renal Cell Carcinoma Is Unlikely an Initial Step in Sarcomatoid Differentiation

Poster:Jana Kaspirkova, Jana Cimická, Barbora Gomolčáková, Ondrej Ondič, Michal Michal. mRNA-Based HPV Test Provides with More Accurate Genotyping Results for HPV16/18/45 Than DNA-Based HPV Test in Cervical Cancer Screening.

107th Annual Meeting of the United States & Canadian Academy of Pathology, March 17-23, 2018, Vancouver Convention Centre, Vancouver, BC, Canada

Poster: Ondič O., Němcová J., Gomolčákova B., Švajdler M., Májek O., Alaghehbandan R., Michal M. The Role of Methylation Silencing of Tumor Suppressor Genes in Cervical HSIL: a Cytologic-Histologic Correlation study.

Poster: Ondič O., Němcová J., Černá K., Alaghehbandan R.. A Possible New Way of Dividing Cervical HSIL Based on the Presence of Diffuse-Type Signal in Dysplastic Epithelium Using HPV DNA or mRNA In Situ Hybridisation Study.