Univerzita Karlova v Praze
1. lékařská fakulta

Habilitační práce

Význam moderních diagnostických metod v gynekopatologii

MUDr. Kristýna Němejcová, Ph.D.

Praha, 2018

Název habilitační práce: Význam moderních diagnostických metod v gynekopatologii

Autor: MUDr. Kristýna Němejcová, Ph.D.

Forma habilitační práce: Soubor vědeckých prací doplněný komentářem

Podpora habilitační práce: Habilitační práce byla vypracována zčásti s podporou grantového projektu AZV 17-28404A a RVO VFN 64165.

Pracoviště: Ústav patologie
1. lékařská fakulta Univerzity Karlovy a Všeobecná fakultní nemocnice v Praze
Studničkova 2
Praha 2
128 00

Seznam použitých zkratek

APA	atypický polypózní adenomyom
CGH	komparativní genomová hybridizace
DNA	deoxyribonukleová kyselina
EBV	virus Ebstein-Barrové
FISH	fluorescenční in situ hybridizace
H&E	hematoxilin eosin
HNF-1β	hepatocytární transkripční faktor 1 beta
HNF1B	gen kódující hepatocytární transkripční faktor 1 beta
ITC	izolované nádorové buňky
LELC	lymfoepitelioma-like karcinom
MPS	masivní paralelní sekvenování
NGS	sekvenování nové generace
PARP	polymeráza polyADP-ribóza
PCR	polymerázová řetězová reakce
SLN	sentinelová uzlina

Seznam publikací, které jsou podkladem habilitační práce

1. Němejcová K, Cibula D, Dundr P. **Expression of HNF-1β in cervical carcinomas: an immunohistochemical study of 155 cases.** Diagn Pathol. 2015;10:8. (IF 2,60)
2. Němejcová K, Rosmusová J, Bártů M, Důra M, Tichá I, Dundr P. **Expression of Glut-1 in Normal Endometrium and Endometrial Lesions.** Int J Surg Pathol. 2017 Aug;25(5):389-396. (IF 0,782)
3. Cibula D, Zikan M, Slama J, Fischerova D, Kocian R, Germanova A, Burgetova A, Dusek L, Dundr P, Gregova M, Nemejcova K.: **Risk of micrometastases in non-sentinel pelvic lymph nodes in cervical cancer.** Gynecol Oncol. 2016 Oct;143(1):83-6. (IF 4.959)
4. Dundr P, Němejcová K, Laco J, Skálová H, Bauerová L, Matěj R, Fischerová D. **Anastomosing Hemangioma of the Ovary: A Clinicopathological Study of Six Cases with Stromal Luteinization.** Pathol Oncol Res. 2017 Oct;23(4):717-722. (IF 1.736)
5. Němejcová K, Kenny SL, Laco J, Škapa P, Staněk L, Zikán M, Kleiblová P, McCluggage WG, Dundr P. **Atypical Polypoid Adenomyoma of the Uterus: An Immunohistochemical and Molecular Study of 21 Cases.** Am J Surg Pathol. 2015; 38:1148-55. (IF 5,145)
6. Němejcová K, Tichá I, Kleiblová P, Bártů M, Cibula D, Dundr P. **Expression, epigenetic and genetic changes of HNF1B in endometrial lesions.** Pathol Oncol Res. 2016 Jul;22(3):523-30. (IF 1,855)
7. Němejcová K, Dundr P, Krajsová I. **Clear cell sarcoma of vulva. A case report.** Cesk Patol. 2016; 52: 215–217.
8. Němejcová K, Hájová N, Tichá I, Bártů M, Dolinská D, Kalist V, Dundr P. **Lymphoepithelioma-like carcinoma of the endometrium: Case report of rare tumour with comprehensive immunohistochemical and molecular analysis.** Přijato k publikaci do Pol J Pathol. (viz. přílohy) (IF 0.990)

Poděkování

Ráda bych poděkovala prof. MUDr. Pavlu Dundrovi, Ph.D., přednostovi Ústavu patologie 1. LF UK a VFN, za pomoc a podporu při vědecké práci. RNDr. Ivaně Tiché, Ph.D. z laboratoře molekulární patologie za odbornou pomoc a spolupráci při řešení molekulární části projektu.

OBSAH

1. ÚVOD

Při bioptickém vyšetření v gynekopatologii, stejně jako v jiných oblastech patologie, vycházíme při mikroskopickém vyšetření ze standarního základního barvení hematoxylin-eosinem (H&E). V některých případech je však přesné určení diagnózy založené pouze na základě morfologických znaků obtížné a problematické a v těchto případech můžeme s výhodou využít moderních diagnostických metod, mezi něž patří zejména imunohistochemická, molekulárně biologická a cytogenetická vyšetření. Význam těchto metod v posledních letech stále stoupá a díky rozvoji imunohistochemických a molekulárně biologických analýz dochází v oblasti gynekopatologie k lepšímu porozumění různým aspektům nenádorových i nádorových onemocnění, a tím v praktické úrovni ke zlepšení nejen diagnostiky, ale i k rozvoji prediktivní patologie umožňující predikování odpovědi nádoru na konkrétní léčbu a tím personalizaci léčebné strategie.

Moderní diagnostické metody nám mohou pomoci rozlišit nejen jednotlivé histologické typy nádorů či lézí, což je důležité zejména s ohledem na terapii i prognózu, ale mohou nám pomoci stanovit biologickou povahu lézí nebo nalézt genetické alterace a epigenetické změny, které by mohly být využitelné při cílené terapii nádorů. V některých případech vedly nové poznatky k většímu porozumění podstaty nádorů či lézí, s následnou změnou klasifikace nebo alespoň otevřely prostor pro diskuzi a další zkoumání, které by následně ke změnám či zpřesnění klasifikace mohly vést. Příkladem jsou serózní karcinomy ovária, kde klinickopatologické a molekulárně biologické analýzy ukázaly, že low grade a high grade serózní karcinomy nepředstavují mofologické spektrum téhož nádoru, který progreduje od lépe diferencovaných forem k hůře diferencovaným, ale že jde o různé entity s odlišným vývojem, které jsou charakterizované jiným typem mutací a z klinického pohledu odlišnou prognózou a reakcí na léčbu. Kromě toho vedlo podrobné vyšetřování vzorků z adnexektomií pacientek s BRCA mutacemi k novému pohledu na primární místo vzniku high grade serózních karcinomů, což

bylo zohledněno ve FIGO i TMN klasifikaci nádorů ovária, děložní tuby a primárních peritoneálních karcinomů a následně i v doporučených postupech pro zpracování bioptického materiálu.

Přesná diagnostika a klasifikace nádorů a lézí má spolu s hodnocením molekulárních a cytogenetických změn zásadní význam při optimalizaci léčebné strategie a individualizace léčby. V posledních letech se pozornost stále více zaměřuje nejen na identifikaci a využití markerů prognostických, ale i na markery prediktivní, což úzce souvisí s rozvojem nových terapeutických možností zejména v oblasti cílené terapie.

To všechno s sebou přináší i neustále se zvyšující nároky na pracoviště patologie dané stále se rozšiřujícícm spektrem dostupných speciálních metod, ať už jde o metody imunohistochemické, cytogenetické, či molekulární. Nejde jen o materiálové náklady, ale i o potřebné přístrojové vybavení a kvalifikovaný personál.

Díky rozvoji moderních diagnostických metod je větší pozornost věnována i bioptickému materiálu, jehož kvalita ovlivňuje výsledek vyšetření. Vzorek odebraný k bioptickému vyšetření by měl být nejen reprezentativní, nezhmožděný a v případě resekátů dobře zorientovatelný, ale v případě, že jsou indikovaná následná imunohistochemická a hlavně molekulárně biologická a cytogenetická vyšetření, hraje zásadní roli tzv. preanalytická fáze odběru, která se týká zejména typu a doby fixace tkáně. Standardně používaným fixativem je pufrovaný roztok 10% formolu (odpovídající 4% formaldehydu), který má neutrální pH.

V některých odběrech pak můžeme narážet na problémy dané malým množstvím diagnostického materiálu, které je pro vyšetření k dispozici. Jde zejména o materiál získaný punkčními (tru-cut) odběry nebo kyretáží, ale se stejným problémem se můžeme setkat i ve větších odběrech či resekátech, pokud je nádor tvořený převážně nekrotickou tkání nebo je nádor či léze zastižena jen okrajově. Zejména v těchto případech jsou kladeny vysoké nároky na vyšetřujícího patologa, který musí být dobře znát jednotlivé histologické typy nádorů a lézí,

včetně jejich možné morfologické ale i imunofenotypické variability. Následná vyšetření pak musí být indikována s rozmyslem, protože zde hrozí riziko postupné ztráty tkáně při zpracování, tzv. „vykrájení“ materiálu.

V tomto kontextu je nutné zmínit, že zcela zásadní roli hraje spolupráce s klinickými lékaři, důležitá je znalost anamnestických dat pacientek, případně dalších relevantních údajů, které by měly zahrnovat i informace o předchozí či probíhají terapii.

2. VYŠETŘENÍ A DIAGNOSTICKÉ METODY V GYNEKOPATOLOGII

Při vyšetření bioptických vzorků v gynekopatologii mohou na základní barvení v indikovaných případech navazovat metody histochemické, imunohistochemické, molekulárně biologické a cytogenetické.

Histochemická vyšetření umožňují prokázat ve vzorku pomocí chemické reakce přítomnost konkrétních látek, jako například glykogenu, železitých iontů (hemosiderin), hlenu, lipidů, melaninu, amyloidu, mikroorganismů a dalších. Jejich praktické využití v diagnostice je však v oblasti gynekopatologie spíše limitované.

Nezastupitelnou roli při gynekopatologickém vyšetření mají metody imunohistochemické. Uplatňují se zejména v diferenciální diagnostice obtížně zařaditelných lézí a nádorů, při klasifikaci nádorů a při určování biologické povahy. U některých typů nádorů je lze využít i při stanovení prognózy a v predikci odpovědi na terapii, například vyšetření hormonálních receptorů u endometroidního karcinomu děložního těla.

Imunohistochemické metody využívají k detekci jednotlivých tkáňových antigenů specifické protilátky, jak monoklonální, tak polyklonální. Monoklonální protilátky jsou produktem jednoho klonu B lymfocytů (hybridomu vzniklého fúzí vybraného klonu B lymfocytů s nádorovou „nesmrtelnou“ plazmatickou buňkou). Polyklonální protilátky jsou produktem mnoha aktivovaných klonů B lymfocytů. Při detekci antigenů jsou nejčastěji využívány tzv. nepřímé dvou a vícestupňové imunohistochemické metody, které umožní značně zesílit signál, a tím zvýšit citlivost reakce. Protože vazba antigenu s protilátkou ve tkáních probíhá bez viditelné reakce, následně se musí vizualizovat pomocí chromogenů, kdy je v případě pozitivní reakce vazba antigenu s protilátkou znázorněna barevným produktem, který lze prohlížet ve světelném mikroskopu.

Při hodnocení preparátů je nezbytné zároveň hodnotit i pozitivní a negativní vnitřní kontrolu, případně využít paralelně zpracovaný kontrolní vzorek, aby nedošlo k falešně pozitivnímu či negativnímu výsledku.

Konkrétní možnosti využití imunohistochemických protilátek v gynekopatologii jsou podrobněji popsány níže v textu, kde využití jednotlivých protilátek demonstrujeme na nádorech ovária (karcinomech i anastomozujícím hemangiomu) a na karcinomech a lézích děložního těla i hrdla [1-6].

Při hodnocení imunohistochemických vyšetření je třeba myslet i na to, že specifita protilátek není stoprocentní, nádory a léze mohou být imunofenotypicky proměnlivé a v řadě případů je proto lépe využívat protilátky v diagnostickém panelu. Jako příklad, kdy je výhodné použít protilátky v diagnostickém panelu, uvádíme algoritmus vyšetření navržený za účelem rozlišení málo diferencovaných dlaždicobuněčných karcinomů a adenokarcinomů děložního hrdla [3].

Kromě toho lze imunohistochemická vyšetření v gynekopatologii využít i k upřesnění nádorového stagingu, například při detekci nádorových metastáz a mikrometastáz v lymfatických uzlinách u karcinomů děložního hrdla, jak ukazujeme ve studii, kde jsme detekovali nádorové metastázy a mikrometastázy u nesentinelových uzlin pacientek, které mají vysoké riziko uzlinového postižení nádorovými metastázami [7].

V posledních letech stále více však nabývá na významu vyšetření bioptických vzorků molekulárně biologickými metodami, které umožňují analýzu nukleových kyselin a proteinů. Vyšetření začíná izolací nukleových kyselin, na níž pak mohou navazovat další metody. Mezi metody využívané v gynekopatologii patří například polymerázová řetězová reakce (PCR), metody využívající expresních DNA čipů, komparativní genomová hybridizace (CGH), metoda přímého Sangerova sekvenování, bisulfitové sekvenování nebo sekvenování nové generace

(NGS, nazývané také masivní paralelní sekvenování (MPS)), sloužící k přesnému určení pořadí bazí.

Molekulárně biologické metody se mohou uplatňovat v diferenciální diagnostice nádorových i nenádorových onemocnění, při určování prognózy, ale i při zjišťování známých, či odhalování nových molekulárních aberací a genových variací v různých lézích či nádorech ženského genitálu, které by mohly eventuálně hrát v budoucnu roli při cílené terapii. Jako příklad práce zaměřené na detekci mutací uvádíme naši studii zaměřenou na výzkum aberací v karcinomech děložního těla, ve které se nám podařilo nalézt několik dosud nepopsaných mutací genu *HNF1B* [5]. Kromě výše uvedeného lze tyto metody využít v prediktivní diagnostice, např. odpovědi na cílenou terapii inhibitory polymerázy polyADP ribózy (PARP inhibitory).

V rutinní diagnostice i k výzkumným účelům se v gynekopatologii využívají i cytogenetické metody, které nejčastěji vychází z klasické hybridizace nukleových kyselin, tj. *in situ* hybridizace (ISH). Tyto metody přispívají zejména ke zlepšení diferenciální diagnostiky problematicky zařaditelných nádorů a lézí. Na našem pracovišti nejvíce využíváme fluorescenční *in situ* hybridizaci (FISH), při které se vizualizují konkrétní geny nebo oblasti chromozomů pomocí fluorescenčně značených sond a výsledný produkt se poté prohlíží ve fluorescenčním mikroskopu. Jako příklad využití moderních cytogenetických metod uvádíme použití FISH při diagnostice světlobuněčného sarkomu vulvy, charakterizovaného translokací genu Ewingova sarkomu (EWS) [8].

Využití metod molekulárně biologických nebo cytogenetických analýz v kombinaci s metodami imunohistochemickými může v některých případech vést k upřesnění lézí a nádorů ženského genitálu, jak dokumentujeme například na skupině atypických polypózních adenomyomů nebo lymfoepitelioma-like karcinomu děložního těla [4, 6].

Význam moderních diagnostických metod v gynekopatologii je demonstrován na několika našich studiích:

2.1. Význam využití imunohistochemických metod v gynekopatologii

Možné využití imunohistochemický metod v diagnostice i při výzkumu gynekologických nádorů a lézí je demonstrován na níže podrobněji popsaných příkladech, které se věnují problematice karcinomů děložního hrdla, nádorů a lézí děložního těla a nádorech ovária.

2.1.1. Využití imunohistochických metod v diferenciální diagnostice nízce diferencovaných adenokarcinomů a dlaždicobuněčných karcinomů děložního hrdla

Rozlišení nízce diferencovaného adenokarcinomu a dlaždicobuněčného karcinomu hrdla založené pouze na histologických znacích je u některých nádorů komplikované a v některých případech dokonce nemožné. Nicméně z terapeutického hlediska je přesná specifikace histologického typu nádoru významná. Proto jsme se pomocí imunohistochemických metod pokusili nalézt vhodné protilátky a stanovit algoritmus vyšetření, který by mohl být nápomocný při diferenciální diagnostice těchto morfologicky nejednoznačných karcinomů hrdla.

Na skupině 155 karcinomů děložního hrdla, zahrnující výše zmíněné nádorové typy včetně karcinomů nízce diferencovaných, jsme porovnávali expresi vybraných protilátek: HNF-1β, D2-40, ER, PR, CEA, p63, p40 a p16. Při výběru jsme se soustředili jednak na protilátky, u kterých už byla exprese v karcinomech hrdla již popsána, ale také markery v této oblasti méně prozkoumané, jako je D2-40 a HNF-1β. Pozitivita protilátky D2-40 již byla dle některých autorů nalezena v podskupině dlaždicobuněčných karcinomů z různých lokalit, ale v karcinomech děložního hrdla do té doby nebyla podrobněji prozkoumána [9-11]. HNF-1β je obecně považovaný za marker světlobuněčných karcinomů, ale v několika recentnějších studiích byla jeho pozitivita nalezena i v jiných typech karcinomů včetně endometroidních, vzácněji i u mucinózních a serózních karcinomů ovária [12-14]. V oblasti děložního hrdla byla exprese HNF-

1β do té doby zmíněna jen ve dvou studiích, které však nebyly primárně cíleny na problematiku exprese tohoto proteinu a byly v nich hodnoceny jen některé vybrané typy adenokarcinomů [15, 16]. Naše předběžné výsledky z limitovaného souboru případů však naznačovaly, že HNF-1β je exprimovaný převážně v adenokarcinomech a nikoli v dlaždicobuněčných karcinomech hrdla. Předpokládali jsme tedy, že pokud by se nám podařilo prokázat odlišnou míru exprese HNF1-β v těchto nádorových typech na větším reprezentativním souboru případů, mohl by tento marker potencionálně využitelný při diferenciální diagnostice málo diferencovaných karcinomů děložního hrdla.

V souladu s našimi předběžnými výsledky a předpoklady se nám expresi HNF-1β podařilo prokázat ve většině (75 %) adenokarcinomů děložního hrdla, oproti tomu u dlaždicobuněčných karcinomů jsme ji zastihli pouze raritně (2 %). Co se týká ostatních markerů, D2-40 byl exprimován přibližně v polovině dlaždicobuněčných karcinomů (54 %), ale v žádném adenokarcinomu. Markery stratifikovaného epitelu p63 a p40 jsme ve stejném rozsahu nalezli ve všech dlaždicobuněčných karcinomech (100 %), ale jen v několika adenokarcinomech (4 %). Protilátku HNF-1β tedy lze považovat za pomocný marker adenokarcinomů děložního hrdla, zatímco p63, p40 a D2-40 za specifické markery dlaždicobuněčných karcinomů ovšem s variabilní senzitivitou. Ostatní protilátky se neukázaly pro diferenciální diagnostiku karcinomů děložního hrdla příliš přínosné.

Protilátky HNF-1β, p63, p40 a D2-40 mají jednotlivě variabilní míru senzitivity, proto se domníváme, že optimálních výsledků pro diferenciální diagnostiku málo diferencovaných karcinomů děložního hrdla lze nejlépe dosáhnout využítím jejich kombinace v diagnostickém panelu [3].

2.1.2. Využití imunohistochemických metod při analýze exprese HNF-1β karcinomech a nenádorových lézích endometria a v korporálním endometriu

V další studii jsme využili imunohistochemických metod k analýze exprese proteinu HNF-1β (hepatocytárního jaderného faktoru 1 beta) ve 320 vzorcích karcinomů, nenádorových endometriálních lézí a v kontrolní skupině 19 světlobuněčných karcinomů ovaria [5].

HNF-1β je transkripční faktor, který hraje klíčovou roli při ontogenezi, reguluje expresi četných genů podílejících se na modulaci buněčného cyklu a pravděpodobně se uplatňuje při karcinogenezi některých nádorů [17, 18]. Zvýšená exprese tohoto markeru byla nalezena ve většině světlobuněčných karcinomů ovaria, pankreatu, ledviny a v hepatocelulárním karcinomu [18-20]. Snížení exprese HNF-1β v buněčných liniích světlobuněčného karcinomu ovaria indukuje apoptózu a exprese tohoto transkripčního faktoru se zdá být v některých případech významnou pro přežívání nádorových buněk [21, 22]. V oblasti ženského genitálu je protein HNF-1β považován za vysoce specifický marker světlobuněčného karcinomu a využívá se v diferenciální diagnostice světlobuněčných karcinomů ovária i endometria [23, 24].

Recentní práce, zabývající se expresí HNF1-β v epitelových nádorech endometria a ovaria, však naznačují, že specifita tohoto markeru pro světlobuněčné karcinomy je zřejmě nižší, než se předpokládalo, protože jeho exprese byla popsána i v dalších typech karcinomů, včetně karcinomů serózních, endometroidních a mucinózních, ve většině typů borderline nádorů ovárií a i v některých nenádorových lézích ženského genitálu [12-14].

V naší práci jsme imunohistochemickými metodami analyzovali expresi HNF-1β v hlavně karcinomech, ale v i nenádorových lézích endometria.

Karcinomy endometria jsou většinou rozdělovány do jednotlivých histologických subtypů na základě morfologických znaků. V některých případech je však přesné určení histologického typu nádoru založené na morfologii obtížné, zejména složité může být odlišení světlobuněčných karcinomů endometria od jiných typů endometriálních karcinomů, které pouze vykazují světlobuněčnou přeměnu. Přesná diagnóza světlobuněčných karcinomů je však velmi

důležitá, protože jde z definice o high grade tumory s odlišnou odpovědí na léčbu i prognózou, než mají některé jiné častější typy karcinomu endometria.

Cílem naší práce bylo provést imunohistochemickou analýzu HNF-1β na karcinomech a lézích korporálního endometria a následně zhodnotit přínos tohoto markeru pro diferenciální diagnostiku i s ohledem na možný prognostický a prediktivní význam. Zároveň jsme se na podskupině endometroidních karcinomů děložního těla pokusili nalézt epigenetické změny a mutace genu *HNF1B*, což je podrobněji popsáno v části věnující se molekulárně biologickým metodám.

V souladu s recentními studiemi jsme imunohistochemicky prokázali, že HNF-1β je senzitivní, ale ne zcela specifický marker pro světlobuněčné karcinomy. Pozitivita tohoto proteinu může být běžně nalezena i v jiných typech nádorů a v nenádorových lézích. Nicméně silnou intenzitu exprese protienu HNF-1β jsme prokázali pouze ve skupině světlobuněčných karcinomů, kromě jednoho případu serózního karcinomu endometria. Domníváme se, že význam intenzity exprese na imunohistochemické úrovni či úrovni exprese mRNA by mohl být předmětem dalších výzkumů. Kromě toho jsme nalezli různou míru exprese HNF-1β v endometroidních karcinomech v závislosti na diferenciaci nádoru. Výsledek exprese HNF-1β v nenádorovém endometriu a v prekancerózních lézích podle přepokladů nesvědčí pro to, že by tento marker mohl být využitelný v diferenciální diagnostice těchto lézí.

Z našich výsledků vyplývá, že HNF-1β může být využit v diferenciální diagnostice nádorů ženského genitálu, ale musíme si být vědomi určitých limitací, zejména nízké specifity markeru pro světlobuněčné karcinomy.

2.1.3. Využití imunohistochemických metod při analýze exprese Glut-1 v korporálním endometriu a karcinomech a lézích endometria

Význam a využití imunohistochemických metod v gynekopatologii si můžeme dále ukázat například na práci, ve které jsme analyzovali expresi Glut-1 ve 336 vzorcích karcinomů i nenádorových lézí děložního těla a v korporálním endometriu v různých fázích cyklu [2].

Glut-1 je membránový glykoprotein, který je spolu s ostatními glukózovými přenašeči zodpovědný za zprostředkování příjmu glukózy do buňky. Zvýšená exprese tohoto markeru byla nalezena u některých maligních nádorů, které mají schopnost přepnout z oxidativní fosforylace na aerobní glykolýzu (Warburgův efekt), což jim přináší selektivní růstovou výhodu [25]. Významně nižší zisk energie při glykolýze oproti oxidativní fosforylaci (2 molekuly ATP versus 32 molekulám ATP) si tyto nádory kompenzují přeprogramováním buněčného metabolismu a jedním z mechanismů je právě zvýšená exprese glukózových transportérů, zejména Glut-1, který je považován za jeden z nejvýznamnějších glukozových transportérů [26, 27].

Podle některých studií je zvýšená exprese tohoto markeru v nádorech zároveň i prognosticky nepříznivým faktorem asociovaným s vyšším stupněm nádorové differenciace (grade) i stádia (stage) a s lymfovaskulární invazí či postižením lymfatických uzlin [28-30]. V nenádorové tkáni a benigních nádorech bývá tento marker většinou negativní nebo pouze slabě pozitivní (kromě pár výjimek, jako jsou např. erytrocyty nebo perineurální buňky)[30-33].

V tomto kontextu je důležitá analýza exprese Glut-1 v přednádorových lézích vzniklých v endometriu, zejména analýza atypické hyperplazie, která je prekursorovou lézí endometroidního karcinomu endometria, ale jejíž odlišení od hyperplazie bez atypií založené pouze na morfologických znacích je v některých případech komplikované [34]. Touto problematikou se zabývalo několik studií s variabilními výsledky [35, 36].

Cílem naší práce bylo tedy analyzovat expresi Glut-1 na velkém souboru případů endometriálních nádorů, ale i dalších lézí a nenádorovém endometriu a vyhodnotit potenciální využití tohoto markeru v diagnostické praxi.

V naší práci se nám na dosud největším souboru případů podařilo potvrdit vysokou míru exprese u karcinomů endometria, a to nejen u endometroidních karcinomů, ale také v dalších typech karcinomů, včetně světlobuněčného a serózního. Dále se na základě výsledků domníváme, že imunohistochemická exprese Glut-1 může být využitelná jako pomocný marker v diferenciální diagnostice mezi hyperplazií atypickou a hyperplazií bez atypií, jejichž rozlišení má zásadní dopad na způsob léčby [2].

2.1.4. Využití imunohistochemických metod při detekci nádorových metastáz a mikrometastáz u nesentinelových uzlin

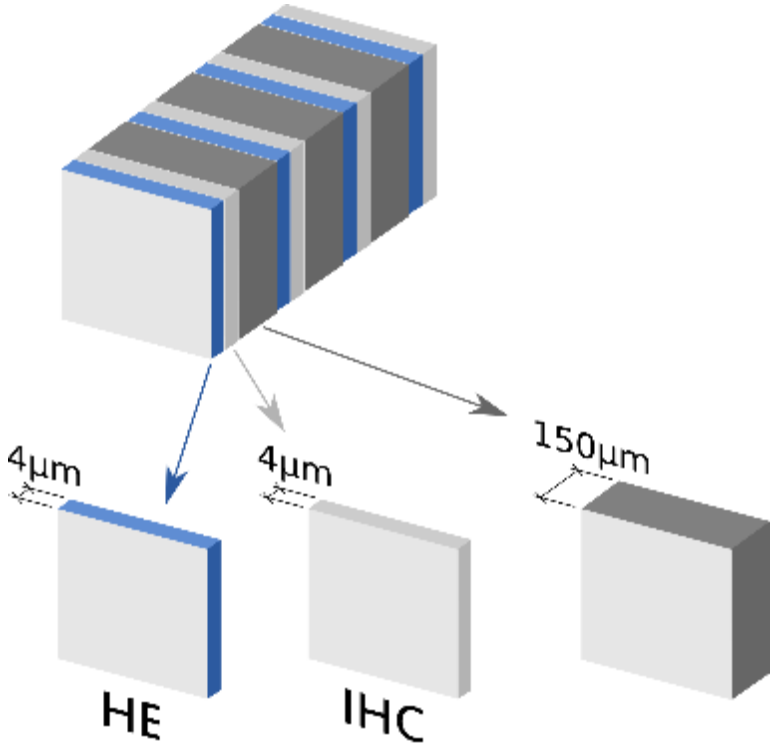
V další práci jsme se zabývali detekcí nádorových metastáz a mikrometastáz u pánevních uzlin pacientek s karcinomy děložního hrdla, které mají vysoké riziko uzlinového postižení nádorovými metastázami [7].

Význam vyšetření sentinelových uzlin (SLN) u karcinomů děložního hrdla byl již potvrzen řadou studií [37-39]. Ultrastagingovým protokolem jsou ale vyšetřované pouze SLN a riziko postižení dalších pánevních (nesentinelových) uzlin mikrometastázami je tudíž neznámé. Cílem naší studie bylo zhodnotit riziko postižení nádorovými mikrometastázami v lymfatických uzlinách pacientek s peroperačně negativními SLN, přičemž do studie byly zařazeny pacietky s vyšším rizikem metastatického postižení, tedy s většími nádory (FIGO IB1, IB2, IIB).

Všechny uzliny od každé pacientky včetně SLN byly nejprve prokrájeny v intervalu dvou mm a dále zpracovány ultrastagingovým protokolem v celém rozsahu. Dle protokolu byly vždy zhotoveny dva po sobě jdoucí řezy v intervalu 150 μm. První z nich byl obarven H&E a druhý protilátkou proti cytokeratinům AE1/AE3 (Obr. 1). Celý proces se opakoval až do úplného

vykrájení uzliny. Takto nastavený protokol nám umožňuje detekovat nádorové mikrometastázy (0,2 až 2,0 mm) i izolované nádorové buňky (ITC) (menší než 0,2 mm, nebo subkapsulární shluky nádorových buněk nepřesahující počet 200 buněk). Podle současných předpokladů představují mikrometastázy pravděpodobně skutečné nádorové postižení, oproti tomu ITC mají zřejmě limitovaný potenciál pro vzdálené šíření nádoru.

Obr.1



Klinická inkluzní kritéria studie splnilo 17 pacientek, u kterých byly ultrastagingovým protokolem zpracovány všechny pánevní lymfatické uzliny. Celkem bylo vyšetřeno 573 pánevních lymfatických uzlin, z čehož 43 tvořily uzliny sentinelové a 530 uzliny nesentinelové. Celkem bylo zhotoveno 5762 skel (2881 H&E a 2881 imunohistochemických preparátů s protilátkou CK AE1/AE3).

Nádorové postižení jsme prokázali celkem v osmi SLN (1 metastázu, 4 mikrometastázy a 3 ITC). Pouze u dvou pacientek byly nalezeny metastázy i v nesentinelových uzlinách (3 mikrometastázy a 1 ITC), přičemž v obou případech nesentinelového postižení byly zároveň

přítomny nádorové mikrometastázy v SLN. U žádné pacientky s ultrastagingově negativní SLN jsme nádorové postižení v nesentinelových uzlinách neprokázali.

Díky moderním vyšetřovacím metodám zahrnujícím imunohistochemické vyšetření ultrastagingovým protokolem jsme v naší skupině „rizikových" pacientek neprokázali při vyšetření SLN „falešnou negativitu". V naší studii se nám tedy podařilo potvrdit nízké riziko pozitivity pánevních lymfatických uzlin u pacientek s ultrastagingově negativní SLN. Jsme si však vědomi limitací daných malým souborem pacientek a význam nádorových metastáz a ITC v lymfatických uzlinách u karcinomu děložního hrdla bude muset být potrvzen v dalších studiích.

2.1.5. Využití imunohistochemických metod při diagnostice vzácného nádoru - anastomozujícího hemangiomu ovária se stromální luteinizací

Dalším příkladem využití kombinace morfologických znaků a imunohistochemického vyšetření je klinickopatologická studie 6 případů anastomozujícího hemangiomu ovária se stromální luteinizací [1]. Jde o vzácný typ nádoru, který byl poprvé popsán v urogenitálním traktu [40].

V ováriu se heminagiomy vyskytují pouze zřídka a konkrétně anastomozujících hemangiomů zde bylo popsáno dosud pouze 8 případů. Při jejich diagnostice se většinou opíráme o morfologické znaky založené na proliferaci anastomozujících cév velikosti kapilár se sinusoidálním uspořádáním připomínajícím červenou pulpu sleziny, které jsou promíseny se středně velkými cévami. Často bývají přítomny i hobnail buňky, tromby, hemoragie a extramedulární hemopoeza.

Kromě toho byla ve všech dosud popsaných případech nalezena různě rozsáhlá stromální luteinizace, která může, pokud je prominentní, vzbuzovat dojem steroidogenního nádoru. Přítomnost luteinizace se pokouší vysvětlit dvě různé teorie. Jedna z nich je založena na principu

tlakových změn v okolí vaskulární léze způsobujících v důsledku stromální luteinizaci, obdobný princip jako u rostoucího folikulu [41, 42]. Druhá teorie se naopak pokouší vysvětlit vznik vaskulární léze na základě působení zvýšených hladin estrogenu na cévy, nicméně proti této verzi svědčí absence exprese estrogenových a progesteronových receptorů v endoteliálních buňkách léze [43].

K potvrzení diagnózy anastomozujícího hemangiomu využíváme imunohistochemické vyšetření potvrzující endoteliální původ léze, pozitivní průkaz protilátek CD31, CD34 nebo fVIII. Diferenciální diagnostika oproti ostatním vaskulárním lézím je většinou založena na morfologických znacích, vyjma Kaposiho sarkomu, k jehož vyloučení využíváme imunohistochemické vyšetření s protilátkou proti HHV8.

Anastomozující hemangiomy ovária jsou vzácné vaskulární léze, které musí být brány v potaz při diagnostice vaskulárních nádorů a lézí ovária a nesmí být zaměněny za ovariální angiosarkom.

2.2. Význam molekulárně biologických a cytogenetických metod v gynekopatologii

V této části uvádíme různé příklady možného využití molekulárně biologických i cytogenetické metod v diagnostice, ale i při detekci známých i neznámých molekulárních aberací a genových variací v různých lézích či nádorech ženského genitálu.

2.2.1. Komplexní imunohistochemická a molekulárně genetická analýza atypických polypózních adenomyomů

Atypické polypózní adenomyomy (APA) jsou vzácné léze děložního těla s rizikem rekurence u konzervativně léčených pacientek [44-46]. Tyto léze bývají nacházeny v souvislosti nebo předcházejí vzniku atypické hyperplazie nebo endometroidního karcinomu [47, 48]. V době

provádění naší studie neexistoval jednotný názor na přístup k pacientkám s APA, což s sebou přinášelo terapeutické problémy zejména s ohledem na fakt, že řada pacientek s APA je v produktivním věku a často si přejí zachovat fertilitu. V tomto kontextu bylo tedy důležité pokusit se určit patogenezi a biologickou povahu léze. Ačkoli bylo v literatuře popsáno asi 230 případů, pouze 2 malé studie se zabývaly některými molekulárními aspekty těchto lézí [49, 50]. Cílem naší studie byla komplexní analýza APA s využitím moderních diagnostických metod se snahou o zhodnocení biologické povahy lézí.

Podařilo se nám shromáždit skupinu 21 těchto vzácných lézí, na které jsme nejprve provedli imunohistochemickou analýzu exprese vybraných protilátek, které zahrnovali HNF-1β, PTEN, mTOR, β-catenin, HNF-1β, Glut-1, MLH1, MSH2, MSH6 a PMS2. Dále jsme pokračovali s vyšetřením mutačního stavu vybraných genů. Cytogenetickou metodou FISH jsme vyšetřeli geny *PTEN* a *TP53* a molekulárně biologickými metodami PCR mutační stav genů *KRAS, BRAF, EGFR.*

Při výběru genů a protilátek jsme se soustředili na ty, u kterých je známá souvislost s vývojem atypické hyperplazie endometria a endometroidního karcinomu, jako jsou například geny *KRAS, PTEN*, proteiny MSI, ale také na některé v této oblasti dosud méně prozkoumané markery jako například HNF-1β, Glut-1 a mTOR.

Naše výsledky ukázaly, že APA sdílejí některé konzistentní imunohistochemické a molekulární znaky, s jakými se setkáváme u atypické hyperplazie a endometroidního karcinomu. Mezi významnější znaky nalezené na imunohistochemické úrovni patří exprese β-cateninu ve skvamózní komponentě lézí, exprese HNF1-β a Glut-1 v glandulární komponentě, dále wild typ exprese p53 a ztráta exprese PTEN, která byla u jedné pacientky doprovázena delecí genu *PTEN* detekovanou metodou FISH. Na molekulární úrovni jsme prokázali mutaci *KRAS* u 5 vzorků, ale u žádného vzorku nebyla nalezena mutace genů *NRAF, BRAF* a *EGFR.* Vzhledem k tomu, že

nebyl prokázán ani deficit v „mismatch repair“ proteinech, domníváme se, že APA nejsou součástí projevu Lynchova syndromu.

Naše výsledky naznačují, že by APA měly být považovány za lokalizovanou formu atypické hyperplazie, tedy prekursor endometroidního karcinomu. Tento závěr je klinicky významný zejména s ohledem na následný terapeutický přístup k často mladým pacientkám, které si přejí zachovat fertilitu.

2.2.2. Molekulární analýza metylace promotoru a mutací genu *HNF1B*

Molekulárně biologické metody jsme využili i v části studie, která navazovala na analýzu exprese HNF-1β v nádorech a lézích korporálního endometria [5]. Vzhledem k tomu, že se transkripční faktor HNF-1β pravděpodobně podílí na karcinogenezi různých typů nádorů, včetně endometroidních karcinomů, věnovali jsme se v této části studie molekulární analýze metylace promotoru a mutací genu *HNF1B* v karcinomech endometria a světlobuněčných karcinomech ovária [18, 19, 51]. Cílem naší studie bylo analyzovat genetické variace genu *HNF1B* v kódujícím regionu i v oblasti promotoru, zhodnocení významu těchto změn a korelace s expresí na úrovni proteinu.

Podle celogenomových studií jsou některé genetické varianty genu *HNF1B* asociovány se zvýšeným nebo naopak sníženým rizikem vzniku různých nádorových typů, včetně endometroidního karcinomu děložního těla a serózního karcinomu ovaria [52-54]. *HNF1B* se může v různých typech nádorů chovat jako tumor supresorový gen nebo jako onkogen, ale přesný mechanismus, kterým se HNF-1β podílí na procesu karcinogeneze, zůstává neznámý a pravděpodobně se v různých typech nádorů liší.

Zvýšená exprese HNF-1β byla nalezena ve většině světlobuněčných karcinomů ovaria, pankreatu, ledviny a v hepatocelulárním karcinomu a downregulace tohoto genu je u světlobuněčných karcinomů ledviny a prostaty asociována s horší prognózou [19, 55].

Epigenetická inaktivace genu obvykle vede ke ztrátě exprese HNF-1β a metylace promotoru byla nalezena u některých buněčných linií karcinomu pankreatu, kolorekta, žaludku a ovaria, ale v oblasti ženského genitálu se této problematice věnovalo pouze několik málo studií [22, 54, 56].

U světlobuněčných karcinomů ovaria je promotor genu *HNF1B* typicky nemetylovaný a genová exprese je ve srovnání s ostatními typy ovariálních karcinomů zvýšena [22, 54]. Snížení exprese HNF-1β v buněčných liniích světlobuněčného karcinomu ovaria indukuje apoptózu a exprese tohoto transkripčního faktoru se zdá být v některých případech významnou pro přežívání nádorových buněk [22].

Naše výsledky byly v souladu s dříve publikovanými studiemi. V žádném světlobuněčném karcinomu ovaria jsme metylaci bisulfitovým sekvenováním neprokázali. Oproti tomu ve skupině endometroidních karcinomů děložního těla, jsme detekovali metylaci promotoru genu *HNF1B* v 13 % a tyto případy zároveň vykazovaly negativitu nebo pouze slabou expresi proteinu HNF-1β.

Dále se nám ve skupině endometroidních karcinomů děložního těla podařilo v kódujícím regionu genu *HNF1B* detekovat čtyři jednonukleotidové sekvenční varianty (1 patogenní nonsense variantu; 1 pravděpodobně patogenní silent variantu a 2 intronové varianty nejasného významu, které by mohly potenciálně vést k aberantnímu sestřihu mRNA a následně aberantnímu proteinovému produktu). Ve světlobuněčných karcinomech ovária jsme nalezli jednu missence variantu, u které bylo prediktivními modely *in silico* potvrzeno, že jde vysoce pravděpodobně o mutaci patogenní.

Jsme si však vědomi limitací plynoucích z malého souboru případů a přesná role *HNF1B* v karcinogenezi, jeho potenciální využití při cílené terapii bude vyžadovat další studie.

2.2.3. Využití molekulárních a cytogenetických metod při diagnostice světlobuněčného sarkomu vulvy

V další práci jsme popsali využití zejména cytogenetické metody FISH v diagnostice světlobuněčného sarkomu vzniklého v oblasti vulvy [8]. Světlobuněčný sarkom, také známý jako melanom měkkých tkání, je vzácný nádor, který tvoří přibližně 1 % nádorů měkkých tkání [57]. Jeho diagnostika je však poměrně obtížná, protože má překryvné fenotypické a imunohistochemické rysy s maligním melanomem. Oba nádory jsou charakterizované expresí S-100 proteinu, HMB-45, Melanu A a mikroftalmického transkripčního faktoru (MiTF), ale světlobuněčný sarkom se oproti malignímu melanomu častěji vyskytuje v hlubokých tkáních bez postižení kožních struktur.

Při diferenciální diagnostice těchto dvou nádorů však můžeme s výhodou využít cytogenetické metody, protože světlobuněčné sarkomy jsou charakterizované přestavbou genu Ewingova sarkomu (EWS). Nejčastější aberací je balancovaná translokace (12;22)(q13;q12), kterou jsme metodou FISH prokázali i v našem případu, a vzácněji může být prokázána translokace (2;22)(q34;q12) [58-60]. V maligních melanomech nebyly tyto přestavby prokázány.

Potenciálně by šlo využít i molekulárně biologické analýzy mutačního stavu genů *BRAF* a *NRAS*, které se naopak vyskytují v maligních melanomech. Mutaci genu *BRAF* lze nalézt až ve 40–60 % všech kožních melanomů a její detekce prediktorem pro terapii BRAF inhibitory. V našem případu jsme však tuto mutaci neprokázali, což podpořilo diagnózu světlobuněčného sarkomu měkkých tkání.

S využitím cytogenetických a imunohistochemických metod jsme tedy diagnostikovali a popsali první případ světlobuněčného sarkomu vzniklého ve vulvě.

2.2.4. Komplexní imunohistochemická a molekulární analýza lymfoepitelioma-like karcinomu

V další práci jsme provedli komplexní imunohistochemickou a molekulární analýzu vzácného nádoru endometria, lymfoepitelioma-like karcinomu (LELC)[61].

Lymfoepiteliální karcinomy (lymfoepiteliomy) jsou nediferencované karcinomy nasofaryngu s prominentním lymfocytárním infiltrátem, většinou asociované s virem Ebstein-Barrové (EBV)[62, 63]. Karcinomy s morfologicky obdobnými rysy vzniklé mimo nasofarynx se označují termínem lymfoepitelioma-like karcinomy. U těchto nádorů je asociace s EBV infekcí variabilní a liší se dle lokality výskytu [63-66].

V ženském genitálu se LELC nejčastěji vyskytují v oblasti děložního hrdla, kde jsou považovány za variantu dlaždicobuněčného karcinomu, a pouze ojediněle v endometriu, kde bylo dosud popsáno pouze šest případů [65-70]. V žádném z nich však nebyla provedena podrobnější molekulární analýza, pouze v jedné studii se autoři zabývali i molekulárním testováním mikrosatelitové instability [68]. Proto jsme náš případ podrobili komplexní imunohistochemické a molekulárně biologické analýze, s cílem lépe objasnit podstatu nádoru.

V našem případu LELC se nám při imunohistochemickém vyšetření podařilo prokázat difúzní expresi p16 a aberantní exprese proteinu p53. Při molekulárním vyšetření metodou NGS jsme nalezli patogenní mutaci genu *TP53* a neprokázali přítomnost mutací v genech *ARID1A* a *PTEN*. Fragmentační analýza prokázala, že jde o nádor mikrosatelitově stabilní (MSS). Tento imunofenotyp i genotyp je charakteristický pro serózní karcinomy endometria.

Histogeneze LELC endometria je neznámá, ale imunohistochemický a molekulárně genetický profil našeho případu naznačuje, že by se mohlo jednat o neobvyklou morfologickou variantu serózního karcinomu endometria. Nicméně k úplnému porozumění histogeneze, biologického chování a prognózy LELC endometria bude zapotřebí dalších studií, což bude jistě komplikované raritním výskytem těchto nádorů.

3. ZÁVĚR

V naší práci jsme se pokusili ukázat, že moderní vyšetřovací metody hrají v oblasti gynekopatologie nenahraditelnou roli a v současné době se některé z nich již staly nedílnou součástí moderních vyšetřovacích postupů. Na příkladech nádorů ale i nenádorových lézí endometria, nádorů ovárií, děložního hrdla a vulvy jsme demonstrovali význam a možné využití speciálních metod v různých oblastech gynekopatologie.

V rámci námi realizovaných výzkumných projektů jsme navrhli panel protilátek, který by mohl napomoci při rozlišení morfologicky obtížně zařaditelných málo diferencovaných karcinomů děložního hrdla. Dále jsme popsali expresi některých protilátek v nádorech, nenádorových lézích i normálních tkáních ženského genitálu, což by mohlo mít v budoucnu přínos pro diferenciální diagnostiku. Imunohistochemickými, molekulárně biologickými a cytogenetickými metodami jsme analyzovali několik dosud méně prozkoumaných lézí a nádorů, jako jsou atypické polypózní adenomyomy děložního těla a lymfoepitelioma-like karcinom endometria. Na základě těchto analýz se domníváme, že by se na atypické polypózní adenomyomy mělo pohlížet jako na lokalizovanou formu atypické hyperplazie, což je důležité zejména s ohledem na fakt, že léze se často vyskytují u mladých žen a dívek přejících si zachování fertility. V případě lymfoepitelioma-like karcinom endometria výsledky imohistochemických a molekulárně biologických analýz ukázaly, že by se mohlo jednat o neobvyklou morfologickou variantu serózního karcinomu endometria. V rámci komplexní studie věnované problematice HNF1B se nám podařilo nalézt několik dosud nepopsaných mutací genu *HNF1B*.

Přestože hrají moderní vyšetřovací metody v gynekopatologii stále větší roli, základem bioptického vyšetření nadále zůstává klasické histologické vyšetření, na které v indikovaných případech tato speciální vyšetření navazují. Stále se rozšiřující spektrum protilátek a poznatky v oblasti molekulární biologie s sebou také přináší zvýšené nároky na znalosti vyšetřujícího

patologa, který s nimi musí být dobře obeznámen. Nelze však zcela opominout ani finanční aspekt, kdy zejména vyšetření molekulárně biologickými a cytogenetickými metodami jsou finančně náročná a jejich využití v rutinní diagnostické praxi naráží na problémy s úhradou.

4. PUBLIKACE IN EXTENSO

4.1. Expression of HNF-1β in cervical carcinomas: an immunohistochemical study of 155 cases.

Němejcová et al. Diagnostic Pathology (2015) 10:8
DOI 10.1186/s13000-015-0245-9

DIAGNOSTIC PATHOLOGY

RESEARCH Open Access

Expression of HNF-1β in cervical carcinomas: an immunohistochemical study of 155 cases

Kristýna Němejcová[1†], David Cibula[2] and Pavel Dundr[1*†]

Abstract

Background: HNF-1β is a commonly used marker in the differential diagnosis of clear cell carcinoma of the ovary and endometrium. Recent studies have found HNF-1β expression to a lesser extent in other ovarian and endometrial tumors including endometrioid, mucinous and, rarely, serous carcinoma. Regarding cervical carcinoma, HNF-1β expression has been mentioned exceptionally in mesonephric and some other types of adenocarcinoma. However, a systematic analysis of HNF-1β expression in cervical carcinomas has not been performed to date.

Methods: We analyzed HNF-1β expression in 155 cervical carcinomas (including 56 adenocarcinomas, 85 squamous cell carcinomas and 14 undifferentiated carcinomas). Expression of HNF-1β was correlated with the expression of other markers including estrogen receptors, progesterone receptors, CEA, p63, p40, p16, and D2-40.

Results: Adenocarcinomas showed expression of HNF-1β in 42/56 cases (75%), CEA in 48/56 cases (85.7%), p63 in 4/56 cases (7.2%), p40 in 2/56 cases (3.6%), estrogen receptors in 9/56 cases (16.1%), progesterone receptors in 5/56 cases (8.9%), p16 in 56/56 (100%) cases, and D2-40 in 0/56 cases (0%). Squamous cell carcinomas showed expression of HNF-1β in 2/85 cases (2.35%), CEA in 77/85 cases (90.6%), p63 and p40 in 85/85 cases (100%), estrogen receptors in 9/85 cases (10.6%), progesterone receptors in 1/85 cases (1.2%), p16 in 84/85 cases (98.8%), and D2-40 in 45/84 cases (53.6%). Undifferentiated carcinomas showed expression of HNF-1β in 2/14 cases (14.3%), CEA in 8/14 cases (57.1%), p16 in 14/14 cases (100%), hormone receptors in 0/13 cases (0%), p63 in 7/14 cases (50%), p40 in 5/14 cases (35.7%), and D2-40 in 1/14 cases (7.1%).

Conclusions: In cervical carcinoma, expression of HNF-1β is mostly restricted to adenocarcinomas and can be used as an auxiliary adenocarcinoma marker in the differential diagnosis of poorly differentiated cervical carcinomas. HNF-1β as an adenocarcinoma marker and p63/p40 and D2-40 as a squamous cell carcinoma markers are highly specific with variable sensitivity. Optimal results can be achieved using these markers in a panel.

Virtual Slides: The virtual slide(s) for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/vs/1348836442160205.

Keywords: HNF-1β, Squamous cell carcinoma, Undifferentiated carcinoma, Immunohistochemistry

Background

Hepatocyte nuclear factor 1 beta (HNF-1β) is a transcription factor that plays a crucial role in the differentiation of visceral endoderm from the primitive endoderm [1,2]. In normal tissues, HNF-1β is expressed in epithelial cells of the urogenital tract, liver, pancreas, gut, and lung [3-5]. However, this marker can be expressed in several types of tumors. In gynecopathology, expression of HNF-1β is commonly used in the differential diagnosis of clear cell carcinomas of the ovary and endometrium. However, recent studies have found HNF-1β expression to a lesser extent in other tumor types including endometrioid, mucinous and, rarely, serous carcinoma and even in some non-neoplastic tissues [6,7]. In cervical carcinoma, expression of HNF-1β has been mentioned in mesonephric and other types of adenocarcinoma [8,9]. However, a systematic analysis of HNF-1β expression in cervical carcinoma has not been performed to date. In our study, we examined HNF-1β expression in invasive carcinomas of the

* Correspondence: pdundr@seznam.cz
† Equal contributors
[1]Department of Pathology, First Faculty of Medicine and General University Hospital, Charles University in Prague, Studnickova 2, Prague 2 12800, Czech Republic
Full list of author information is available at the end of the article

uterine cervix. Expression of this marker was correlated with expression of other markers including estrogen receptors, progesterone receptors, CEA, p63, p40, p16, and D2-40.

Methods

In total, 155 specimens were included in the study, including 56 adenocarcinomas (46 endocervical adenocarcinomas, usual type; 8 mucinous carcinomas, NOS; 2 mucinous carcinomas, intestinal type), 86 squamous cell carcinomas and 13 undifferentiated carcinomas. All cases were selected from files of our department (Department of Pathology, the First Faculty of Medicine and General University Hospital, Charles University in Prague). In all cases, formalin-fixed, paraffin-embedded tissue blocks were available for subsequent immunohistochemical analysis. Tissue blocks containing only a small amount of tumor or otherwise inadequate samples were excluded. Selected cases represented routine diagnostic surgical specimens including 37 endocervical curettage, 8 punch biopsy, 19 cone biopsy, and 91 hysterectomy specimens. In compliance with the Helsinki Declaration, the project has been approved by Ethics Committee of General University Hospital in Prague.

Immunohistochemical analysis

Immunohistochemical analysis was performed using the avidin-biotin complex method with antibodies against the following antigens: HNF-1β (polyclonal, dilution 1:500, Sigma-Aldrich, Prestige Antibodies, St. Louis, United States), estrogen receptor (clone GF11, dilution 1:50, Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), progesterone receptor (clone 16, dilution 1:200, Novocastra), CEA (clone II-7, dilution 1:100, Dako, Glostrup, Denmark), p63 (clone 4A4, dilution 1:50, Diagnostic BioSystems, Pleasanton, USA), P40 (polyclonal, dilution 1:50, BioSystems), P16 (clone E6H4, CINtec® Histology Kit, Roche mtm Laboratories AG, Manheim, Germany), and D2-40 (clone D2-40, dilution 1:100, Dako). Antigen retrieval was performed including pretreatment in 0.01 M citrate buffer (pH 6.0) for 40 min in a water bath at 98°C for progesterone receptors and CEA. Heat-induced epitope retrieval was done in 0.01 citrate buffer (pH 6.1) for HNF-1β, and in 0.01 citrate buffer (pH 9.0) for estrogen receptors, p63 and p40. All antibodies were processed manually, except for p16, which was stained on a Ventana Benchmark immunostainer (CINtec® Histology Kit, Roche mtm laboratories AG, Manheim, Germany).

Immunohistochemical results were semiquantitatively assessed and graded on a four-tier scale based on the percentage of positive cells: 0 = <5%; 1 = 5-29%; 2 = 30-59%; 3 = >60% positive cells. For HNF-1β, estrogen receptors, progesterone receptors, p63, and p40 only nuclear staining was regarded as positive. For p16, nuclear and cytoplasmic staining was considered as positive. Positivity of CEA and D2-40 was defined as distinct membrane staining. Moreover, the staining intensity of HNF-1β was assessed as weak, moderate or strong.

Results

Patients' age ranged from 23 to 86 years (mean 53.7; median 55.0). The 56 ACAs included 46 endocervical adenocarcinomas, usual type, 8 mucinous carcinomas, NOS, and 2 mucinous carcinomas, intestinal type). Thirty-seven cases were moderately differentiated, 15 cases were poorly differentiated, and 4 cases were well differentiated. In the group of 86 SCC, 2 cases were well differentiated, 49 cases moderately differentiated and 34 cases poorly differentiated. We focused only on "usual" types of endocervical adenocarcinoma and other types of cervical adenocarcinoma (including serous, endometrioid, and clear cell) were excluded from the study. However, 4 cases of clear cell adenocarcinoma were used as a positive control for HNF-1β staining (all 4 cases showed strong 3+ positivity).

Immunohistochemistry

All the results are summarized in Table 1. Table 2 summarizes antibodies which can be used in the differential diagnosis of ACA and SCC. Figure 1 shows HNF-1β expression in cervical carcinomas.

Adenocarcinomas

Expression of HNF-1β was found in 42/56 ACAs (75%). Twenty-seven cases were 3+ positive, eight cases showed 2 + positivity, and seven cases were positive only focally 1+. Intensity of staining was highest in the group of 3+ positive cases (2 lesions showed strong positivity, 17 lesions showed moderate positivity and 8 lesions weak positivity). In the group of 2+ and 1+ positive lesions, the intensity of staining varied between weak to moderate.

Simultaneous expression of p63 and p40 was found in 2/56 tumors (3.6%). Two other cases were p63 weakly positive (1+) without simultaneous expression of p40 (both of them with weak intensity of staining). Estrogen receptors were positive in 9/56 cases (16.1%), two cases strongly 3+, one 2+ and remaining 6 weakly 1+ positive. In 5 of these cases was also found simultaneous expression of progesterone receptors. CEA was positive in 48/56 cases (85.7%) of ACAs and p16 was positive in all 56 ACAs. None of the ACAs showed D2-40 positivity.

Squamous cell carcinomas

SCC showed expression of HNF-1β in 2/85 cases (2.35%). These cases expressed HNF-1β in approximately 70% and 30% of tumor cells, respectively. Both cases were simultaneously strongly p16, p63, and p40 positive, and showed

Table 1 Immunohistochemical findings in cervical carcinomas

		HNF-1β		ER		PR		CEA		p16		p63		p40		D2-40	
		pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg	pos	neg
ACA		42 (75%)	14 (25%)	9 (16.1%)	47 (83.9%)	5 (8.9%)	51 (91.1%)	48 (85.7%)	8 (14.3%)	56 (100%)	0 (0%)	4 (7.2%)	52 (92.8%)	2 (3.6%)	54 (96.4%)	0 (0%)	56 (100%)
(56 cases)	3+	27 (48.2%)		2 (3.6%)		3 (5.4%)		31 (55.4%)		52 (92.9%)		0		0			
	2+	8 (14.3%)		1 (1.8%)		1 (1.8%)		13 (23.2%)		2 (3.6%)		1 (1.8%)		1 (1.8%)			
	1+	7 (12.5%)		6 (10.7%)		1 (1.8%)		4 (7.1%)		2 (3.6%)		3 (5.4%)		1 (1.8%)			
SCC		2 (2.35%)	83 (97.65)%	9 (10.6%)	76 (89.4)%	1 (1.2%)	84 (98.8%)	77 (90.6%)	8 (9.4%)	84 (98.8%)	1 (1.2%)	85 (100%)	0 (0%)	85 (100%)	0 (0%)	45 (53.6%)	39 (46.4%)
(85 cases)	3+	1 (1.2%)		3 (3.5%)		0		13 (15.3%)		83 (97.6%)		83 (97.6%)		83 (97.6%)		15 (17.9%)	
	2+	1 (1.2%)		2 (2.4)%		0		15 (17.6%)		0		2 (2.4%)		2 (2.4%)		15 (17.9%)	
	1+	0		4 (4.7%)		1 (1.2%)		49 (57.6%)		1 (1.2%)		0		0		15 (17.9%)	
UC		2 (14.3%)	12 (85.7%)	0 (0%)	14 (100%)	0 (0%)	14 (100%)	8 (57.1%)	6 (42.9%)	14 (100%)	0 (0%)	7 (50%)	7 (50%)	5 (35.7%)	9 (64.3%)	1 (7.1%)	13 (92.9%)
(14 cases)	3+	1 (7.1%)						1 (7.1%)		13 (92.9%)		4 (28.6%)		4 (28.6%)		0	
	2+	0						2 (14.3%)		0		1 (7.1%)		0		1 (7.1%)	
	1+	1 (7.1%)						5 (35.7%)		1 (7.1%)		2 (14.3%)		1 (7.1%)		0	

ACA = adenocarcinomas, SCC = squamous cell carcinomas UC = undifferentiated carcinomas, Pos = positive, Neg = negative. Immunohistochemical results of HNF-1β, estrogen receptors, progesterone receptors, CEA, p63, p40, p16, and D2-40 were semiquantitatively assessed and graded on a four-tier scale based on the percentage of positive cells: 0 = <5%; 1 = 5-29%; 2 = 30-59%; 3 = >60% positive cells.

Table 2 Summary of antibodies which can be used in differential diagnosis of ACA and SCC

	ACA	SCC
HNF-1β	++	-
	(75%)	(2%)
p63/p40	–+	+++
	(7% / 4%)	(100%)
CEA	+++	+++
	(86%)	(91%)
ER	+–	+–
	(16%)	(11%)
PR	–+	-
	(9%)	(1%)
D2-40	-	++
	(0%)	(54%)

ACA - adenocarcinoma; SCC - squamous cell carcinoma.
In brackets is a percentage of positive cases in our study.

1+ and 2+ expression of CEA. All SCCs were p63 and p40 positive. 9/85 (10.6%) SCCs were estrogen receptors positive and one of them was progesterone receptors positive 1/85 (1.2%). CEA was positive in 77/85 cases (90.6%). P16 showed positivity in all but one SCC. The negative case was HNF-1β, estrogen receptors, progesterone receptors and CEA negative, and strongly 3+ p63 and p40 positive. D2-40 was positive in 45/84 cases (53.6%). In one case there was not enough material for analysis.

Undifferentiated carcinomas

Undifferentiated carcinomas showed expression of HNF-1β in 2/14 cases (14.3%). In one case (3+) the positivity was strong in approximately 70% of tumor cells. In second case (1+) the positivity was strong in approximately 10% of tumor cells. Both cases were estrogen receptor and progesterone receptor negative. The former was simultaneously CEA, p63, and p40 negative and showed p16 1+ positivity, and the latter was CEA 2+ positive, p16 3+ positive, p63 1+ positive and p40 negative. Alcian blue staining at pH 2.5 was negative in both cases. Expression of CEA was positive in 8/14 cases (57.14%). All tumors were p16 positive and hormone receptor negative. P63 was positive in 7/14 cases (50%) and p40 was positive in 5/14 (35.7%) cases. P63 and p40 were simultaneously positive in 5 cases (4 were 3+ and 1 was 1+). Two other p63 positive cases (1+ and 2+) were p40 negative. Only one of p63/p40 3+ positive cases was simultaneously D2-40 positive. Other 12/13 cases were D2-40 negative.

Non-neoplastic tissue

Non-neoplastic squamous epithelium was found in immunohistochemically examined slides in 59 cases. In all these cases, HNF-1β was negative in this epithelium. Non-neoplastic glands or superficial columnar epithelium was found in 33 cases. 21/33 cases (63.6%) were HNF-1β positive, 19 cases with weak and 2 cases with moderate intensity of staining.

Discussion

The hepatocyte nuclear factor 1 (HNF-1) transcription factor family includes HNF-1 (also known as HNF-1α, Tcf-1), and variant isoforms of the HNF-1 (vHNF-1) (also known as HNF-1β, Tcf-2, LFB3). These transcription factors are expressed in a different spatio-temporal manner in the yolk sac endoderm, and in the developing kidney, liver, and pancreas [10]. HNF-1β is also expressed in the developing neural tube, lungs, and entire urogenital system [3-5]. Functions of HNF-1 family proteins are essential for different stages of ontogenesis. HNF-1β is crucial in the differentiation of visceral endoderm from the primitive endoderm and is essential for formation of kidney tubules, intrahepatic bile ducts and gallbladder, and specification of pancreatic primordium [1,2]. In humans, mutations in the HNF-1β gene are associated with a number of diseases associated with defects in kidney development and a complex syndrome known as renal cysts and diabetes (RCAD), characterized by multiple abnormalities of the kidney, male and female genital tract, and by early-onset diabetes, pancreatic hypoplasia, and liver dysfunction [11,12].

In normal tissues, HNF-1β is expressed in epithelial cells of the urogenital tract, liver, pancreas, gut, and lung [3-5]. In tumors, mutations and epigenetic inactivation of the HNF-1β gene has been shown to be involved in the development of several cancer [13-15]. Methylation of the HNF-1β gene promoter was found in some cancer cell lines derived from pancreatic, colorectal, gastric, and ovarian tumors [16]. Tumor cell lines with a mutation in HNF-1β usually show a loss of protein expression as detected by immunohistochemistry [17]. On the contrary, some tumors show up-regulation of HNF-1β and expression of this protein is found in most clear cell carcinomas of pancreas, endometrium, ovary and kidney [6,13,14,18,19]. It has been shown that down-regulation of HNF-1β in clear-cell renal cell carcinoma is associated with tumor progression and poor prognosis [15]. However, the precise role of HNF-1β in carcinogenesis as well as the importance of molecular targeting of this protein for therapeutic purposes remains unknown.

Regarding to the expression of HNF-1β in non-neoplastic tissue and neoplasms of the female genital tract, only a few studies have analyzed HNF-1β expression in endometriosis, normal endometrium, and tumors of cervix, endometrium and ovary. Most of these studies found that expression of HNF-1β is mostly restricted to clear cell

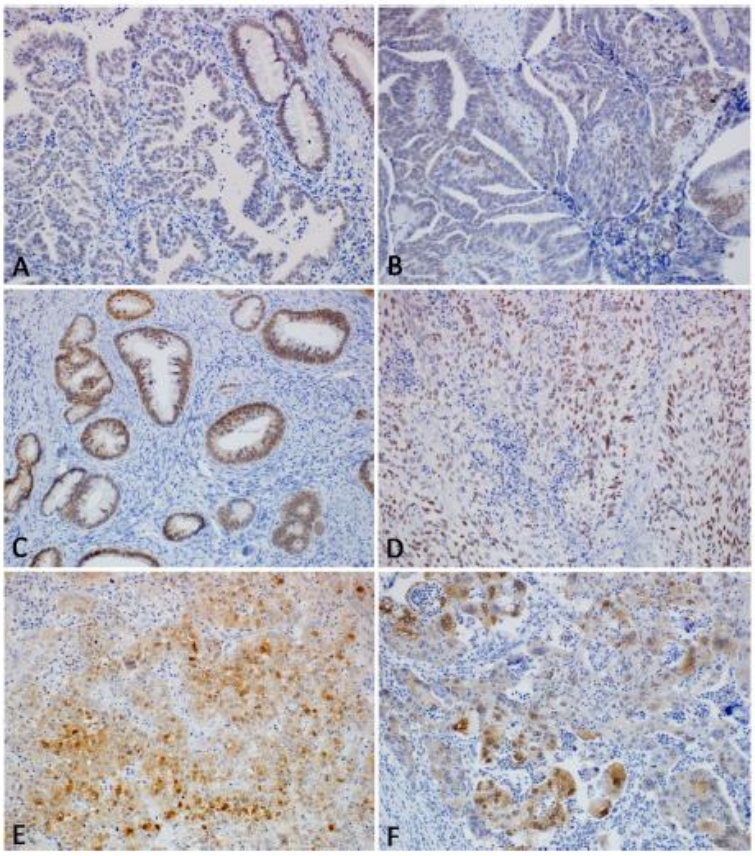


Figure 1 HNF-1β expression in cervical carcinomas. (A) Weak nuclear expression of HNF-1β in adenocarcinoma. Note moderate HNF-1β expression in normal endocervical glands (upper right corner) (200x). **(B)** Moderate expression of HNF-1β in adenocarcinoma (200x). **(C)** Well differentiated adenocarcinoma showing strong expression of HNF-1β (200x). **(D)** Strong expression of HNF-1β in squamous cell carcinoma (200x). **(E)** Focal nuclear expression of HNF-1β in squamous cell carcinoma. Note non-specific cytoplasmic staining in some tumor cells (200x). **(F)** Focal nuclear expression of HNF-1β in undifferentiated carcinoma. Note non-specific cytoplasmic staining in some tumor cells (200x).

adenocarcinoma and concluded that this marker is specific for clear cell adenocarcinoma [6,13,17,18]. However, in other studies, the authors described HNF-1β expression not only in clear cell adenocarcinoma, but also in other tumor types including serous, endometrioid and mucinous carcinoma, and most types of borderline tumors [8,20-22]. Some recent studies have found expression of HNF-1β in some cases of endometriosis (particularly atypical or with inflammatory changes) and in normal endometrium, especially in the secretory phase or gestational state [22,23]. Expression of HNF-1β was not found in the ovarian surface epithelium. However, one study described its expression in some ovarian inclusion cysts [22].

Expression of HNF-1β in carcinoma of the uterine cervix has been mentioned only in two studies. One of them focused on the HPV status and immunohistochemical profiles of unusual histologic subtypes of endocervical adenocarcinoma [8]. This study examined 26 cases of various subtypes of cervical adenocarcinomas. HNF-1β was positive in 7/9 (78%) clear cell carcinoma, in 2/5 (40%) of usual type of endocervical adenocarcinoma, in 3/11 (27%) of gastric-type of endocervical adenocarcinoma, in 3/3 (100%) minimal deviation adenocarcinoma, in 1/1 (100%) mesonephric adenocarcinoma, in 1/1 (100%) serous adenocarcinoma, and in 1/1 (100%) malignant mixed Müllerian tumor. In a second study, focused on the immunohistochemical analysis of seven mesonephric adenocarcinomas, expression of HNF-1β was found in 3/7 cases (42.8%) [9].

Distinguishing between poorly differentiated adenocarcinoma and squamous cell carcinoma of the uterine cervix

can be difficult and in some cases is almost impossible based only on histological features. Clinically, however, this distinction is important and can modify therapeutic decisions, in particular because of squamous cell carcinoma radiosensitivity. Immunohistochemistry can be of help in poorly differentiated tumors, particularly with antibodies against p63 or p40 as markers of stratified epithelium. Moreover, some papers also describe the expression of D2-40 in a subset of squamous cell carcinomas [24-26]. Other markers such as CEA, cytokeratin 7, as well as the estrogen and progesterone receptors are not helpful in distinguishing these tumors [27,28].

In our study, we found expression of HNF-1β in 42/56 cases of adenocarcinoma (75%) and in 2 only /85 cases of SCC (2.35%). Expression D2-40 was positive in 45/84 SCC (53.6%) and 0/56 of ACAs. Expression of p63 and p40 was found in a coordinate staining pattern in 2/56 adenocarcinoma (3.6%) and 85/85 SCC (100%). Moreover, weak expression of p63 without expression of p40 was found in other two adenocarcinoma cases. Regarding undifferentiated carcinomas, we propose that these tumors can be subclassified based on the immunohistochemical profile into three groups: possible adenocarcinoma (2/14 cases; 14.3%) characterized by positivity for HNF-1β and negativity of p40 and D2-40; possible SCC (5/14 cases; 35.7%) characterized by positivity for p40/p63, variable expression of D2-40 and negativity for HNF-1β; undifferentiated carcinoma, NOS (7/14 cases; 50%) characterized by HNF-1β, p63/p40, and D2-40 negativity.

Conclusions

Based on our results, expression of HNF-1β is mostly restricted to adenocarcinomas and can be used as an auxiliary adenocarcinoma marker in the differential diagnosis of poorly differentiated cervical carcinomas. According to our results, HNF-1β can be considered an adenocarcinoma marker while p63/p40 and D2-40 are highly specific markers of SCC with variable sensitivity. Optimal results can be achieved by using these markers in a panel. Limitation of the utility of HNF-1β includes expression in benign glands, which preclude use of this marker in the differential diagnosis of benign lesions and well differentiated p16 negative types of cervical adenocarcinoma. Also, the positivity of HNF-1β in a subset of endometrioid adenocarcinoma prevents the use of this marker in the differential diagnosis between endometrioid and endocervical type of adenocarcinoma.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KN carried out the immunohistochemical analysis, collected and interpreted data, prepared the design of the study, and drafted the manuscript. DC collected and interpreted data, participated in the conception and design of the study, and helped to draft the manuscript. PD conceived of the study, helped with immunohistochemical analysis and participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by IGA MZ CR project NT14001-3/2013, by Charles University in Prague (Project PRVOUK-P27/LF1/1, UNCE 204024, and SVV 260022), and by the project BBMI LM2010004.

Author details

[1]Department of Pathology, First Faculty of Medicine and General University Hospital, Charles University in Prague, Studnickova 2, Prague 2 12800, Czech Republic. [2]Oncogynecological Centre, Department of Obstetrics and Gynecology, First Faculty of Medicine and General University Hospital, Charles University in Prague, Studnickova 2, Prague 2 12800, Czech Republic.

Received: 11 February 2015 Accepted: 9 March 2015
Published online: 25 March 2015

References

1. Barbacci E, Chalkiadaki A, Masdeu C, Haumaitre C, Lokmane L, Loirat C, et al. HNF1β/TCF2 mutations impair transactivation potential through altered co-regulator recruitment. Hum Mol Genet. 2004;13:3139–49.
2. Cereghini S. Liver-enriched transcription factors and hepatocyte differentiation. FASEB J. 1996;10:267–82.
3. De Simone V, De Magistris L, Lazzaro D, Gerstner J, Monaci P, Nicosia A, et al. LFB3, a heterodimer-forming homeoprotein of the LFB1 family, is expressed in specialized epithelia. EMBO J. 1991;10:1435–43.
4. Haumaitre C, Reber M, Cereghini S. Functions of HNF1 family members in differentiation of the visceral endoderm cell lineage. J Biol Chem. 2003;278:40933–42.
5. Lazzaro D, De Simone V, De Magistris L, Lehtonen E, Cortese R. LFB1 and LFB3 homeoproteins are sequentially expressed during kidney development. Development. 1992;114:469–79.
6. Yamamoto S, Tsuda H, Aida S, Shimazaki H, Tamai S, Matsubara O. Immunohistochemical detection of hepatocyte nuclear factor 1β in ovarian and endometrial clear-cell adenocarcinomas and nonneoplastic endometrium. Hum Pathol. 2007;38:1074–80.
7. Kajihara H, Yamada Y, Shigetomi H, Higashiura Y, Kobayashi H. The dichotomy in the histogenesis of endometriosis-associated ovarian cancer: clear cell-type versus endometrioid-type adenocarcinoma. Int J Gynecol Pathol. 2012;31:304–12. doi:10.1097/PGP.0b013e318243a97b.
8. Park KJ, Kiyokawa T, Soslow RA, Lamb CA, Oliva E, Zivanovic O, et al. Unusual endocervical adenocarcinomas: an immunohistochemical analysis with molecular detection of human papillomavirus. Am J Surg Pathol. 2011;35:633–46. doi:10.1097/PAS.0b013e318215346b9.
9. Kenny SL, McBride HA, Jamison J, McCluggage WG. Mesonephric adenocarcinomas of the uterine cervix and corpus: HPV-negative neoplasms that are commonly PAX8, CA125, and HMGA2 positive and that may be immunoreactive with TTF1 and hepatocyte nuclear factor 1-β. Am J Surg Pathol. 2012;36:799–807. doi:10.1097/PAS.0b013e31824a72c6.
10. Cereghini S, Ott MO, Power S, Maury M. Expression patterns of vHNF1 and HNF1 homeoproteins in early postimplantation embryos suggest distinct and sequential developmental roles. Development. 1992;116:783–97.
11. Bellanné-Chantelot C, Chauveau D, Gautier JF, Dubois-Laforgue D, Clauin S, Beaufils S, et al. Clinical spectrum associated with hepatocyte nuclear factor-1β mutations. Ann Intern Med. 2004;140:510–7.
12. Lokmane L, Haumaitre C, Garcia-Villalba P, Anselme I, Schneider-Maunoury S, Cereghini S. Crucial role of vHNF1 in vertebrate hepatic specification. Development. 2008;135:2777–86. doi: 10.1242/dev.023010.
13. Kato N, Tamura G, Motoyama T. Hypermethylation of hepatocyte nuclear factor-1β (HNF-1β) CpG island in clear cell carcinoma of the ovary. Virchows Arch. 2008;452:175–80.
14. Kim L, Liao J, Zhang M, Talamonti M, Bentrem D, Rao S, et al. Clear cell carcinoma of the pancreas: histopathologic features and a unique biomarker hepatocyte nuclear factor-1beta. Mod Pathol. 2008;21:1075–83. doi:10.1038/modpathol.2008.95.
15. Buchner A, Castro M, Hennig A, Popp T, Assmann G, Stief CG, et al. Downregulation of HNF-1B in renal cell carcinoma is associated with tumor

progression and poor prognosis. Urology. 2010;76:507. doi:10.1016/j.urology.2010.03.042.
16. Terasawa K, Toyota M, Sagae S, Ogi K, Suzuki H, Sonoda T, et al. Epigenetic inactivation of TCF2 in ovarian cancer and various cancer cell lines. Br J Cancer. 2006;94:914–21.
17. Tsuchiya A, Sakamoto M, Yasuda J, Chuma M, Ohta T, Ohki M, et al. Expression profiling in ovarian clear cell carcinoma: identification of hepatocyte nuclear factor-1 β as a molecular marker and a possible molecular target for therapy of ovarian clear cell carcinoma. Am J Pathol. 2003;163:2503–12.
18. Kato N, Sasou S, Motoyama T. Expression of hepatocyte nuclear factor-1β (HNF-1β) in clear cell tumors and endometriosis of the ovary. Mod Pathol. 2006;19:83–9.
19. Kato N, Motoyama T. Hepatocyte nuclear factor-1beta(HNF-1beta) in human urogenital organs: its expression and role in embryogenesis and tumorigenesis. Histol Histopathol. 2009;24:1479–86.
20. Fadare O, Liang SX. Diagnostic utility of hepatocyte nuclear factor 1-beta immunoreactivity in endometrial carcinomas: lack of specificity for endometrial clear cell carcinoma. Appl Immunohistochem Mol Morphol. 2012;20:580–7. doi:10.1097/PAI.0b013e31824973days.
21. Kalloger SE, Köbel M, Leung S, Mehl E, Gao D, Marcon KM, et al. Calculator for ovarian carcinoma subtype prediction. Mod Pathol. 2011;24:512–21. doi:10.1038/modpathol.2010.215.
22. Tomassetti A, De Santis G, Castellano G, Miotti S, Mazzi M, Tomasoni D, et al. Variant HNF1 modulates epithelial plasticity of normal and transformed ovary cells. Neoplasia. 2008;10:1481–92.
23. Worley MJ, Welch WR, Berkowitz RS, Ng SW. Endometriosis-associated ovarian cancer: a review of pathogenesis. Int J Mol Sci. 2013;14:5367–79. doi:10.3390/ijms14035367.
24. Dumoff KL, Chu C, Xu X, Pasha T, Zhang PJ, Acs G. Low D2-40 immunoreactivity correlates with lymphatic invasion and nodal metastasis in early-stage squamous cell carcinoma of the uterine cervix. Mod Pathol. 2005;18:97–104.
25. Chuang WY, Yeh CJ, Wu YC, Chao YK, Liu YH, Tseng CK, et al. Tumor cell expression of podoplanin correlates with nodal metastasis in esophageal squamous cell carcinoma. Histol Histopathol. 2009;24:1021–7.
26. Yuan P, Temam S, El-Naggar A, Zhou X, Liu DD, Lee JJ, et al. Overexpression of podoplanin in oral cancer and its association with poor clinical outcome. Cancer. 2006;107:563–9.
27. Toki T, Yajima A. Immunohistochemical localization of carcinoembryonic antigen (CEA) in squamous cell carcinoma of the uterine cervix: prognostic significance of localization pattern of CEA. Tohoku J Exp Med. 1991;165:25–32.
28. Shen K, Yueng W, Ngan H. Estrogen and progesterone receptors in normal cervix and primary cervical carcinoma. Chin Med J (Engl). 1994;107:648–52.

4.2. Expression of Glut-1 in Normal Endometrium and Endometrial Lesions.

Original Article

International Journal of Surgical Pathology
1–8

DOI: 10.1177/1066896916683510
ijs.sagepub.com
SAGE

Expression of Glut-1 in Normal Endometrium and Endometrial Lesions: Analysis of 336 Cases

Kristýna Němejcová, MD, PhD[1], Jana Rosmusová, MD[1], Michaela Bártů, MD[1], Miroslav Důra, MD[1,2], Ivana Tichá, PhD[1], and Pavel Dundr, MD, PhD[1]

Abstract

Background: Glucose transporter-1 (Glut-1) is a membrane glycoprotein that is, together with other glucose transporters, responsible for the regulation of glucose uptake. An increased expression of this protein seems to be a general feature of several malignant tumors that are able to reprogram their metabolism and switch from oxidative phosphorylation to aerobic glycolysis. **Methods:** We performed comprehensive immunohistochemical analysis of Glut-1 expression in 336 endometrial samples, including tumors, nontumor lesions, and normal tissues. **Results:** Expression of Glut-1 was found in 87% of endometrioid carcinomas (160/184 cases), 100% of serous carcinomas (29/29 cases), 100% of clear cell carcinomas (17/17 cases), 50% of polyps with atypical hyperplasia (8/16 cases), 12.5% of polyps with non-atypical hyperplasia (3/24 cases), 77% of hyperplasias with atypias (10/13 cases), 9% of hyperplasias without atypias (1/11 cases), 87% of secretory endometrium samples (13/15 cases), and in none of the nonsecretory endometrium samples (0/27 cases). In endometrioid carcinomas, Glut-1 was expressed in a marked geographical pattern. In nontumor lesions, its expression was more common in atypical hyperplasia and polyps with atypical hyperplasia compared with polyps with non-atypical hyperplasia and hyperplasias without atypia (P = .00032). **Conclusion:** Our study confirms the high expression of Glut-1 not only in endometrioid carcinomas but also in other carcinomas of endometrium including clear cell and serous types. Glut-1 expression can be used as a surrogate marker in differential diagnosis between hyperplasia with and without atypia. Because of common Glut-1 expression in malignant tumors, therapeutic strategies influencing this protein or its signaling pathways can be beneficial.

Keywords

endometrium, hyperplasia, clear cell carcinoma, serous carcinoma, endometrioid carcinoma, Glut-1, immunohistochemistry

Introduction

In most mammalian cells, the uptake of glucose is mediated by members of Glut (SLC2A) family of membrane transport proteins. This family includes 14 Glut proteins; however, only Glut-1-4 are well established with known substrates.[1] In tumor tissue, one of the most important transporters largely responsible for glucose uptake is glucose transporter-1 (Glut-1). An increased expression of this protein can be detected in several malignant tumors and seems to constitute an adverse prognostic factor associated with higher tumor grade, higher stage, as well as lymphovascular space/lymph node involvement.[2-4] Contrasting with these findings, with the exception of some cells such as erythrocytes, perineurial cells, trophoblastic cells, reactive germinal centers, squamous epithelium, renal tubules, and some endothelial cells, immunohistochemically proven expression of Glut-1 in nontumor tissue and benign tumors is relatively rare and usually weak.[4,5] Nevertheless, there are several studies in which Glut-1 expression was detected by western blot also in all normal and nonneoplastic tissues; however, the intensities of Glut-1 expression were significantly lower than those found in neoplastic tissue.[6-8] Currently, only a few studies have analyzed endometrial lesions and focused mostly on endometrial and cervical carcinoma.[3,9,10] Glut-1 has been demonstrated to be expressed to different extents in both tumor and nontumor tissues. Nevertheless, all studies are limited by the small size of the examined sample set. The goals of the present study were (*a*) to analyze the

[1]Department of Pathology, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic
[2]Department of Dermatovenerology, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic

Corresponding Author:
Pavel Dundr, Department of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Studnickova 2, Prague 2 12800, Czech Republic.
Email: pdundr@seznam.cz

Table 1. Characteristics of 294 Patients With Different Endometrial Lesions and 42 Controls.

	EC									Normal Endometrium		
	G1	G2	G3	SC	CCC	HPA	HP	HA	H	Sec.	Pro.	Atr.
Total	77	78	29	29	17	16	24	13	11	15	16	11
Age (mean)	63	68	69	67	67	61	55	67	60	34	36	63
LVSI	16	27	14	15	10	NA	NA	NA	NA	NA	NA	NA
LN metastases	2/20	9/31	4/16	12/20	6/13	NA	NA	NA	NA	NA	NA	NA
Distant metastases	0	0	2	4	1	NA	NA	NA	NA	NA	NA	NA
Stage IA	40	31	9	5	4	NA	NA	NA	NA	NA	NA	NA
Stage IB	22	25	10	4	2	NA	NA	NA	NA	NA	NA	NA
Stage II	8	10	3	3	4	NA	NA	NA	NA	NA	NA	NA
Stage IIIA	4	3	1	2	0	NA	NA	NA	NA	NA	NA	NA
Stage IIIB	0	1	0	0	0	NA	NA	NA	NA	NA	NA	NA
Stage IIIC	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA
Stage IIIC1	2	8	3	9	5	NA	NA	NA	NA	NA	NA	NA
Stage IIIC2	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA
Stage IVA	0	0	1	0	0	NA	NA	NA	NA	NA	NA	NA
Stage IVB	0	0	2	4	1	NA	NA	NA	NA	NA	NA	NA

Abbreviations: EC, endometrioid carcinomas; G1, well differentiated carcinoma; G2, moderately differentiated carcinoma; G3, poorly differentiated carcinoma; SC, serous carcinoma of endometrium; CCC, endometrial clear cell carcinoma; HPA, polyps with atypical hyperplasia; HP, polyps with non-atypical hyperplasia; HA, hyperplasias with atypias; H, hyperplasias without atypias; Sec., secretory; Pro., proliferative; Atr., atrophic; LN metastases, metastases to the lymph nodes (number of cases with positive lymph node/total number cases where lymphadenectomy was performed); NA, not applicable.

expression of Glut-1 in 336 different endometrial tumors, nontumor lesions, and normal tissues, which is, so far, the most extensive published sample set; and (*b*) to find potential use of Glut-1 expression in the question of differential diagnostics between typical and atypical hyperplasia.

Material and Methods

Formalin-fixed paraffin-embedded tissue blocks were obtained from the archive files processed at our department. A review of the hematoxylin and eosin–stained slides was performed for all the involved cases. In total, 336 formalin-fixed paraffin-embedded specimens were selected for further immunohistochemical analysis, comprising of 184 endometrioid carcinomas (ECs), 29 serous carcinomas (SCs), 17 clear cell carcinomas (CCCs), 16 polyps with atypical hyperplasia, 24 polyps with non-atypical hyperplasia, 13 hyperplasias with atypias, 11 hyperplasias without atypias, and 42 samples of normal endometrium in various phases of the endometrial cycle (16 proliferative endometrium, 15 secretory endometrium, and 11 atrophic endometrium).

The patients' characteristics are summarized in Table 1. The mean age of all the patients was 62 years (range = 20-90 years), the mean age of patients with endometrial carcinomas was 66 years (range = 28-90 years), and the mean age of patients with endometrial lesions and normal endometrium was 53 years (range = 20-86 years). Endometrial carcinomas were classified according to the criteria of the International Federation of Gynecology and Obstetrics. The cases of endometrial carcinomas included 89 patients with stage IA, 63 with stage IB, 28 with stage II, 10 with stage IIIA, 1 with stage IIIB, 27 with stage IIIC, 1 with stage IVA, and 7 with stage IV (4 of the curettage samples could not be classified because the following surgical procedure was not performed within our institution and biopsy reports were not available to us). Histological type and grade of all tumors were assessed according to the World Health Organization classification.[11] EC comprised 77 well-differentiated (G1), 78 moderately differentiated (G2), and 29 poorly differentiated (G3) tumors. Lymphovascular invasion was present in 82 cases, 57/184 (31%) ECs, 10/17 (59%) CCCs, and 15/29 (52%) serous carcinomas. A lymphadenectomy procedure was performed in 100 patients, and in 33 cases metastases to the lymph node(s) were found (predominantly in SCs). Distant metastases were found in 7 patients comprising 4 with SC, 2 with grade 3 EC, and 1 with CCC. In compliance with the Helsinki Declaration, the project has been approved by Ethics Committee of General University Hospital in Prague.

Immunohistochemical Analysis

Immunohistochemical analysis was performed using the avidin-biotin complex method with antibody against the Glut-1 (polyclonal, 1:100; Cell Marque, Rocklin, CA). Antigen retrieval was performed including pretreatment in

Table 2. H-Score Scale and Calculation.

	IHC Staining Glut-1	H-Score Scale
0	Negative	0-29
1+	Weak positivity	30-99
2+	Moderate positivity	100-199
3+	Strong positivity	200-300

Abbreviations: IHC, immunohistochemical staining; H-score = [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)].

0.01 M citrate buffer (pH 9.0) for 40 minutes in a water bath at 98°C. Only the membranous staining was regarded as positive. The expression of Glut-1 was then evaluated by 2 independent pathologists. Immunohistochemical results were assessed semiquantitatively with the use of an H-score method as described previously.[12] The H-score combines the percentage of positive cells and staining intensity level (weak 1+, moderate 2+, strong 3+). The score for each sample was calculated using the following formula: [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)]. The final score ranged from 0 to 300. Finally, we classified the samples on the basis of a discriminatory threshold (Tables 2 and 3), which we assessed as follows: specimens with scores 0 to 29, 30 to 99, 100 to 199, and 200 to 300 were classified as having negative, weak, moderate, and strong positivity, respectively.

Statistical Analysis

Software STATISTICA 10 (StatSoft, Tulsa, OK) was used for data analysis. The χ^2 test was applied to evaluate differences in Glut-1 expression and other qualitative variables between different groups of samples. All tests were 2-sided, and a P value <.05 was considered as significant.

Results

Immunohistochemical Findings

All the results are summarized in Table 4. Figure 1 shows representative examples of the Glut-1 expression in normal endometrium and endometrial tumors.

An expression of Glut-1 was found in 160/184 (87%) of all ECs with more pronounced expressions found in less differentiated tumors; however, the difference in Glut-1 expression among groups with no, week, moderate, and strong Glut-1 expression is not statistical significant in our sample set (P = .13004). ECs showed positivity of Glut-1 in 63/77 (82%) of grade 1 tumors, 69/78 (89%) of grade 2 tumors, and 28/29 (96.5%) of grade 3 tumors. Only 24/184 (13%) cases were negative. In the group of well-differentiated ECs, the expression was weak in 31/77 (40%) cases, moderate in 29/77 (38%) cases, and strong in 3/77 (4%) cases. Fourteen of 77 (18%) cases were negative. In moderately differentiated ECs, the expression was weak in 31/78 (40%) cases, moderate in 34/78 (44%) cases, and strong in 4/78 (5%) cases. Nine out of 78 (12%) cases were negative. In poorly differentiated tumors, the expression was weak in 7/29 (24%) cases, moderate in 18/29 (62%) cases, and strong in 3/29 (10%) cases. Only 1 case from this group (1/29; 3%) was negative. Positive stromal cells staining was found in the total of only 8 out of 184 cases of ECs, with a weak to moderate intensity of staining.

Serous carcinomas were Glut-1 positive in all cases (29/29; 100%). The expression was weak in 6 cases, moderate in 17 cases, and strong in 6 cases. Two cases demonstrated weak positivity located also in stromal cells. Different pattern of distribution of Glut-1 expression was found in 4/29 (14%) cases, where the positivity was found predominantly in papillary surface, without apparent relation to areas of necrosis or distance from stromal tissue.

Clear cell carcinomas showed positivity of Glut-1 in all 17/17 (100%) cases. Positivity was weak in 4 cases, moderate in 6 cases, and strong in 7 cases. There was an apparent type of zonation found in 6 cases, but similarly to SCs the positivity of staining was mostly pronounced along the surface portions of tumors without any relation to necrosis or distance from the tumor stroma. No case of CCC showed positivity of stromal cells.

To summarize, the expression of Glut-1 in tumor samples was detected in 206/230 (90%) of all endometrial carcinomas. Interestingly, we found much stronger expression (concerning both the intensity and extensity) of Glut-1 in areas found distant from the tumor stroma, as well as in necrotic areas. This finding was especially pronounced in well and moderately differentiated ECs, where this type of expression was detected in 145/184 (79%) cases. Regarding other tumor types, Glut-1 expression was rather heterogeneous and without the presence of apparent zonation.

Samples of nontumor endometrial lesions and normal endometrium showed positivity in 35/106 (33%) cases. Precancerous lesions were Glut-1 positive in 18/29 (62%) cases, including 8/16 (50%) polyps with atypical hyperplasia and 10/13 (77%) hyperplasias with atypias. The expression was weak in 14 cases and moderate in 4 cases.

In hyperplastic endometrium without atypias (hyperplasias and polyps), Glut-1 positivity was found in 4/35 (11%) cases, including weak expression in 3/24 (12.5%) polyps with non-atypical hyperplasia and 1/11 (9%) hyperplastic endometrium. All of the 4 positive samples showed only weak positivity. The one positive case of hyperplasia without atypias (H-score 90) showed a few isolated scattered glands with nuclear enlargement, rounded and focally distinctive nucleoli; however, these attributes were not sufficient enough to classify the lesion as atypical hyperplasia. In some cases, we also found a weak to moderate positivity

Table 3. Discriminatory Thresholds for Negativity and Positivity in Hyperplastic Endometrial Lesions[a].

H-Score	HPL Positive	HPL Total	Positivity %	AHPL Positive	AHPL Total	Positivity %	Sensitivity %	Specificity %
50	2	35	6	10	29	34	34	94
45	2	35	6	12	29	41	41	94
40	2	35	6	14	29	48	48	94
35	4	35	11	16	29	55	55	89
30	**4**	**35**	**11**	**18**	**29**	**62**	**62**	**89**
25	6	35	17	20	29	69	69	83
20	9	35	26	20	29	69	69	74
15	11	35	31	21	29	72	72	69
10	14	35	40	23	29	79	79	60

Abbreviations: HPL, hyperplastic lesion without atypias (polyps with non-atypical hyperplasia + hyperplasias without atypias); AHPL, atypical hyperplastic lesion (polyps with atypical hyperplasia + hyperplasias with atypias).
[a]Most suitable threshold is indicated in boldface.

Table 4. Summary of Immunohistochemical Results[a].

	EC									Normal Endometrium		
Glut-1	G1	G2	G3	SC	CCC	HPA	HP	HA	H	Sec.	Pro.	Atr.
Total	77	78	29	29	17	16	24	13	11	15	16	11
Positive	63	69	28	7	17	8	3	10	1	13	0	0
3+	3	4	3	6	7	0	0	0	0	0	0	0
2+	29	34	18	17	6	1	0	3	0	3	0	0
1+	31	31	7	6	4	7	3	7	1	10	0	0
Negative	14	9	1	0	0	8	21	3	10	2	16	11

Abbreviations: CCC, endometrial clear cell carcinoma; SC, serous carcinoma of endometrium; EC, endometrioid carcinoma; HPA, polyps with atypical hyperplasia; HP, polyps with non-atypical hyperplasia; HA, hyperplasias with atypias; H, hyperplasias without atypias; Sec., secretory; Pro., proliferative; Atr., atrophic; G1, well-differentiated carcinoma; G2, moderately differentiated carcinoma; G3, poorly differentiated carcinoma.
[a]Immunohistochemical results were assessed on the basis of discriminatory thresholds: specimens with scores 0 to 29, 30 to 99, 100 to 199, and 200 to 300 were classified, respectively, as having negative, weak 1+, moderate 2+, or strong 3+ positivity.

of stromal cells (3/24 hyperplastic endometrium and 3/11 polyps with non-atypical hyperplasia).

Samples from normal endometrium showed positivity of Glut-1 in 0/16 (0%) cases of proliferative endometrium, in 13/15 (87%) cases of secretory endometrium (10 cases weak and 3 cases moderate positivity), and in 0/11 (0%) of atrophic endometrium. Regarding the secretory endometrium, the intensity of staining appeared to be mostly weak to moderate. In some cases of proliferative and secretory endometrium, the review also found a weak to moderate positivity of stromal cells (9/16 proliferative and 10/15 secretory endometrium samples).

A few cases of nontumor endometrial lesions and normal endometrium showed positivity of Glut-1 located within the surface epithelium. These findings were pronounced mostly in proliferative endometrium (6/16; 37.5% cases), polyps with atypical hyperplasia (3/16; 19% cases), and polyps with non-atypical hyperplasia (11/24; 46% cases). However, we did not consider these cases to be truly positive.

Discussion

Changes in tumor glucose metabolism seem to represent a very important metabolic aspect of several tumors. When focusing on glucose metabolism, the characteristic feature of malignant tumors is their ability to reprogram glucose metabolism in such a way that it causes a shift from oxidative phosphorylation pathway to glucose aerobic glycolysis (Warburg's effect).[13,14] This feature represents a selective growth advantage for several reasons, including resistance to apoptosis, acidification of tumor microenvironment favoring the selection of more aggressive and invasive tumor cells, and much higher speed of ATP production. However, the process of glycolysis when compared to oxidative phosphorylation actually results in significantly lower production of ATP molecules (2 ATPs vs 32 ATPs).[15]

Nevertheless, tumor cells are able to compensate for this energy deficit by upregulating glucose transporters expression, especially Glut-1, which allows for an increased glucose uptake.[16] The mechanism of reprogramming the cellular metabolism are various and include mitochondrial damage, stabilization of hypoxia-inducible factor-1α (HIF-1α), inactivation of tumor suppressors such as p53 and PTEN, and upregulation of several oncogenes of the PI3K/Akt/mTOR signaling pathway.[17,18] The impact of these changes is complex and includes not only the upregulation of glucose transporters but also resistance to apoptosis and promotion of further proliferation. The most important glucose transporter in tumor cells seems to be Glut-1.

The expression of Glut-1 is influenced by certain growth factors such as insulin-like growth factor-1 and tumor necrosis factor-α, and downstream effectors of mTOR, particularly HIF-1α and c-myc.[19,20] These effectors also play important role in the adaptive cellular response to hypoxia.[21]

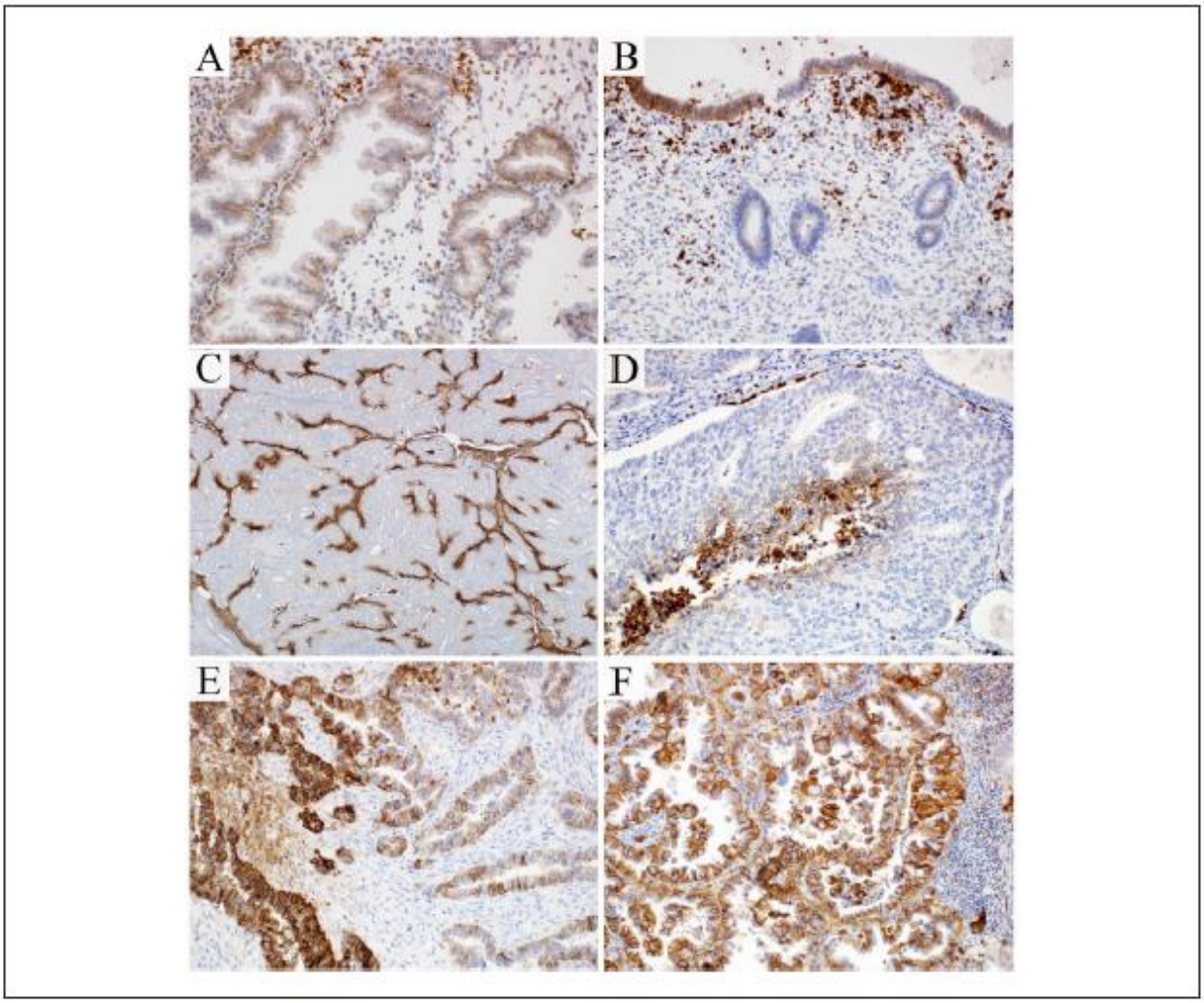


Figure 1. Glut-1 expression in endometrial lesions. (A) Secretory endometrium showing weak Glut-1 expression (200×). (B) Negativity of Glut-1 in proliferative endometrium. Note the expression of Glut-1 in superficial cells (100×). (C) Glut-1 expression in endometrioid carcinoma (G2). Note the zonation pattern of positivity (20×). (D) Higher magnification showing Glut-1 expression in endometrioid carcinoma (G2). Note the positivity of tumor cells in areas of necrosis distant from the tumor stroma (200×). (E) Glut-1 expression in endometrial serous carcinoma (200×). (F) Glut-1 expression in clear cell carcinoma (200×).

Increased expression of Glut-1 was already described in several malignant tumors, including lung, breast, pancreatic, esophageal, colorectal, and head and neck cancers.[22-27] Concerning the female genital tract, the studies of Glut-1 expression to date focused predominantly on endometrial and cervical lesions,[3,28,29] but the expression has been analyzed also in different types of ovarian tumors.[30] For example, an absence of Glut-1 staining in cervical cancer significantly increased metastasis-free survival but did not affect disease-free survival or progression-free survival.[28] The prognostic significance of Glut-1 expression in endometrial cancer is equivocal. In one study, the expression of Glut-1 was correlated with shorter disease-free survival.[31] However, another study failed to find any prognostic significance of this expression.[32] In these studies, the evaluated tumors were classified only as positive or negative without further subclassification based on the extent of Glut-1 expression. The prognostic value of Glut-1 expression in endometrial carcinomas should be, however, correlated with the extent and intensity of staining or carefully thresholded, because several studies reported at least focal Glut-1 expression in up to 100% of tumor cases of all types of endometrial carcinoma.[10,29] The protein expression was assessed immunohistochemically and further confirmed by western blot assay and in one study also by mRNA expression analysis.[6-8] Based on these data, the expression of

Glut-1 can be used as a prognostic factor, but the extent of described positivity should also be considered.[6,33] Moreover, there is a statistically significant difference in the expression of Glut-1 among various endometrial lesions.[34] Normal endometrium shows positive Glut-1 expression only rarely and if so in limited extent, which is mostly varied between 0% and 3.3% of samples.[3,10,34] However, in one study the authors reported immunofluorescence detection of Glut-1 in all of the examined samples of normal endometrium.[6] In our study, we have found an expression of Glut-1in 87% of secretory endometrium samples, but in none of the proliferative endometrium samples. In nonneoplastic tissue, Glut-1 can be detected in 0% to 24% of hyperplastic endometrium without atypias, but the percentage increases up to 71% when atypical hyperplasias are concerned.[10,34] According to one study, an expression of Glut-1 alone, however, does not seem to constitute an independent marker of increased risk of recurrence.[35] Interestingly, a few reports described an increased Glut-1 expression in those tumor areas close to necrotic regions or distant from stromal (mesenchymal) tissue, possibly correlating the expression with areas of hypoxia.[10]

Glut-1 expression could be potentially used not only as a prognostic marker but also as a diagnostic surrogate marker to aid with distinction between typical and atypical hyperplasia, which is in concordance with our results.[10] However, the sensitivity and specificity in this setting is variable according to previous studies.[10,36] This can be also influenced by the considerable interobserver and intraobserver variations in diagnosis of atypical hyperplasia.[37,38] Due to the above-mentioned variability in diagnosis of atypical hyperplasia, Glut-1 could be potentially used rather as an indicator of hyperplasia with higher risk of EC development.[10] However, further studies specifically devoted to this issue are needed.

Based on the fact that aerobic glycolysis found in transformed tumor cells represents a selective growth advantage, interference with aerobic glycolysis represents a potentially effective strategy to influence cancer cells. Seeing as the expression of Glut-1 in malignant tumors seems to be rather common, this protein and components of its signaling pathway Akt/mTOR, which influence its expression, seem to represent the best potential targets. In a study focused on cell cultures of lung and breast cancers, it has been shown that specific antibodies aimed against Glut-1 inhibited proliferation by 50% and 75%, respectively.[39] Moreover, increased apoptosis and potentiation of chemotherapy effects were noted in this study. The mTOR pathway inhibitors (rapamycin analogues) seem to be effective in patients with advanced endometrial cancer and inhibition of the PI3K/Akt/mTOR pathway may represent a possible therapeutic approach.[40,41] Another therapeutic approach includes competitive inhibition of glucose uptake and utilization with the use of a glucose analogue 2-deoxy-D-glucose (2DG).[42]

Our results are similar to several previously published studies.[3,36] In normal endometrium, we found Glut-1 expression only in samples of secretory endometrium. Regarding the potential use of Glut-1 expression in the differential diagnostics of different hyperplasias, Glut-1 expression was found in 11% of hyperplasias without atypia and in 62% of atypical hyperplasias (Table 3). We then attempted to assess a threshold for discriminating between these 2 groups based on the H-score of Glut-1 expression. We found only a weak expression in 4 (11%) positive cases out of 35 hyperplasias without atypia. Moreover, one of these cases showed few isolated scattered glands displaying nuclear atypias not quite sufficient to classify the lesion as atypical hyperplasia. In Glut-1 positive atypical hyperplasias the expression was weak in 14/29 (48%) cases and moderate in 4/29 (14%) cases. Based on our results, if we compare the H-score of Glut-1 expression found in hyperplasias with and without atypias, the best cutoff point for their discrimination seems to be a score of 30, which showed the best sensitivity and specificity in our sample set (Table 3).

We therefore surmise that expression of Glut-1 can be used as a surrogate marker in the distinction between hyperplasias with and without atypias. However, the extent of expression should be carefully thresholded. Even though such a conclusion is in agreement with the results of some previous studies,[10,34] we are well aware of the small sample set of hyperplasia samples in our study, and more data are needed for a definite confirmation of our conclusions.

In contrast with other studies, the extent of Glut-1 expression in ECs was not strongly correlated with increased grade and stage of the tumors. By applying the H-score and threshold at 30, we observed a trend of increased expression of Glut-1 with higher grade of ECs ($P = .13004$). When the discriminatory threshold was set at 99, the samples divided into 2 groups where all negative and weak positive cases (H-score 0-99) are considered as negative and all cases with moderate and strong Glut-1 positivity (H-score 100-300) are considered as positive, and the trend of higher Glut-1 expression in less differentiated tumors is stronger ($P = .01784$). We found positivity in up to 89% of well-differentiated and moderately differentiated ECs (82% in G1 and 89% in G2 ECs, mostly weak and moderate positivity), compared to 96.5% positivity in G3 ECs and 100% positivity in SCs and CCCs. Higher staining intensity was also observed in a group of high-grade carcinomas, including endometrioid grade 3, SCs and CCCs of the endometrium. In most tumors with endometrioid differentiation (79%), the expression showed geographical pattern, mostly restricted to areas distant from supportive stroma, in the center of tumor nests, especially pronounced in well-differentiated and moderately differentiated tumors. This was, however, different in the group of tumors with serous or clear cell differentiation,

where the expression of Glut-1 was more heterogeneous with variable extent and without apparent zonation relating to necrosis or distance from stromal tissue.

We observed some weak to moderate positivity of Glut-1 in stromal cells, mainly in nonneoplastic tissue (27%) and then predominantly in some cases of secretory and proliferative endometrium; however, the same positivity was present in only a few carcinomas (4%). Glut-1 positivity of stromal cells was also previously described in a study devoted to endometrial pregnancy changes, which showed increased Glut-1 expression on both mRNA and protein level, present throughout the process of decidualization.[43]

In conclusion, we performed a comprehensive analysis of Glut-1 expression in normal endometrium and in various endometrial lesions on the largest series of cases reported to date. Because the expression of Glut-1 is common in malignant tumors, therapeutic strategies influencing this protein or its signaling pathways could be beneficial. Our study confirms high expression of Glut-1 not only in ECs but also in other carcinomas of endometrium including clear cell and serous types. From the diagnostic point of view, Glut-1 expression can be used as a surrogate marker in differential diagnosis between hyperplasia with and without atypia.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Ministry of Health, Czech Republic—Conceptual Development of Research Organization 64165, General University Hospital in Prague, Czech Republic, by Charles University in Prague (Project PRVOUK-P27/LF1/1, UNCE 204024, and SVV UK 260256/2016), by research project LM 2015089, and by OPPK (Research Laboratory of Tumor Diseases, CZ.2.16/3.1.00/24509).

References

1. Thorens B, Mueckler M. Glucose transporters in the 21st century. *Am J Physiol Endocrinol Metab*. 2010;298:E141-E145.
2. Iwasaki K, Yabushita H, Ueno T, Wakatsuki A. Role of hypoxia-inducible factor-1alpha, carbonic anhydrase-IX, glucose transporter-1 and vascular endothelial growth factor associated with lymph node metastasis and recurrence in patients with locally advanced cervical cancer. *Oncol Lett*. 2015;10:1970-1978.
3. Ma X, Hui Y, Lin L, Wu Y, Zhang X, Liu P. Clinical significance of COX-2, GLUT-1 and VEGF expressions in endometrial cancer tissues. *Pak J Med Sci*. 2015;31:280-284.
4. Younes M, Lechago LV, Somoano JR, Mosharaf M, Lechago J. Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers. *Cancer Res*. 1996;56:1164-1167.
5. Voldstedlund M, Dabelsteen E. Expression of GLUT1 in stratified squamous epithelia and oral carcinoma from humans and rats. *APMIS*. 1997;105:537-545.
6. Goldman NA, Katz EB, Glenn AS, et al. GLUT1 and GLUT8 in endometrium and endometrial adenocarcinoma. *Mod Pathol*. 2006;19:1429-1436.
7. Krzeslak A, Wojcik-Krowiranda K, Forma E, et al. Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. *Pathol Oncol Res*. 2012;18:721-728.
8. Kuiper C, Molenaar IG, Dachs GU, Currie MJ, Sykes PH, Vissers MC. Low ascorbate levels are associated with increased hypoxia-inducible factor-1 activity and an aggressive tumor phenotype in endometrial cancer. *Cancer Res*. 2010;70:5749-5758.
9. Jo MS, Choi OH, Suh DS, et al. Correlation between expression of biological markers and [F]fluorodeoxyglucose uptake in endometrial cancer. *Oncol Res Treat*. 2014;37:30-34.
10. Wang BY, Kalir T, Sabo E, Sherman DE, Cohen C, Burstein DE. Immunohistochemical staining of GLUT1 in benign, hyperplastic, and malignant endometrial epithelia. *Cancer*. 2000;88:2774-2781.
11. Kurman RJ, Carcangiu ML, Herrington CS, Young RH, eds. *World Health Organization. Classification of Tumours of the Female Reproductive Organs*. 4th ed. Lyon, France: IARC Press; 2014:123-131.
12. Hirsch FR, Varella-Garcia M, Bunn PA, et al. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J Clin Oncol*. 2003;21:3798-3807.
13. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
14. Klement RJ, Kammerer U. Is there a role for carbohydrate restriction in the treatment and prevention of cancer? *Nutr Metab (Lond)*. 2011;8:75.
15. Annibaldi A, Widmann C. Glucose metabolism in cancer cells. *Curr Opin Clin Nutr Metab Care*. 2010;13:466-470.
16. Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol*. 2005;202:654-662.
17. Pelicano H, Xu RH, Du M, et al. Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechanism. *J Cell Biol*. 2006;175:913-923.
18. Robey RB, Hay N. Is Akt the "Warburg kinase"? Akt-energy metabolism interactions and oncogenesis. *Semin Cancer Biol*. 2009;19:25-31.
19. Altomare DA, Khaled AR. Homeostasis and the importance for a balance between AKT/mTOR activity and intracellular signaling. *Curr Med Chem*. 2012;19:3748-3762.
20. Osthus RC, Shim H, Kim S, et al. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem*. 2000;275:21797-21800.
21. Greijer AE, van der Groep P, Kemming D, et al. Up-regulation of gene expression by hypoxia is mediated predominantly

by hypoxia-inducible factor 1 (HIF-1). *J Pathol*. 2005;206: 291-304.
22. Brown RS, Wahl RL. Overexpression of Glut-1 glucose transporter in human breast cancer. An immunohistochemical study. *Cancer*. 1993;72:2979-2985.
23. Carvalho KC, Cunha IW, Rocha RM, et al. GLUT1 expression in malignant tumors and its use as an immunodiagnostic marker. *Clinics (Sao Paulo)*. 2011;66:965-972.
24. Haber RS, Rathan A, Weiser KR, et al. GLUT1 glucose transporter expression in colorectal carcinoma: a marker for poor prognosis. *Cancer*. 1998;83:34-40.
25. Nagase Y, Takata K, Moriyama N, Aso Y, Murakami T, Hirano H. Immunohistochemical localization of glucose transporters in human renal cell carcinoma. *J Urol*. 1995;153:798-801.
26. Ogawa J, Inoue H, Koide S. Glucose-transporter-type-I-gene amplification correlates with sialyl-Lewis-X synthesis and proliferation in lung cancer. *Int J Cancer*. 1997;74: 189-192.
27. Yamamoto T, Seino Y, Fukumoto H, et al. Over-expression of facilitative glucose transporter genes in human cancer. *Biochem Biophys Res Commun*. 1990;170:223-230.
28. Airley R, Loncaster J, Davidson S, et al. Glucose transporter glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. *Clin Cancer Res*. 2001;7:928-934.
29. Idrees MT, Schlosshauer P, Li G, Burstein DE. GLUT1 and p63 expression in endometrial intraepithelial and uterine serous papillary carcinoma. *Histopathology*. 2006;49:75-81.
30. Cantuaria G, Fagotti A, Ferrandina G, et al. GLUT-1 expression in ovarian carcinoma: association with survival and response to chemotherapy. *Cancer*. 2001;92:1144-1150.
31. Seeber LM, Horree N, van der Groep P, van der Wall E, Verheijen RH, van Diest PJ. Necrosis related HIF-1alpha expression predicts prognosis in patients with endometrioid endometrial carcinoma. *BMC Cancer*. 2010;10:307.
32. Sadlecki P, Bodnar M, Grabiec M, et al. The role of hypoxia-inducible factor-1 alpha, glucose transporter-1, (GLUT-1) and carbon anhydrase IX in endometrial cancer patients. *Biomed Res Int*. 2014;2014:616850.
33. Lee DW, Chong GO, Lee YH, et al. Role of SUVmax and GLUT-1 expression in determining tumor aggressiveness in patients with clinical stage I endometrioid endometrial cancer. *Int J Gynecol Cancer*. 2015;25:843-849.
34. Ashton-Sager A, Paulino AF, Afify AM. GLUT-1 is preferentially expressed in atypical endometrial hyperplasia and endometrial adenocarcinoma. *Appl Immunohistochem Mol Morphol*. 2006;14:187-192.
35. Sebastiani V, Visca P, Botti C, et al. Fatty acid synthase is a marker of increased risk of recurrence in endometrial carcinoma. *Gynecol Oncol*. 2004;92:101-105.
36. Xiong Y, Xiong YY, Zhou YF. Expression and significance of beta-catenin, Glut-1 and PTEN in proliferative endometrium, endometrial intraepithelial neoplasia and endometrioid adenocarcinoma. *Eur J Gynaecol Oncol*. 2010;31:160-164.
37. Kendall BS, Ronnett BM, Isacson C, et al. Reproducibility of the diagnosis of endometrial hyperplasia, atypical hyperplasia, and well-differentiated carcinoma. *Am J Surg Pathol*. 1998;22:1012-1019.
38. Skov BG, Broholm H, Engel U, et al. Comparison of the reproducibility of the WHO classifications of 1975 and 1994 of endometrial hyperplasia. *Int J Gynecol Pathol*. 1997;16:33-37.
39. Rastogi S, Banerjee S, Chellappan S, Simon GR. Glut-1 antibodies induce growth arrest and apoptosis in human cancer cell lines. *Cancer Lett*. 2007;257:244-251.
40. Gadducci A, Tana R, Cosio S, Fanucchi A, Genazzani AR. Molecular target therapies in endometrial cancer: from the basic research to the clinic. *Gynecol Endocrinol*. 2008;24:239-249.
41. Oza AM, Pignata S, Poveda A, et al. Randomized phase II trial of ridaforolimus in advanced endometrial carcinoma. *J Clin Oncol*. 2015;33:3576-3582.
42. Zhang D, Li J, Wang F, Hu J, Wang S, Sun Y. 2-Deoxy-D-glucose targeting of glucose metabolism in cancer cells as a potential therapy. *Cancer Lett*. 2014;355:176-183.
43. Frolova A, Flessner L, Chi M, Kim ST, Foyouzi-Yousefi N, Moley KH. Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells. *Endocrinology*. 2009;150:1512-1520.

4.3. Risk of micrometastases in non-sentinel pelvic lymph nodes in cervical cancer.

Gynecologic Oncology 143 (2016) 83–86

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Risk of micrometastases in non-sentinel pelvic lymph nodes in cervical cancer

D. Cibula [a,*], M. Zikan [a], J. Slama [a], D. Fischerova [a], R. Kocian [a], A. Germanova [a], A. Burgetova [b], L. Dusek [c], P. Dundr [d], M. Gregova [d], K. Nemejcova [d]

[a] Gynecologic Oncology Center, Department of Obstetrics and Gynecology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic
[b] Department of Radiology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic
[c] Institute for Biostatistics and Analyses, Masaryk University, Brno, Czech Republic
[d] Department of Pathology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic

HIGHLIGHTS

- Sensitivity of SLN ultrastaging is high for the presence of both macrometastases and micrometastases in non-SLN pelvic lymph nodes.
- Intraoperative pathologic SLN evaluation has high false negative rate in tumors at high risk of LN involvement.
- SLN status does not represent the status of the parametrial LNs.

ARTICLE INFO

Article history:
Received 21 May 2016
Received in revised form 28 June 2016
Accepted 9 July 2016
Available online 12 July 2016

Keywords:
Sentinel lymph node
Pathologic ultrastaging
Micrometastasis

ABSTRACT

Objective. A high sensitivity of sentinel lymph nodes (SLN) for pelvic lymph node (LN) staging has been repeatedly shown in patients with cervical cancer. However, since only SLN are evaluated by pathologic ultrastaging, the risk of small metastases, including small macrometastases (MAC) and micrometastases (MIC), in non-SLN is unknown. This can be a critical limitation for the oncological safety of abandoning a pelvic lymphadenectomy.

Methods. The patients selected for the study had cervical cancer and were at high risk for LN positivity (stage IB–IIA, biggest diameter ≥ 3 cm). The patients had no enlarged or suspicious LN on pre-operative imaging; SLNs were detected bilaterally and were negative on intra-operative pathologic evaluation. All SLNs and all other pelvic LNs were examined using an ultrastaging protocol and processed completely in intervals of 150 μm.

Results. In all, 17 patients were enrolled into the study. The mean number of removed pelvic LNs was 30. A total of 573 pelvic LNs were examined through ultrastaging protocol (5762 slides). Metastatic involvement was detected in SLNs of 8 patients (1× MAC; 4× MIC; 3× ITC) and in non-SLNs in 2 patients (2× MIC). In both cases with positive pelvic non-SLNs, there were found MIC in ipsilateral SLNs. No metastasis in pelvic non-SLNs was found by pathologic ultrastaging in any of the patients with negative SLN Side-specific sensitivity was 100% for MAC and MIC. There was one case of ITC detected in non-SLN, negative ipsilateral SLN, but MIC in SLN on the other pelvic side.

Conclusions. After processing all pelvic LNs by pathologic ultrastaging, there were found no false-negative cases of positive non-SLN (MAC or MIC) and negative SLN. SLN ultrastaging reached 100% sensitivity for the presence of both MAC and MIC in pelvic LNs.

1. Introduction

The reliability of sentinel lymph node (SLN) evaluation for pelvic lymph node (LN) staging has been assessed in many single institutional studies, in large retrospective cohorts, and also in prospective multicenter studies [1–4]. In the only prospective trial, which involved pathologic SLN ultrastaging, and in which the primary end point was post-operative morbidity, there was no false-negative case for patients with bilaterally detected SLN [3]. In the largest retrospective study to date, on 645 patients, the sensitivity in an identical subgroup of patients, with bilateral detection of SLN, reached 97% [2]. In all of these studies, pathologic ultrastaging was

* Corresponding author at: Gynecologic Oncology Center, Department of Obstetrics and Gynecology, Apolinarska 18, Prague 2 120 00, Czech Republic.
E-mail address: d_cibula@yahoo.com (D. Cibula).

http://dx.doi.org/10.1016/j.ygyno.2016.07.101
0090-8258/

solely used to process SLNs. The risk of MIC in non-SLN has not been assessed in any of these studies.

Whereas pelvic LNs are, during standard pathologic evaluation, mostly processed in 2 to 3 mm slices, pathologic ultrastaging of SLNs includes additional levels in very small intervals, usually 150–250 μm. This protocol enables the detection of small metastases, which could otherwise be missed: both small macrometastases (MAC), and, especially, micrometastases (MIC). Micrometastases are not a rare finding in early-stage cervical cancer; they are found in 10–15% patients and their prevalence increases with tumor size and stage of disease, as well as the prevalence of MAC [3,5–7]. Even though the prognostic importance of low-volume disease (micrometastases (MIC) and isolated tumor cells (ITC)) has not been established yet, in a large retrospective study the presence of MIC but not ITC was associated with significantly decreased overall survival, and the survival was not different from those with MAC in LN [5].

Currently, the possibility of abandoning systematic pelvic lymphadenectomy and replacing it with SLN biopsy is broadly discussed. Even though prospective controlled trials are only being initiated, the first papers are appearing, and they report small cohorts relying solely on SLN biopsy [8,9].

The primary aim of this pilot study was to evaluate the risk of MIC in pelvic LN in patients with negative SLN. All removed LN, including non-SLN and all SLN from both sides, were processed by pathologic ultrastaging. The risk of LN involvement was increased by the selection of cases with larger tumors.

2. Methods

2.1. Selection of patients

Patients with a high risk of LN involvement but negative intraoperative pathologic SLN assessment were enrolled in the study. The following inclusion criteria were used: a) squamous cancer, adenocancer, or adenosquamous cancer of the uterine cervix confirmed by histology; b) bulky cervical tumor (≥ 3 cm of the largest diameter); c) no bulky or suspicious LNs on preoperative imaging; d) planned surgical treatment, including LN staging. Only those patients with bilateral SLN detection and negative intraoperative pathologic SLN evaluation were included.

2.2. Surgery

A combined technique with both radioactive tracer (99Tc, long protocol, application 12 h before surgery) and blue dye (application at the beginning of the surgery) was used for SLN detection either by laparoscopy or by laparotomy. The application technique was modified in cases with large tumors, as previously published (application into the residual stroma by a spinal needle, continuous control of vaginal leak when injected into the necrotic tissue) [10]. All identified SLNs were submitted for intraoperative pathologic evaluation according to a standard protocol (see below). Lymph node staging continued with a systematic pelvic lymphadenectomy. Lymph nodes were removed from 7 standard regions in the pelvis (external iliac left and right, obturator left and right, common iliac left and right, presacral). Patients with MAC detected on intraoperative assessment were excluded from further analysis. All pelvic LNs, including SLNs and non-SLNs, were processed according to the pathological protocol for SLN ultrastaging (see below).

2.3. Pathologic processing

At the time of surgery, the SLNs were cut along their longest axis and both halves of each node were examined with frozen sectioning techniques. SLNs with a diameter of <3 mm were processed as a whole and examined in the frozen section. All patients with MAC were excluded from further analysis.

After that, SLNs as well as all other non-SLNs were fixed in 10% formalin. After fixation, all LNs were sliced at 2 mm intervals and embedded in paraffin. All LNs were further examined by the ultrastaging protocol in its entirety. This protocol consisted of 2 consecutive sections (4 μm thick) obtained in regular 150 μm intervals, which were cut from each paraffin block until there was no lymph node tissue left. The first section was stained with H&E and the second section was examined immunohistochemically with antibody against cytokeratins (AE1/AE3, 1:50 dilution; Dako, Glostrup, Denmark) (Fig. 1).

The presence of MAC, MIC, and ITC was recorded and classified according to the TNM system. Macrometastasis was defined as a metastasis > 2 mm in diameter, MIC as a metastasis between 0.2 and 2 mm, and ITC as individual tumor cells or small clusters of cells <0.2 mm in diameter.

2.4. Statistics

Standard summary statistics were applied in the analyses; the median supported by the 5th–95th percentile range or by the min–max range for continuous variables and absolute and relative frequencies for categorical variables. The diagnostic power of examinations was assessed on the basis of Receiver Operating Characteristics curves. The ROC analysis was performed using a ROC calculator for the AUC computation and testing (SPSS Inc., 2012) and MedCalc 11.1.0.0 (MedCalc Software 1993–2009) was used for the computation of sensitivity and specificity. The significance of the ROC analysis was based on the calculated area under the curve (AUC), with a corresponding 95% confidence interval. The computation was based on binormal assumption.

The predictive power of the assessed examination was described by sensitivity, specificity, negative and positive predictive value, overall accuracy, and ROC-derived area under curve; all measures of predictive power were supplied by 95% confidence intervals and statistical significance.

Analyses were performed using SPSS 21 (IBM Corporation, 2012).

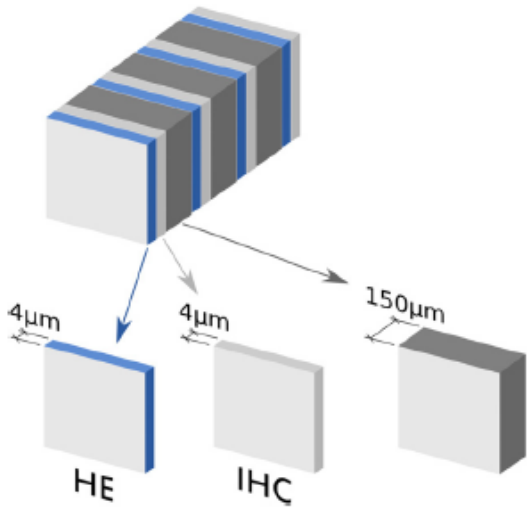


Fig. 1. Protocol for pathologic processing of SLNs and all pelvic lymph nodes. The ultrastaging protocol consisted of processing of each lymph node in the whole. Two consecutive sections (4 μm-thick) were obtained in regular 150 μm intervals. The first section was stained with H&E and the second section was examined immunohistochemically with antibody against cytokeratins (AE1/AE3).

3. Results

In all, 26 patients fulfilled the pre-operative inclusion criteria and were referred for radical surgery including LN staging. Intra-operatively, 9 patients were excluded due to unilateral SLN detection [3] or detection of MAC from frozen section [6]. 17 patients were enrolled in the final analysis. Clinical characteristics of the patients are displayed in Table 1. The large number of pelvic LNs removed from each patient (minimum 15, median 30) reflects a systematic approach to pelvic lymphadenectomy.

A total of 573 pelvic LNs were examined by ultrastaging protocol, which encompasses 5762 slides analyzed (2881H&E + 2881 AE1/AE3). Of these, 43 were SLNs and 530 were non-SLNs.

Metastases were found in 6 SLNs by ultrastaging; 1 of them was MAC (2.25 mm), 2 were MIC (0.5–1.89 mm) and ITC were detected in 3 SLNs (in 2nd–4th series).

In non-SLNs, 3 LNs with MIC were found in 2 patients. In both these patients, MIC were simultaneously detected in SLNs. In the first case, a MIC of 1.6 mm in 3rd–9th series was found in the obturator fossa; in the latter case, 3 isolated MIC were detected in 1 LN from the obturator fossa of 0.39–0.49 mm (in 9th–10th series), and ITC in 1 presacral LN (in the 4th series). A summary of the SLN and non-SLN status after ultrastaging is given in Table 2. The sensitivity of SLN ultrastaging was 100% for all types of metastases in pelvic LNs if counted per patient. The laterality of different types of metastases is shown separately for SLN and non-SLN in Table 3. The side-specific sensitivity of SLN ultrastaging reached 100% for detection of both MIC and MAC in pelvic LNs. In one case, ITC was detected in non-SLN on the side with negative SLN, while MIC was found in SLN and non-SLN on the other side of the pelvis.

In addition, in 2 patients there were MAC detected in parametrial LNs. In 1 patient with MIC in SLN, 3 MAC were found in the right and left parametrial LNs (6 mm, 5 mm, and 5 mm in diameter). In the second case without involvement of any other LN (SLN or non-SLN), MAC was found in LN from the left parametrium (2.7 mm in diameter).

4. Discussion

In a carefully selected group of 17 patients with early-stage but high-risk cervical cancer, we found in SLN through final ultrastaging ITC in 3 cases, MIC in 4 cases and MAC in 1 case, despite negative intraoperative SLN evaluation. All removed LNs, including SLN and pelvic non-SLN, were processed by pathologic ultrastaging. We found 100% sensitivity of SLN ultrastaging for both MAC and MIC in pelvic LNs. Not one single case was found with MIC in non-SLN and negative SLN. There was one side-specific false-negative case for the presence of ITC in a patient with negative ipsilateral SLN but MIC in SLN and non-SLN on the other side of the pelvis.

The main limitation of this study is the small size of the cohort. With a median of 32 obtained LNs, 1 patient represents assessing >300 pathological slides. The assessment of all LNs according to the protocol for ultrastaging cannot be applied in high numbers of patients due to the financial cost. Patients at high risk of LN involvement were selected to overcome the small size of the cohort. Only patients who had tumors larger than 3 cm with a deep stromal invasion were enrolled in the study; furthermore, in these patients SLN was bilaterally detected and all removed SLN were negative on intra-operative evaluation. The correct selection criteria is reflected in the high proportion of patients with positive LNs. Final ultrastaging revealed metastatic involvement of SLN in almost 50% of all patients (8/17), despite a negative intra-operative result. As expected, the majority of cases had MIC or ITC; in 1 case only did the patient have a small MAC (2.25 mm).

A similar methodology of SLN and all other pelvic LNs processing has been used in the past, but only in a few small studies. As early as in 2004, French authors published a cohort of 26 patients with IA1–IB1 stages, in which the intra-operative assessment was negative [11]. They detected 2 cases of MIC in SLN. More importantly, they found a MIC in non-SLN in 3 cases with negative SLN ultrastaging. The false-negative rate reached 60%. This study remains the only one that showed false-negative SLN ultrastaging for detection of MIC. A hypothetical explanation could be the limited experience with SLN detection in the years 2001–2002, when the study was carried out. Another possible limitation could have been the enrollment of patients in whom SLN was detected only unilaterally. It is currently well established that staging must be side-specific and SLN status on one side of the pelvis does not represent LN status in the other one.

In another French study, 13 patients with bilateral SLN detection and negative intra-operative SLN evaluation were included [12]. Metastatic involvement was detected in SLN in 5 patients (2× MAC, 1× MIC, 2× ITC); all other LNs were metastasis-free. The number of obtained LNs from pelvic lymphadenectomy (mean 8 per patient) reflected a limited extent of lymph node staging. Two years later a Canadian group published data from a cohort of 23 patients. Patients with low-risk cases for LN involvement were enrolled [13]. The main inclusion criterion was negative SLN ultrastaging. Not one single metastasis was detected in any of the non-SLNs or LNs in these patients. Japanese authors presented a small cohort in 2009 [14]. Cases with bilateral SLN detection were enrolled in the study, in which all pelvic lymph nodes were negative through a routine pathological examination. Metastases were

Table 1
Basic characteristics of patients (N = 17).

	Characteristics[a]
Age	48; 45 (32; 69)
Tumor volume (mm³)	38,470; 23,250 (1080; 236,778)
Stage	
IB1 (>3 cm)	N = 8 (47%)
IB2	N = 7 (41%)
IIB	N = 2 (12%)
Histological type	
Adeno	N = 6 (35.3%)
Squamous	N = 9 (52.9%)
Adenosquamous	N = 2 (11.8%)
LVSI	
Yes	N = 10 (58.8%)
Number of SLN per pt	2.5; 2 (2; 5)
Number of PLN per pt	30.4; 30 (15; 50)

[a] Mean; median (min; max) for continuous variables and absolute and relative frequency for categorical variables.

Table 2
SLN and non-SLN pelvic lymph node status after ultrastaging (N = 17).

SLN	Non-SLN	N (%)
neg	neg	9 (53%)
ITC	neg	3 (18%)
MIC	neg	2 (12%)
MAC	neg	1 (6%)
MIC	**MIC**	**2 (12%)**

Table 3
Laterality of LN positivity (N = 17).

Left		Right		N (%)
SLN	PLN	SLN	PLN	
neg	neg	neg	neg	9 (52.9%)
ITC	neg	neg	neg	2 (11.8%)
MIC	MIC	neg	ITC	1 (5.9%)
MIC	neg	neg	neg	1 (5.9%)
neg	neg	ITC	neg	1 (5.9%)
neg	neg	MAC	neg	1 (5.9%)
neg	neg	MIC	MIC	1 (5.9%)
neg	neg	MIC	neg	1 (5.9%)

detected in SLNs in 2 cases (1× MIC, 1× ITC) and no metastases were reported in pelvic non-SLN. The main weakness of all cited papers was the enrollment of low-risk patients, which is reflected by the small portion of patients with positive LNs from the final pathology (9/72 cases).

Especially for the situation in which SLN is detected on one side of the pelvis only, the so-called side-specific sensitivity is of importance. It expresses the agreement from the staging between SLN and non-SLN on each side of the pelvis separately. It is obvious from Table 3 that ITC were found in our study in one case on the side of the pelvis where SLN ultrastaging was negative. Side-specific sensitivity was, however, 100% for the detection of both MIC and MAC.

Data on the prognostic significance of MIC and ITC in patients with cervical cancer are limited so far since the size of the cohorts in the majority of studies did not allow for evaluation of the oncological outcome. In the largest retrospective series to date, which included 645 patients with SLN biopsy, MAC, MIC, and ITC were detected by SLN ultrastaging combined with staging of all other pelvic LN in 21%, 7%, and 4%, respectively [5]. The presence of MAC and MIC was associated with a significant decrease of overall survival but no difference in survival was found between patients with negative LN and with ITC. It is hypothesized that MIC represents a truly small metastatic involvement, while ITC can be a different entity with a limited potential for the development of distant disease spread.

As an additional finding, positive parametrial LNs were detected in 2 cases. In one patient, there was simultaneously found a MIC in SLN, while in the other patient, all pelvic LNs were metastasis-free. Even though there were only 2 such cases, it adds to the growing evidence that the results of SLN staging do not represent the status of parametrial LNs and that patients with larger tumors are not suitable candidates for abandoning parametrectomy, even if the pelvic LNs are negative [15, 16].

This study does not allow making a definite conclusion due to the small number of patients; it brings, however, another important piece of evidence on the small risk of pelvic LN positivity in patients with negative SLN ultrastaging. A significant feature of this study was the careful selection of patients at high risk of LN involvement. Metastatic involvement, mostly the presence of MIC, was detected in SLN in almost half of the patients, despite negative intraoperative evaluation. There was no false-negative case for the presence of either MAC or MIC. Also side-specific sensitivity reached 100% for both MAC and MIC, while in one case ITC was detected in non-SLN in the patient with negative ipsilateral SLN and the presence of MIC on the other side of the pelvis.

Disclosure statement

The authors declare that there are no conflicts of interest.

Acknowledgement

This work was supported by the Czech Health Research Council (16-31643A), by Charles University in Prague (UNCE 204024 and PRVOUK-P27/LF1/1) and by the project of the Ministry of Health of the Czech Republic (MZ CR – RVO VFN64165).

References

[1] L. Rob, H. Robova, M.J. Halaska, et al., Current status of sentinel lymph node mapping in the management of cervical cancer, Expert. Rev. Anticancer. Ther. 13 (2013) 861–870.
[2] D. Cibula, N. Abu Rustum, L. Dusek, et al., Bilateral ultrastaging of sentinel lymph node in cervical cancer: lowering the false-negative rate and improving the detection of micrometastasis, Gynecol. Oncol. 127 (3) (2012) 462–466.
[3] F. Lecuru, P. Mathevet, D. Querleu, et al., Bilateral negative sentinel nodes accurately predict absence of lymph node metastasis in early cervical cancer: results of the SENTICOL study, J. Clin. Oncol. 29 (13) (2011) 1686–1691.
[4] C. Altgassen, H. Hertel, A. Brandstadt, C. Kohler, M. Durst, A. Schneider, Multicenter validation study of the sentinel lymph node concept in cervical cancer: AGO study group, J. Clin. Oncol. 26 (2008) 2943–2951.
[5] D. Cibula, N. Abu-Rustum, L. Dusek, et al., Prognostic significance of low volume sentinel lymph node disease in early-stage cervical cancer, Gynecol. Oncol. 124 (3) (2012) 496–501.
[6] S.E. Lentz, L.I. Muderspach, J.C. Felix, W. Ye, S. Groshen, C.A. Amezcua, Identification of micrometastasis in histologically negative lymph nodes of early-stage cervical cancer patients, Obstet. Gynecol. 103 (2004) 1204–1210.
[7] E. Darai, R. Rouzier, M. Ballester, E. Barranger, C. Coutant, Sentinel lymph node biopsy in gynaecological cancers: the importance of micrometastasis in cervical cancer, Surg. Oncol. 17 (2008) 227–235.
[8] L. Gortzak-Uzan, W. Jimenez, S. Nofech-Mozes, et al., Sentinel lymph node biopsy vs. pelvic lymphadenectomy in early stage cervical cancer: is it time to change the gold standard? Gynecol. Oncol. 116 (2010) 28–32.
[9] H. Niikura, S. Okamoto, T. Otsuki, et al., Prospective study of sentinel lymph node biopsy without further pelvic lymphadenectomy in patients with sentinel lymph node-negative cervical cancer, Int. J. Gynecol. Cancer 22 (2012) 1244–1250.
[10] Slama J, Dundr P, Dusek L, Fischerova D, Pinkavova I, Zikan M, Vrzackova P, Kojanova M, Cibula D. Sentinel lymph node status in patients with locally advanced cervical cancers and impact of neoadjuvant chemotherapy. Gynecol Oncol. (May);125(2): 303–6.
[11] P. Marchiolè, A. Buénerd, J.Y. Scnazec, D. Dargent, P. Mathevet, Sentinel lymph node biopsy is not accurate in predicting lymph node status for patients with cervical carcinoma, Cancer 100 (10) (2004 May 15) 2154–2159.
[12] E. Barranger, A. Cortez, F. Commo, O. Marpeau, S. Uzan, E. Darai, P. Callard, Histopathological validation of the sentinel node concept in cervical cancer, Ann. Oncol. 15 (6) (2004 Jun) 870–874.
[13] I. Popa, M. Plante, M.C. Renaud, M. Roy, B. Têtu, Negative sentinel lymph node accurately predicts negative status of pelvic lymph nodes in uterine cervix carcinoma, Gynecol. Oncol. 103 (2) (2006 Nov) 649–653.
[14] S. Okamoto, H. Niikura, K. Yoshinaga, S. Nagase, T. Takano, K. Ito, N. Yaegashi, Detection of micrometastases in cervical cancer with a system that evaluates both sentinel and nonsentinel lymph nodes, Int. J. Gynecol. Cancer 19 (4) (2009 May) 708–711.
[15] M. Frumovitz, E.D. Euscher, M.T. Deavers, P.T. Soliman, K.M. Schmeler, P.T. Ramirez, C.F. Levenback, "Triple injection" lymphatic mapping technique to determine if parametrial nodes are the true sentinel lymph nodes in women with cervical cancer, Gynecol. Oncol. 127 (3) (2012 Dec) 467–471.
[16] P. Benedetti-Panici, F. Maneschi, G. D'Andrea, G. Cutillo, C. Rabitti, M. Congiu, F. Coronetta, A. Capelli, Early cervical carcinoma: the natural history of lymph node involvement redefined on the basis of thorough parametrectomy and giant section study, Cancer 88 (10) (2000 May 15) 2267–2274.

4.4. Anastomosing Hemangioma of the Ovary: A Clinicopathological Study of Six Cases with Stromal Luteinization.

Pathol. Oncol. Res. (2017) 23:717–722
DOI 10.1007/s12253-016-0186-y

ORIGINAL ARTICLE

Anastomosing Hemangioma of the Ovary: A Clinicopathological Study of Six Cases with Stromal Luteinization

Pavel Dundr [1] · Kristýna Němejcová [1] · Jan Laco [2] · Helena Skálová [1] · Lenka Bauerová [1] · Radoslav Matěj [1,3] · Daniela Fischerová [4]

Received: 20 April 2016 / Accepted: 29 December 2016 / Published online: 3 January 2017

Abstract We report six cases of anastomosing hemangioma of the ovary. All lesions were unilateral and arose in 43 to 81 year old females. In all but one patient, the tumor was asymptomatic and represented incidental finding. The exception was a tumor associated with massive ascites and elevated CA 125. The tumors were, on cut section, spongy and dark violet in color. The size of tumors ranged from 0.5 to 3.5 cm. All lesions showed the same histological features and consisted of capillary sized anastomosing vessels with sinusoid-like pattern intermingled with sporadic medium sized vessels. Interestingly, in all cases there were areas of luteinized cells at the tumor periphery, which ranged from rare small nests to multiple and commonly confluent areas. In one tumor, components of mature adipose tissue were present. Immunohistochemically, all tumors were CD31 and CD34 positive. Other markers examined were negative, including; estrogen receptor, progesterone receptor, androgen receptor, and D2–40. Proliferative activity (Ki-67 index) was very low in all cases. Anastomosing hemangioma is a rare entity, only 8 lesions occurring in ovary has been described from its initial description in 2009. We report six additional cases with their clinicopathological correlation.

Keywords Anastomosing hemagioma · Capillary hemangioma · Ovary · Stromal luteinization · Urogenital tract

Part of this work was presented on 27th European Congress of Pathology, Belgrad, Serbia, 5–9 September 2015.

✉ Pavel Dundr
pdundr@seznam.cz

[1] Department of Pathology, First Faculty of Medicine Charles University and General University Hospital in Prague, Studnickova 2, 12800 Prague 2, Czech Republic

[2] The Fingerland Department of Pathology, Charles University, Faculty of Medicine and University Hospital Hradec Kralove, Hradec Kralove, Czech Republic

[3] Department of Pathology and Molecular Medicine, Thomayer Hospital, Prague, Czech Republic

[4] Gynecologic Oncology Center, Department of Obstetrics and Gynecology, First Faculty of Medicine Charles University and General University Hospital in Prague, Prague, Czech Republic

Introduction

Anastomosing hemangioma is a rare recently described entity initially reported in the urogenital tract on a series of 6 cases occurring in kidney and testis [1]. Since that, about 50 cases have been described in kidney, and sporadic cases have been reported in other organs of genitourinary tract including testis, spermatic cord, uterus, and urinary bladder [2–4]. However, this entity is not specific for urogenital tract and has been reported in other locations including liver, gastrointestinal tract (colon, small bowel), mesentery, adrenal gland, retroperitoneum, and soft tissues [5–7]. Regarding the ovary, only 8 cases of anastomosing hemangioma have been described in this location to date [2, 7, 8]. We report a series of 6 additional cases of anastomosing hemangioma of the ovary emphasizing their clinicopathological and morphological features, including a common finding of stromal luteinization.

Material and Methods

The archive files of participating departments were searched for vascular lesions of the ovary. Cavernous lesions and non-neoplastic vascular proliferation were excluded. Finally, we identified six cases of capillary-type hemangioma which were

included in the study. Histologic review of the hematoxylin and eosin-stained slides was performed in all cases and formalin-fixed paraffin-embedded (FFPE) tissue blocks were selected for immunohistochemical analysis. Immunohistochemical analysis was performed using the avidin-biotin complex method with antibody against the CD31 (clone JC70A, dilution 1:25, Dako, Glostrup, Denmark), CD34 (clone QBEND 10, dilution 1:50, Dako), D2–40 (clone D2–40, dilution 1:100, Dako), CD68 (clone PGM1, dilution 1:25, Dako), α-inhibin (clone R1, ready-to-use, Dako), calretinin (clone DAKCalret1, dilution 1:50, Dako), estrogen receptor (ER, clone 6F11, 1:200, Novocastra, Newcastle, UK), progesterone receptor (PR, clone 16, dilution 1:100, Novocastra), androgen receptor (AR, clone AR 441, dilution 1:100, NeoMarkers, Fremont, CA), and Ki-67 (clone Mib-1, 1: 50, Dako). Antigen retrieval was performed by including pretreatment in 0.01 M citrate buffer (pH 6.0) for 40 min in a water bath at 98 °C for CD31, CD34, D2–40, progesterone receptor and androgen receptor. Antigen heat-induced epitope retrieval was performed in 0.01 M citrate buffer (pH 9.0) for CD68, α-inhibin, calretinin, Ki-67, and estrogen receptor.

Results

The clinicopathological features are summarized in Table 1.

Clinical Findings

The tumors occurred in female patients aged 43 to 81 years (mean 66 years (SD = 11.4), median 68.5 years). Three cases involved the right ovary, 2 cases the left ovary, and in one case the laterality was unknown. The size ranged from 0.5 cm to 3.5 cm in the largest diameter. In all but one case, the tumors represented incidental findings and were clinically asymptomatic. The only symptomatic case was a tumor 3.5 cm in largest diameter associated with massive ascites (2.5 l) and elevated serum CA 125 (470 U/mL; normal value is 0–35 U/mL) (Figs. 1, 2, 3, 4 and 5).

Pathological Findings

Grossly, the tumors were in all cases relatively well demarcated spongy lesions confined to ovary. On cut section the tumors were dark violet in color with one exception (case No. 3) with a component of yellowish tissue 0.8 × 0.5 cm. Microscopically, all tumors showed typical features of anastomosing hemangioma and consisted of anastomosing proliferation of capillary-sized vessels intermingled with rare medium-sized vessels. The sinusoid-like architecture closely resembled splenic red pulp. The tumor cells showed mild nuclear variability without apparent atypia. Mitoses were absent. In all cases, scattered hobnail cells were present. Common findings were fibrin thrombi and hemorrhage. Numerous mononuclear cells were present inside the vascular spaces. In all cases areas of stromal luteinization consisting of large cells with eosinophilic or clear finely vacuolated cytoplasm can be found. In one case (case No. 5), rare Reinke crystalloids were present. The luteinized areas ranged from small and rare groups of cells (case No. 2), multiple larger groups (case No. 6) to large confluent group of cells (cases No. 1, 3, 4, 5). In all cases, these areas were located at the periphery of the vascular tumors with only rare luteinized cells intermingled with the tumor vessels. Moreover, in one tumor there was an area (0.8 × 0.5 cm) consisting of mature adipose tissue component intermingled with nests of luteinized cells.

Immunohistochemical Analysis

Immunohistochemically, the tumor cells showed in all cases expression of CD31 and CD34. Other markers examined were negative, including; estrogen receptor,

Table 1 Clinicopathological features of anastomosing hemangioma

Case No.	Age	Site	Size (cm)	Clinical manifestation	Follow-up	Stromal luteinization
1	66	ovary	0.5	incidental finding (HEBA because of metrorrhagia)	25 months, NED	confluent, periphery
2	43	left ovary	1.3 × 0.3	incidental finding (HEUA because of leiomyomas and benign ovarian cyst)	4 months, NED	rare small group of cells
3	69	right ovary	1.5 × 0.8	incidental finding (HEBA because of leiomyomas)	52 months, NED	confluent, periphery
4	81	right ovary	3.5 × 3 × 2.5	incidental finding (HEBA because of metrorrhagia due to adenomyosis)	NA	confluent, periphery
5	68	left ovary	3.5 × 3 × 1.8	ovarian tumor, ascites, increased CA125 (470 U/ml)	NA	confluent, mostly periphery
6	69	right ovary	1.2 × 0.8	suspected ovarian tumor (patient under surveillance because of lymphoma)	13 months, NED	multiple small nodules, periphery

HEBA hysterectomy with bilateral salpingo-oophorectomy, *HEUA* hysterectomy with unilateral salpingo-oophorectomy, *NA* not available, *NED* no evidence of disease

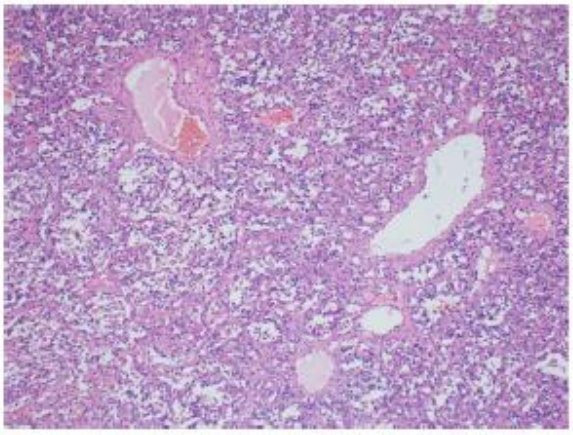


Fig. 1 Hemangioma of the ovary consisting of anastomosing small vessels and few larger medium-sized vessels (case no. 6, HE, 100 x)

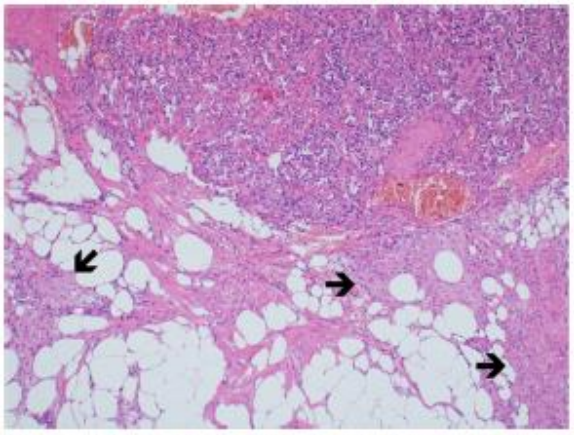


Fig. 3 Anastomosing hemangioma with component of adipose tissue. Note the luteinized cells intermingled with adipocytes (arrows) (case no. 3, HE, 100 x)

progesterone receptor, androgen receptor, and D2–40. The luteinized cells were positive with antibodies against α-inhibin and calretinin. CD68 was positive in mononuclear cells located in vascular spaces. Proliferative activity (Ki-67 index), even though estimated in areas of "hot spots", was in all cases very low (case No. 4 < 1%; case No. 1, 3 and 5 < 2%; case No. 2 and 6 < 4%).

Discussion

Hemangiomas of the ovary are rare lesions, less than 90 cases have been reported in the literature to date [2, 7–37]. The lesions are usually small and asymptomatic, but ovarian hemangiomas can be associated with pseudo-Meigs syndrome and a yet unnamed syndrome consisting of unilateral or bilateral hemangioma, elevation of CA 125, and massive ascites [13, 14, 20, 22, 36]. Histologically, more than half of reported cases were cavernous type and remaining cases were capillary type or mixed. Hemangiomas or florid vascular proliferation resembling hemangiomas can rarely arise in teratoma [38–41]. Based on the reported features of capillary hemangiomas described prior to the definition of anastomosing hemangioma in 2009, or after that but classified only as a capillary hemangioma, we believe that most of these cases should be classified as anastomosing hemangioma [9, 14, 18, 20, 25, 33]. To this regard, anastomosing hemangioma seems to be the most common type of capillary hemangioma occurring in ovary. Anastomosing hemangioma was initially described in the urogenital tract, however other reports described its occurrence in other organs and tissues and this entity is not specific for genitourinary organs [5–7]. The morphology of anastomosing hemangioma is

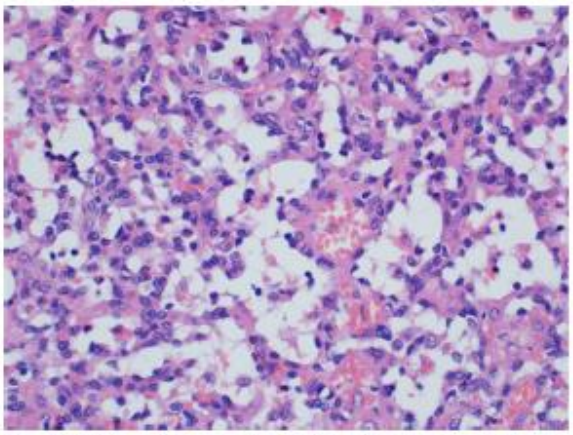


Fig. 2 Anastomosing hemangioma with mild nuclear variability and scattered hobnail cells (case no. 6, HE, 400 x)

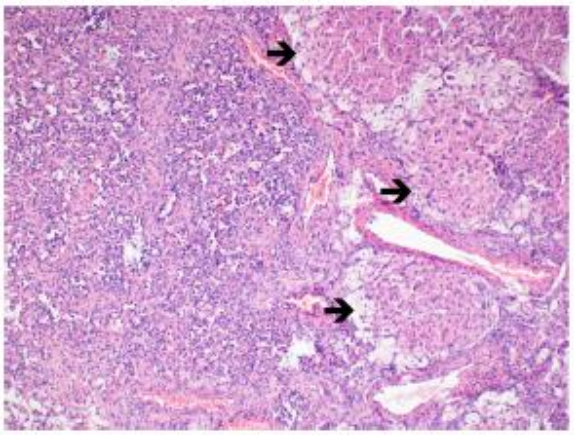


Fig. 4 Anastomosing hemangioma (left) with luteinized cells at the periphery (arrows) (case no. 5, HE, 100 x)

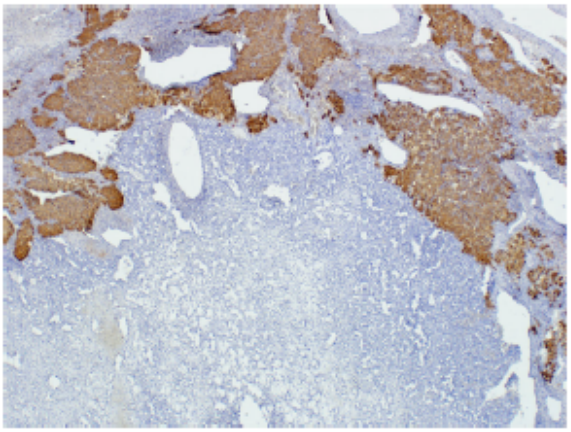


Fig. 5 Anastomosing hemangioma at the periphery with sheets of luteinized cells (case no. 5, α-inhibin, 40 x)

quite characteristic and encompasses non-lobular proliferation of capillary sized vessels with sinusoid-like arrangements resembling red pulp of the spleen, which are intermingled with moderate-sized "feeding" and "draining" vessels. Hobnail cells are common, as well as thrombi, extramedullary hematopoiesis and areas of hemorrhage. There is some variability in nuclei but nuclear atypias are generally absent. Mitoses are absent or rare.

The differential diagnosis of anastomosing hemangioma encompasses especially low grade angiosarcoma. In contrast to anastomosing hemangioma, angiosarcoma is characterized by presence of nuclear atypia, increased mitotic and proliferative activity, and common multilayering of tumor cells or presence of solid areas. Moreover, necrosis is commonly present. Necrosis can be present in the anastomosing hemangioma as well, usually in the center of the lesion together with other regressive changes. Nevertheless, the misdiagnosis of anastomosing hemangioma with well differentiated angiosarcoma could be a common event, at least according to the literature data [1, 4]. Other lesions, which can be confused with ovarian hemangioma, include epithelioid and infantile hemangioendothelioma, and non-tumorous vascular proliferation of ovarian hilus [42, 43]. Hemangioendothelioma is characterized by a focal component with solid or cord-like architecture. Moreover, cells with eosinophilic vacuolated cytoplasm representing the earliest stage of lumen formation are present. Cases of anastomosing hemangioma located in adipose tissue, either primarily or for example in kidney hilus, usually show infiltrative growth at the periphery and tumor vessels are intermingled with mature adipocytes. However, the lipomatous component can be on rare occasions a part of the tumor arising in area where adipose tissue does not normally occur [34]. This component was found in one of our cases, representing a substantial part of the tumor. In these cases, the differential diagnosis also includes myelolipoma. Moreover, in cases with prominent stromal luteinization, the differential diagnosis includes also steroidogenic tumors. Stromal luteinization is not a rare finding in ovarian hemangiomas [8, 10, 25, 32, 36]. There are two theories explaining such changes [10, 32]. The most probable hypothesis is that the non-functioning vascular tumors behave similarly like enlarged follicles causing pressure on the adjacent tissue leading to the development of theca-like luteinized stromal cells. The other hypothesis is that preexisting hyperestrogenic state from stromal luteinization stimulates development of ovarian hemangioma due to the growth stimulatory effects of estrogens on vessels. However, the absence of estrogen and progesterone receptors in endothelial cells in all our cases as well as in other studies concerning their expression, suggests that ovarian hemangiomas may occur independently of hormonal stimulation [16, 25, 35]. In addition, we assessed expression of androgen receptors, which were also negative. In our cases, luteinized cells were present in all cases. In one case, the luteinized cells were rare and formed a small group. However, in other 5 cases these areas were much more prominent. Interestingly, in one case we have found rare Reinke crystalloids, so these cells can be classified as Leydig cells.

In conclusion, anastomosing hemangioma of the ovary is rare vascular lesion, which can be misdiagnosed as an ovarian angiosarcoma. Awareness of this entity is essential for achieving a correct diagnosis. Moreover, the possibility of stromal luteinization should be considered, to avoid misdiagnosis with steroid cell tumor with pronounced stromal vascularization or mixed vascular-steroidogenic tumor.

Acknowledgements This work was supported by Ministry of Health, Czech Republic (Project RVO 64165), by Charles University in Prague (Project PRVOUK-P27/LF1/1, PRVOUK P37/11, UNCE 204024, and SVV UK 260256/2016), BBMRI_CZ LM2015089 and BBMRI_CZ LM2010004, and by OPPK (Research Laboratory of Tumor Diseases, CZ.2.16/3.1.00/24509).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Montgomery E, Epstein JI (2009) Anastomosing hemangioma of the genitourinary tract: a lesion mimicking angiosarcoma. Am J Surg Pathol 33(9):1364–1369. doi:10.1097/PAS.0b013e3181ad30a7
2. Kryvenko ON, Gupta NS, Meier FA, Lee MW, Epstein JI (2011) Anastomosing hemangioma of the genitourinary system: eight cases in the kidney and ovary with immunohistochemical and ultrastructural analysis. Am J Clin Pathol 136(3):450–457. doi:10.1309/AJCPJPW34QCQYTMT

3. Omiyale AO (2015) Anastomosing hemangioma of the kidney: a literature review of a rare morphological variant of hemangioma. Ann Transl Med 3(11):151. doi:10.3978/j.issn.2305-5839.2015.06.16
4. Tao LL, Dai Y, Yin W, Chen J (2014) A case report of a renal anastomosing hemangioma and a literature review: an unusual variant histologically mimicking angiosarcoma. Diagn Pathol 9:159. doi:10.1186/s13000-014-0159-y
5. John I, Folpe AL (2016) Anastomosing hemangiomas arising in unusual locations: a Clinicopathologic study of 17 soft tissue cases showing a predilection for the Paraspinal region. Am J Surg Pathol. doi:10.1097/PAS.0000000000000627
6. Lin J, Bigge J, Ulbright TM, Montgomery E (2013) Anastomosing hemangioma of the liver and gastrointestinal tract: an unusual variant histologically mimicking angiosarcoma. Am J Surg Pathol 37(11):1761–1765. doi:10.1097/PAS.0b013e3182967e6c
7. O'Neill AC, Craig JW, Silverman SG, Alencar RO (2016) Anastomosing hemangiomas: locations of occurrence, imaging features, and diagnosis with percutaneous biopsy. Abdom Radiol (NY). doi:10.1007/s00261-016-0690-2
8. Metodiev D, Ivanova V, Omainikova B, Boshnakova T (2015) Ovarian anastomosing hemangioma with stromal luteinization: a case report. Akush Ginekol (Sofiia) 54(9):58–61
9. Akbulut M, Bir F, Colakoglu N, Soysal ME, Duzcan SE (2008) Ovarian hemangioma occurring synchronously with serous papillary carcinoma of the ovary and benign endometrial polyp. Ann Saudi Med 28(2):128–131
10. Anand MS, Shetty S, Mysorekar VV, Kumar RV (2012) Ovarian hemangioma with stromal luteinization and HCG-producing mononucleate and multinucleate cells of uncertain histogenesis: a rare coexistence with therapeutic dilemma. Indian J Pathol Microbiol 55(4):509–512. doi:10.4103/0377-4929.107793
11. Comunoglu C, Atasoy L, Baykal C (2010) Ovarian hemangioma occurring synchronously with contralateral mature cystic teratoma in an 81-year-old patient. Ups J Med Sci 115(4):297–299. doi:10.3109/03009734.2010.502602
12. Correa-Rivas MS, Colon-Gonzalez G, Lugo-Vicente H (2003) Cavernous hemangioma presenting as a right adnexal mass in a child. P R Health Sci J 22(3):311–313
13. Erdemoglu E, Kamaci M, Ozen S, Sahin HG, Kolusari A (2006) Ovarian hemangioma with elevated CA125 and ascites mimicking ovarian cancer. Eur J Gynaecol Oncol 27(2):195–196
14. Gehrig PA, Fowler WC Jr, Lininger RA (2000) Ovarian capillary hemangioma presenting as an adnexal mass with massive ascites and elevated CA-125. Gynecol Oncol 76(1):130–132. doi:10.1006/gyno.1999.5648
15. Grant JW, Millward-Sadler GH (1986) Haemangioma of the ovary with associated endometrial hyperplasia. Case report Br J Obstet Gynaecol 93(11):1166–1168
16. Gucer F, Ozyilmaz F, Balkanli-Kaplan P, Mulayim N, Aydin O (2004) Ovarian hemangioma presenting with hyperandrogenism and endometrial cancer: a case report. Gynecol Oncol 94(3):821–824. doi:10.1016/j.ygyno.2004.06.021
17. Gunes HA, Egilmez R, Dulger M (1990) Ovarian haemangioma. Br J Clin Pract 44(12):734–735
18. Gupta R, Singh S, Nigam S, Khurana N (2006) Benign vascular tumors of female genital tract. Int J Gynecol Cancer 16(3):1195–1200. doi:10.1111/j.1525-1438.2006.00523.x
19. Jurkovic I, Dudrikova K, Boor A (1999) Ovarian hemangioma. Cesk Patol 35(4):133–135
20. Kaneta Y, Nishino R, Asaoka K, Toyoshima K, Ito K, Kitai H (2003) Ovarian hemangioma presenting as pseudo-Meigs' syndrome with elevated CA125. J Obstet Gynaecol Res 29(3):132–135
21. Kim SS, Han SE, Lee NK, Choi KU, Joo JK, Suh DS, Kim HY, Kim KH (2015) Ovarian cavernous hemangioma presenting as a large growing mass in a postmenopausal woman: a case report and review of the literature. J Menopausal Med 21(3):155–159. doi:10.6118/jmm.2015.21.3.155
22. Koh LW, Sun YL, Koh PH, Chiu HY, Chen SY, Huang MH (2007) Ovarian capillary hemangioma presenting as pseudo-Meigs' syndrome: a case report. J Minim Invasive Gynecol 14(3):367–369. doi:10.1016/j.jmig.2006.10.030
23. Loverro G, Cormio G, Perlino E, Vicino M, Cazzolla A, Selvaggi L (1998) Transforming growth factor-beta1 in hemangioma of the ovary. Gynecol Obstet Investig 46(3):210–213
24. McBurney RP, Trumbull M (1955) Hemangioma of the ovary with ascites. Miss Doct 32(10):271–274
25. Miliaras D, Papaemmanouil S, Blatzas G (2001) Ovarian capillary hemangioma and stromal luteinization: a case study with hormonal receptor evaluation. Eur J Gynaecol Oncol 22(5):369–371
26. Mirilas P, Georgiou G, Zevgolis G (1999) Ovarian cavernous hemangioma in an 8-year-old girl. Eur J Pediatr Surg 9(2):116–118. doi:10.1055/s-2008-1072225
27. Mitra B, Sengupta S, Rai A, Mehta J, Quader AR, Roy S, Borges A (2013) Ovarian haemangioma: A rare case report. Int J Surg Case Rep 4(11):981–984. doi:10.1016/j.ijscr.2013.07.029
28. M'Pemba Loufoua-Lemay AB, Peko JF, Mbongo JA, Mokoko JC, Nzingoula S (2003) Ovarian torsion revealing an ovarian cavernous hemangioma in a child. Arch Pediatr 10(11):986–988
29. Ozana M, Formanek P, Sula F, Silhan J (1994) Ovarian hemangioma with torsion. Ceska Gynekol 59(1):18–20
30. Pethe VV, Chitale SV, Godbole RN, Bidaye SV (1991) Hemangioma of the ovary–a case report and review of literature. Indian J Pathol Microbiol 34(4):290–292
31. Rivasi F, Philippe E, Walter P, de Marco L, Ludwig L (1996) Ovarian angioma. Report of 3 asymptomatic cases. Ann Pathol 16(6):439–441
32. Savargaonkar PR, Wells S, Graham I, Buckley CH (1994) Ovarian haemangiomas and stromal luteinization. Histopathology 25(2):185–188
33. Schoolmeester JK, Greipp PT, Keeney GL, Soslow RA (2015) Ovarian hemangiomas do not harbor EWSR1 rearrangements: Clinicopathologic characterization of 10 cases. Int J Gynecol Pathol 34(5):437–444. doi:10.1097/PGP.0000000000000171
34. Tran TA, Pernicone P (2012) Anastomosing hemangioma with fatty changes of the genitourinary tract: a lesion mimicking angiomyolipoma. Cent European J Urol 65(1):40–42. doi:10.5173/ceju.2012.01.art13
35. Uppal S, Heller DS, Majmudar B (2004) Ovarian hemangioma–report of three cases and review of the literature. Arch Gynecol Obstet 270(1):1–5. doi:10.1007/s00404-003-0584-8
36. Yamawaki T, Hirai Y, Takeshima N, Hasumi K (1996) Ovarian hemangioma associated with concomitant stromal luteinization and ascites. Gynecol Oncol 61(3):438–441. doi:10.1006/gyno.1996.0170
37. Ziari K, Alizadeh K (2016) Ovarian hemangioma: a rare case report and review of the literature. Iran J Pathol 11(1):61–65
38. Akbulut M, Zekioglu O, Terek MC, Ozdemir N (2009) Florid vascular proliferation in mature cystic teratoma of the ovary: case report and review of the literature. Tumori 95(1):104–107
39. Baker PM, Rosai J, Young RH (2002) Ovarian teratomas with florid benign vascular proliferation: a distinctive finding associated with the neural component of teratomas that may be confused with a vascular neoplasm. Int J Gynecol Pathol 21(1):16–21
40. Itoh H, Wada T, Michikata K, Sato Y, Seguchi T, Akiyama Y, Kataoka H (2004) Ovarian teratoma showing a predominant hemangiomatous element with stromal luteinization: report of a case and review of the literature. Pathol Int 54(4):279–283. doi:10.1111/j.1440-1827.2004.01621.x
41. Pongsuvareeyakul T, Settakorn J, Khorana J, Khunamornpong S (2015) Ovarian teratoma with predominant hemangiomatous

component in early childhood. J Pediatr Adolesc Gynecol 28(1): e1–e3. doi:10.1016/j.jpag.2014.02.013

42. Illueca C, Machado I, Garcia A, Covisa A, Morales J, Cruz J, Traves V, Almenar S (2011) Uncommon vascular tumor of the ovary. Primary ovarian epithelioid hemangioendothelioma or vascular sarcomatous transformation in ovarian germ cell tumor? Arch Gynecol Obstet 284(6):1589–1591. doi:10.1007/s00404-011-2036-1
43. Prus D, Rosenberg AE, Blumenfeld A, Udassin R, Ne'eman Z, Young RH, Ariel I (1997) Infantile hemangioendothelioma of the ovary: a monodermal teratoma or a neoplasm of ovarian somatic cells? Am J Surg Pathol 21(10):1231–1235

4.5. Atypical Polypoid Adenomyoma of the Uterus: An Immunohistochemical and Molecular Study of 21 Cases.

ORIGINAL ARTICLE

Atypical Polypoid Adenomyoma of the Uterus

An Immunohistochemical and Molecular Study of 21 Cases

Kristýna Němejcová, MD, Sarah L. Kenny, MB,† Jan Laco, PhD,‡ Petr Škapa, MD,§ Libor Staněk, MBiolSci,* Michal Zikán, PhD,‖ Petra Kleiblová, PhD,¶# W. Glenn McCluggage, FRCPath,† and Pavel Dundr, MD, PhD**

Abstract: Atypical polypoid adenomyoma (APA) is an uncommon uterine lesion that commonly recurs after local excision and is occasionally associated with or precedes the development of atypical hyperplasia or endometrioid adenocarcinoma. Despite the fact that about 230 cases have been reported in the literature, only 2 studies of 6 and of 7 cases have investigated the molecular aspects; as such, molecular alterations that occur in APA remain largely unknown. We undertook a comprehensive immunohistochemical and molecular analysis of 21 cases of APA in 17 patients (including 4 recurrent/persistent lesions). The analyzed genes were *PTEN* and *TP53* (by fluorescence in situ hybridization) and *KRAS*, *BRAF*, *EGFR*, and *NRAS* (all by polymerase chain reaction). Immunohistochemical staining was performed for PTEN, p53, mTOR, β-catenin, HNF-1β, and GLUT1 and for the mismatch-repair proteins MLH-1, MSH-2, MSH-6, and PMS-2. In most cases, there was nuclear expression of β-catenin in squamous morules and expression of HNF-1β, mTOR, and GLUT1 in the glandular component. All cases exhibited "wild-type" expression of p53. A common finding was loss of PTEN expression (6/19 cases). In 1 of these cases, loss of PTEN expression was accompanied by *PTEN* deletion. Mutation of the *KRAS* gene was found in 5/19 cases. Intact mismatch-repair protein expression was present in all cases, and *TP53* abnormalities or mutations of *EGFR*, *NRAS*, or *BRAF* genes were not found. Given the association with atypical hyperplasia and endometrioid adenocarcinoma and the shared immunohistochemical and molecular features, we feel that, conceptually, APA is best regarded as analogous to a localized form of atypical hyperplasia.

Key Words: uterus, atypical polypoid adenomyoma, atypical hyperplasia, molecular pathology, immunohistochemistry

(*Am J Surg Pathol* 2015;00:000–000)

From the *Department of Pathology, First Faculty of Medicine and General University Hospital; ‡The Fingerland Department of Pathology, Faculty of Medicine and University Hospital Hradec Kralove; §Department of Pathology and Molecular Medicine, Second Faculty of Medicine and Faculty Hospital in Motol; ‖Department of Obstetrics and Gynecology, Oncogynecological Centre, First Faculty of Medicine and General University Hospital; ¶Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine; #Institute of Biology and Medical Genetics, First Faculty of Medicine, General University Hospital in Prague, Charles University in Prague, Prague, Czech Republic; and †Department of Pathology, Royal Group of Hospitals Trust, Belfast, Northern Ireland, United Kingdom.

Conflicts of Interest and Source of Funding: Supported by Ministry of Health, Czech Republic (IGA MZ CR project NT14001-3/2013), by Charles University in Prague (Project PRVOUK-P27/LF1/1, PRVOUK P37/11, UNCE 204024, and SVV 260022), and by Bank of Biological Material project BBM LM2010004. The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

Correspondence: Pavel Dundr, MD, PhD, Department of Pathology, First Faculty of Medicine and General University Hospital, Charles University in Prague, Studnickova 2, Prague 2, 12800, Czech Republic (e-mail: pdundr@seznam.cz).

Atypical polypoid adenomyoma (APA) is an uncommon uterine lesion first described in 1981.[1] It usually occurs in premenopausal women and most commonly involves the endometrium of the lower uterine segment or the upper endocervix as a polypoid mass, although all parts of the uterine corpus can be involved. As many patients with APA are in the reproductive age group, fertility preservation is a common issue. APA is regarded as a benign lesion, but in conservatively treated patients it is managed by curettage or polypectomy, recurrence/persistence is common, and there have been reports of the associated synchronous or metachronous occurrence of atypical hyperplasia (AH) or endometrioid adenocarcinoma (EC) either within the APA or the nonpolypoid endometrium; as such, management may be problematic.[2–4] In this context, it is very important to determine the molecular events associated with the pathogenesis of APA and related to its biological potential. However, despite the fact that approximately 230 cases have been reported in the literature, only 2 studies comprising 6 and 7 cases have investigated the molecular aspects of APA.[5,6]

In this study, we undertook a comprehensive immunohistochemical and molecular analysis of 21 cases of APA in 17 patients. We specifically concentrated on those molecular events and proteins involved in the pathogenesis of AH and EC given the association with APA.

MATERIALS AND METHODS

Cases

The cases were derived from the pathology archives of the institutions to which the authors are affiliated.

Twenty-one cases from 17 patients were included. Histologic review of the hematoxylin and eosin–stained slides was performed. In all cases, formalin-fixed, paraffin-embedded tissue blocks were available for immunohistochemical and molecular analysis. Six of the patients had recurrent/persistent lesions after curettage or polypectomy. However, in 1 of these, only material from the recurrent/persistent lesion was available (patient 7). In 4 other patients, there was enough material from the primary and recurrent/persistent lesions for the analysis (patients 4, 5, 6, and 9). One patient also developed a second recurrence (in this patient, material from the first recurrence was not available) (patient 11).

Immunohistochemical Analysis

Immunohistochemical staining was performed using the avidin-biotin complex method with antibodies against the following antigens: β-catenin (clone β-catenin-1, 1:400; Dako, Glostrup, Denmark), mTOR (phosphorylated, clone 7C10, 1:50; Cell Signaling Technology Inc., Danvers, MA), GLUT1 (polyclonal, 1:100; Cell Marque, Rocklin, CA), HNF-1β (polyclonal, 1:500; Sigma-Aldrich, Prestige antibodies, St Louis), p53 (clone DO7, 1:50; Immunotech, QC, Canada), PTEN (clone SP218, 1:200; Spring Bioscience, Pleasanton, CA), MLH-1 (clone G168-15, 1:50; Zytomed Systems, Berlin, Germany), MSH-2 (clone FE 11, 1:50, Zytomed Systems), MSH-6 (clone FE 11, 1:50; Zytomed Systems), and PMS-2 (clone EPR3947, ready-to-use; Zytomed Systems). Antigen retrieval was performed. This comprised pretreatment in 0.01 M citrate buffer (pH 6.0) for 40 minutes in a water bath at 98°C for β-catenin, mTOR, p53, PTEN, MSH-1, and MSH-6. Heat-induced epitope retrieval was performed in 0.01 M citrate buffer (pH 6.1) for HNF-1β and in 0.01 M citrate buffer (pH 9.0) for GLUT1, PMS-2, and MLH-1.

β-catenin, HNF-1β, PTEN, mTOR, and GLUT1 immunohistochemistry was semiquantitatively assessed and graded on a 5-tier scale on the basis of the percentage of positive cells: 0 = < 5%; 1+ = 5% to 10%; 2+ = 11% to 50%; 3+ = 51% to 75%; and 4+ = > 75% positive cells. For β-catenin, HNF-1β, and GLUT1, the assessment was performed separately for glandular and squamous morular components. Expression of the mismatch-repair (MMR) proteins MLH-1, MSH-2, MSH-6, and PMS-2 was classified as negative (no nuclear staining of epithelial cells) or positive (convincing nuclear staining of any proportion of epithelial cells—in practice, many nuclei were positive in all cases). p53 protein expression was assessed as "wild-type" or "mutation-type." "Mutation-type" staining was regarded as diffuse intense nuclear positivity of >75% of epithelial cells or totally negative staining with a positive internal control in the form of weak nuclear staining of a proportion of stromal cells, whereas "wild-type" staining comprised focal, weak staining of a proportion of the epithelial nuclei.[7,8] Only nuclear staining was regarded as positive for HNF-1β, p53, MLH-1, MSH-2, MSH-6, PMS-2, PTEN, and β-catenin (cytoplasmic/membranous staining occurs normally and was disregarded). Cytoplasmic staining for mTOR and membranous staining for GLUT1 were interpreted as positive.

Fluorescence In Situ Hybridization

Numerical aberrations of *TP53* and *PTEN* genes were analyzed using the fluorescence in situ hybridization (FISH) method with probes ZytoLight SPEC TP53 Dual Color Probe (ZytoVision, Germany) and PTEN Dual Color Probe (ZytoVision). The assay procedure was conducted according to the manufacturer's instructions. Briefly, the slides with 2 to 3-μm-thick sections of paraffin-embedded tissue were deparaffinized in xylene, then pretreated in 0.2 M HCl and subsequently in an NaSCN solution at 80°C. The next step was the proteolytic treatment. Protease II (25 mg) in a 50 mL saline solution at pH 2.0 was used with a digestion time of 60 minutes. Then, the sections were fixed in buffered formalin. In the next step, we applied the FISH probe, sealed it with a liquid rubber cement, codenatured the specimen and the DNA probe for 1 minutes at 75°C, and then hybridized it overnight in a ThermoBrite system (Dako) at 37°C.

In each sample, a minimum of 100 nuclei (cutoff for numerical aberrations was 10% of positive nuclei) were evaluated using the Olympus Provis AX70 microscope (Olympus, Tokyo, Japan).

Isolation of Nucleic Acid

Sections of formalin-fixed, paraffin-embedded tissue were used for DNA isolation using standard procedures. Approximately seven 10-μm-thick sections from each sample were deparaffinized in xylene. The DNA was then extracted using the QIAamp DNA mini kit (Qiagen, Hamburg, Germany).

Analysis of the *KRAS* and *EGFR* Genes

KRAS and EGFR StripAssay Kit (VienaLab Diagnostics GmbH, Vienna, Austria) was used according to the manufacturer's instructions, with robotic hybridization system Bee20 (Bee Robotics Ltd, North Wales, UK). The *EGFR* gene was tested for mutations in exons 18, 19, 20, and 21, and the *KRAS* gene was tested for mutations in exons 2 and 3 (codon 12, 13, 61).

Analysis of the *NRAS* Gene

NRAS Mutation Analysis Kit (EntroGen, Los Angeles) was used for *NRAS* gene mutations in exon 2 (codon 12/13) and exon 3 (codon 61) according to the manufacturer's instructions using Light Cycler LC480 (Roche, Basel, Switzerland).

Analysis of the *BRAF* Gene

The Cobas 4800 BRAF V600 Mutation Test (Roche) was used for the analysis of V600 mutations in exon 15 of the *BRAF* gene according to the manufacturer's instructions.

RESULTS

The age of the patients ranged from 29 to 53 years (mean 41 y; median 44 y). Clinical data, including follow-up, were available in 13/17 patients. In 6/13 patients with an available clinical history, the patients presented with abnormal uterine bleeding. Five lesions were asymptomatic and detected incidentally during radiologic inves-

tigations. In 2/13 patients, the lesion was discovered during investigations for infertility.

Seven patients underwent a hysterectomy (3 after preceding curettage or polypectomy; however, material from the prehysterectomy biopsy was available in only 2 patients). Four patients underwent a hysteroscopy with resection of the lesion by electrosurgical loop, and 2 underwent curettage (2 of these 6 patients subsequently had a hysterectomy).

Subsequently, 6/13 patients with available clinical history were diagnosed with a recurrent/persistent lesion from 1 to 8 months after first diagnosis. One patient exhibited 2 recurrences (3 and 8 mo after the first diagnosis).

Histologically, all lesions showed the typical morphologic features of APA and consisted of irregular endometrioid-type glands, often with a complex architectural pattern, arranged haphazardly within a fibromyomatous stroma. The degree of architectural complexity varied among the lesions. Squamous morules, in 1 case with central necrosis, were present in all but 2 cases. The glandular epithelium exhibited mild to moderate nuclear atypia. Neither AH nor EC were present in any case. Figure 1 shows representative images of APAs included in the study.

Immunohistochemical Findings

The results of the immunohistochemical analysis in the epithelial component are summarized in Table 1. The staining pattern of the stromal cells was as expected without any unusual patterns. Representative images of immunohistochemical results are shown in Figure 1.

β-catenin

Nuclear staining was present in 13/17 patients (17/21 lesions). There were differences between the glandular and squamous morular elements. In all but 1 case the staining was stronger and more extensive in squamous morules. Of 19 lesions with squamous morules, 4 were negative, 5 showed 3+ positivity, and 10 exhibited 4+ positivity. The glandular component was negative in 9 cases, 10 cases were 1+ positive, 1 case was 2+ positive, and 1 case was 3+ positive.

mTOR

The expression of mTOR could be assessed in 15/17 patients (19/21 lesions). Of all analyzed samples, 2 were negative. Positivity was restricted mostly to the glandular component. The intensity of staining was weak or moderate in 15 cases and strong in 2. Of 17 positive cases, 7 were classified as 4+, 5 as 3+, 4 as 2+, and 1 as 1+.

PTEN

The expression of PTEN could be assessed in 16/17 patients (19/21 samples). Of these samples, 6 (from 5 patients) were negative with only focal positivity in up to 5% tumor cells. Two lesions were classified as 1+, 4 as 2+, 5 as 3+, and 2 as 4+. The staining intensity was mostly weak or moderate. Normal endometrium was present in 10/21 samples. Diffuse PTEN expression was found in all cases, weak to moderate in glands and moderate to strong in stromal cells.

GLUT1

The expression of GLUT1 could be assessed in all 17 patients (21 cases). All cases showed some degree of positivity. The expression differed between glandular and squamous morular components. The glandular component was positive in all but 1 case. In contrast, the squamous morular component was negative in 6/18 cases. Ten lesions showed the same or higher positivity in the squamous morular compared with the glandular components. The glandular component was classified as: 1 case negative, 3 cases 1+, 9 cases 2+, 6 cases 3+, and 2 cases 4+. The squamous morular component was: 6 cases negative, 2 cases 1+, 7 cases 2+, 1 case 3+, and 2 cases 4+.

HNF-1β

The nuclear expression of HNF-1β was present mostly in the glandular component. Only 1 lesion was focally positive in <5% of the squamous morular component and was classified as negative. The staining intensity was usually weak or moderate. All but 5 cases were positive in the glandular component: 1 case 1+, 4 cases 2+, 6 cases 3+, and 5 cases 4+. In many cases, there was weak cytoplasmic staining that was regarded as nonspecific.

MMR Proteins (MLH-1, MSH-2, MSH-6, PMS-2)

Nuclear staining for the MMR proteins could be assessed in 18/21 cases for MLH-1 and MSH-2, 12/21 cases for PMS-2, and 10/21 cases for MSH-6. In 3 cases, there was no nuclear staining either in the lesion or in normal tissues with MLH-1, MSH-2, and MSH-6 and in 1 of these cases also with PMS-2; these cases were excluded from the analysis because of technical limitations. Two of these cases belonged to the group of recurrent/persistent lesions and showed positive staining in a subsequent biopsy. Of the analyzed cases, many nuclei were positive for the MMR proteins in all lesions. Staining was similar in the glandular and squamous morular elements.

p53 Protein

All cases exhibited weak nuclear positivity of scattered cells, in keeping with "wild-type" expression.

Molecular Analysis

The results of the molecular analysis are summarized in Table 2.

PTEN Gene

Numerical changes of the *PTEN* gene could be analyzed in all 21 samples. *PTEN* deletion was detected in 3 patients. In 1 of these patients, *PTEN* deletion detected by FISH was accompanied by loss of PTEN immunohistochemical expression. This patient had a recurrent/persistent lesion, and loss of PTEN expression accompanied by *PTEN* deletion was present in both the primary and recurrent/persistent lesion. In the other 2 patients, *PTEN* deletion

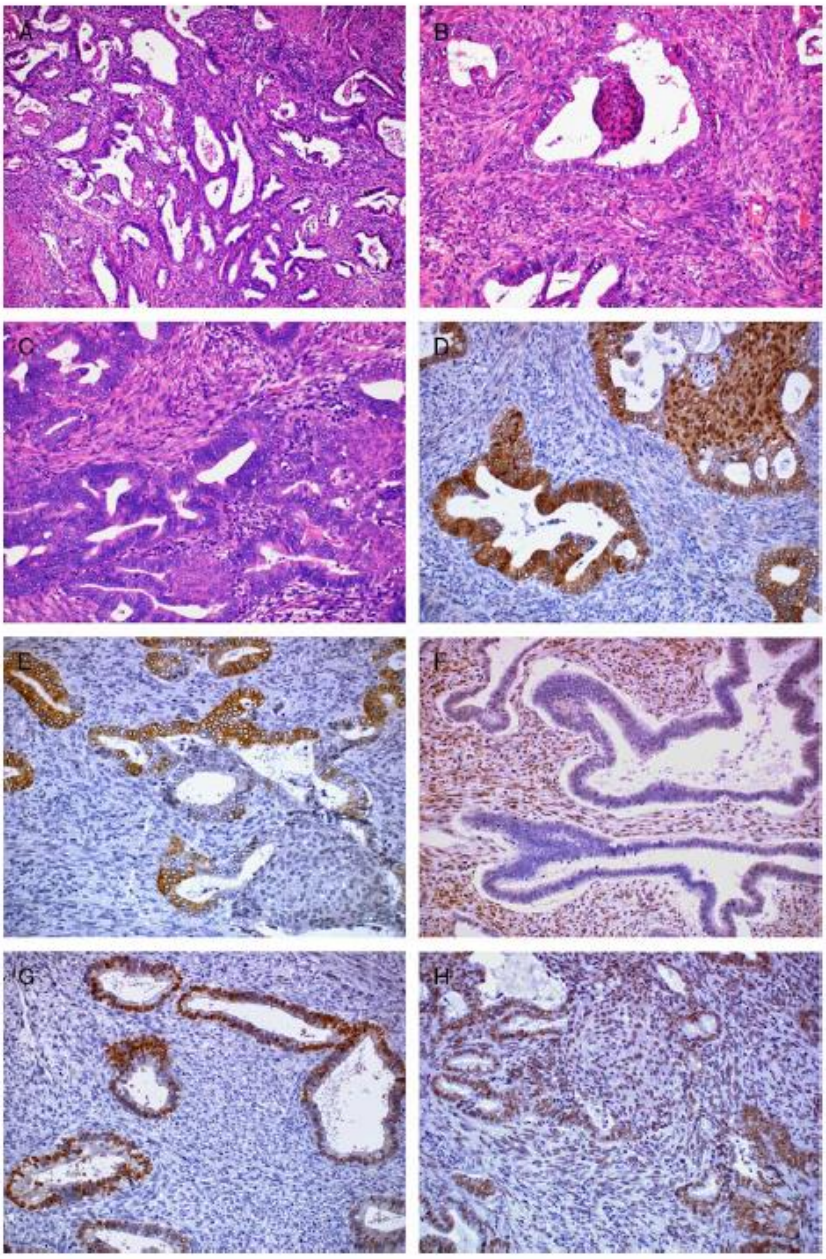


FIGURE 1. Morphologic and immunohistochemical features in cases of APA. A, Typical features of APA with haphazardly arranged irregular endometrioid-type glands (HE). B, Glands with squamous morule located within a fibromyomatous stroma (HE). C, Case with complex architectural pattern and moderate nuclear atypia (HE). D–H, Results of immunohistochemical staining showing nuclear expression of β-catenin mostly restricted to squamous morule (D); positivity of mTOR restricted to the glandular component (E); PTEN with negative glands and positivity in stromal cells); HNF-1β with nuclear expression in some glandular cells (G) and nuclear expression of MLH-1 (H). HE indicates hematoxylin and eosin.

fibromuscular rather than usual "endometrial-type." If an endometrial glandular proliferation arises within this stroma, the stroma could in some way prevent the glandular proliferation spreading outside this region and therefore result in a localized polypoid lesion.

APA may be misdiagnosed as endometrial hyperplasia, including AH, because of the architecturally complex glands and the cytologic atypia. Typically, the glands in AH (and other endometrial hyperplasias) are surrounded by a dense proliferative endometrioid-type stroma, whereas in APA the stroma is leiomyomatous or fibromyofibromatous. APA may also be misdiagnosed as EC, including myoinvasive EC, especially on a curettage, biopsy, or polypectomy specimen. Marked cytologic atypia favors a myoinvasive adenocarcinoma, as, in APA, there is usually no more than mild to moderate cytologic atypia. The leiomyomatous or fibromyomatous stromal component of APA grows in short interlacing fascicles, in contrast to the elongated fibers of the normal myometrium. In curettings or biopsy from an APA, there are usually also fragments of normal background endometrium, and with an EC it would be unusual on biopsy to obtain only myoinvasive neoplasm without free tumor fragments.

APA is generally regarded as a benign lesion. However, in conservatively managed patients, treated by curettage or polypectomy, recurrence/persistence is common. In different studies, the rate varies but recurrence/persistence has been observed in up to 45% of cases.[1,9–12] In our study, a recurrence/persistence developed in 6/13 (46%) patients with an available clinical history. In a meta-analysis of 136 cases of APA published in 2006, the recurrence/persistence rate was 30%.[13] Moreover, this meta-analysis showed that APA not uncommonly coexists with endometrial hyperplasia and that 8.8% of APAs are associated with the development of EC. The hyperplasia or EC may involve the APA or the nonpolypoid endometrium. Other cases of APA coexisting with or preceding the development of EC have been reported.[2–4] On the basis of these data, cases of APA should be carefully evaluated clinically and histologically, and this should not be regarded as an unequivocally benign lesion.[14] Because of the fact that many patients with APA are in the reproductive age group, a request for fertility sparing management is common. However, patients with APA who wish to preserve fertility and are managed by local excision without hysterectomy should be carefully monitored and followed up.

Given the significant risk for recurrence/persistence after conservative management and the risk of development of AH and EC, it is important to know the underlying molecular events associated with APA. Only 2 small studies of 6 and 7 cases investigating the molecular events have been reported to date.[5,6] The first study analyzed microsatellite instability (MSI) by polymerase chain reaction in 5 different microsatellite loci (BAT25, BAT26, D2S123, D5S346, and D17S250) and looked for *MLH-1* promoter hypermethylation and *CTNNB1* mutations.[5] Immunohistochemistry for MLH-1, MSH-2, and β-catenin was also performed. The results revealed *MLH-1* promoter hypermethylation in 2 cases and nuclear expression of β-catenin in 5. However, *CTNNB1* mutation was not found in any case. In contrast, in the second study, mutations of *CTNNB1* gene were detected in all 7 cases.[6] The discrepant results could possibly be related to the fact that in the latter study the epithelial component was microdissected, and the samples were not "contaminated" by the stromal component. The second study that was concerned mainly with the assessment of proliferative activity showed overexpression of p21 and cyclin D1 in both the epithelial and stromal components.[6] The authors concluded that APAs are lesions with decreased cell proliferation and that the activation of β-catenin signaling seems to be essential for the morular phenotype and the architectural features of the glandular component.

Given the paucity of information regarding the molecular events in APA, the goal of our study was to analyze molecular and immunohistochemical features in a large series of cases and to compare the results with the literature pertaining to AH and EC. The analyzed genes and proteins were selected because of the molecular alterations involved in the development of EC, which, as discussed, may be associated with APA.[15–24] Molecular changes occurring in type II endometrial carcinomas (the prototype of which is serous carcinoma) were not analyzed, except for *TP53*. We also examined the expression of some other markers, including GLUT1, mTOR, and HNF-1β.

KRAS mutations have been implicated in the development of AH and EC. Mutations of this gene have been reported in about 20% of EC and some cases of AH.[25,26] In our study, mutation of the *KRAS* gene was found in 4/16 (25%) patients. About 20% of ECs and some endometrial hyperplasias have been reported to be *BRAF* mutated.[27] However, in our study no *BRAF* mutations were found.

The majority of ECs have altered PTEN immunohistochemical expression, either reduced or complete absence. Mutation or loss of PTEN expression has been implicated as early markers of endometrial carcinogenesis, which also occurs in precursor lesions, and it was hypothesized that this can be used as a biomarker identifying lesions that are more likely to progress into a carcinoma.[28] However, later studies showed that loss of PTEN expression was found not only in lesions that progressed into a carcinoma but also in a similar percentage of lesions that did not progress, and ultimately the loss of PTEN expression has not proven to be useful as a progression marker.[29] Moreover, loss of PTEN expression can also be observed in normal endometrium, in both the secretory and proliferative phases.[28] In addition, it is well known that there is a relatively poor correlation between loss of PTEN protein expression and the presence of *PTEN* mutation.[30] This could be related to the occurrence of epigenetic changes that are not detectable by sequencing. In our study, loss of PTEN expression was identified in 5/16 patients. In 1 of these patients, the loss

of PTEN expression was accompanied by *PTEN* deletion detected by FISH. *PTEN* deletion occurred in 2 other cases in which immunohistochemical expression was maintained. This may be explained by the fact that *PTEN* deletion accompanied by loss of protein expression affects only some glands. It has been shown that PTEN phosphatase is a negative regulator of mTOR, and AH and EC are commonly associated with increased mTOR signaling.[31,32] However, little is known about mTOR expression in endometrial lesions. In our study, phosphorylated (activated) mTOR expression was identified in 17/19 lesions.

MSI is found in about 25% to 30% of type I endometrial carcinomas.[33–36] Most sporadic MSI endometrial carcinomas arise secondary to *MLH-1* promoter methylation, but a proportion have Lynch syndrome secondary to mutation in 1 of the MMR genes. It has been shown that in some patients with Lynch syndrome, MSI can be demonstrated in endometrial hyperplasia several years before the development of EC.[37] The expression of MMR proteins in normal endometrium, endometrial hyperplasias, and carcinomas has been evaluated in many studies.[37–39] MSI and loss of MMR protein expression has been observed in a subset of EC, which frequently show lower uterine segment location, mucinous differentiation, intratumoral heterogeneity, undifferentiated/dedifferentiated morphology, and prominent lymphocytic infiltration.[37,40–42] In our study, loss of MMR protein expression was not found in any case. Therefore, MSI is unlikely to occur in the development of APA, and this lesion is not likely to be a component of Lynch syndrome.

Mutations and epigenetic inactivation of *HNF-1β* gene have been shown to be involved in the development of several tumors. Tumor cell lines with *HNF-1β* mutation usually show loss of protein expression as detected by immunohistochemistry. In contrast, some tumors show upregulation of HNF-1β, and strong expression of this protein is found in most clear cell carcinomas of the ovary and other sites in the female genital tract.[43,44] However, this marker is not specific for clear cell carcinoma and can be expressed in other tumors including EC.[45] Regarding the expression of HNF-1β in non-neoplastic tissues, studies have described positive staining in endometriosis and in normal endometrium, especially gestational or secretory endometrium.[46,47] In our study, we found expression of HNF-1β in the glandular component of APA in 16/21 samples. The expression of GLUT1 in normal and pathologic endometrium has been analyzed in only a few studies. Preferentially, the expression of this protein has been demonstrated in AH and EC, and this has been suggested as a surrogate marker for distinguishing between non-AH and AH.[48] However, in 1 study the authors identified GLUT1 expression in some cases of non-AH.[49,50] In our study, we found GLUT1 expression in the glandular component of APA in 20/21 samples.

In summary, we have shown that APAs exhibit consistent immunohistochemical and molecular features, especially the nuclear expression of β-catenin in squamous morules, the expression of HNF-1β, phosphorylated mTOR and GLUT1 in the glandular component, and "wild-type" p53 protein expression. Loss of PTEN expression and *KRAS* mutation also occurs in some cases. The immunophenotype and molecular events are similar to those found in AH and EC. On the basis of our results, and in agreement with previously published clinical and morphologic data, we believe that APA represents a lesion with an increased risk of developing AH and EC. Given the association with AH and EC and the shared immunohistochemical and molecular features, we feel that, conceptually, APA is best regarded as analogous to a localized form of AH.

REFERENCES

1. Mazur MT. Atypical polypoid adenomyomas of the endometrium. *Am J Surg Pathol*. 1981;5:473–482.
2. Fukunaga M, Endo Y, Ushigome S, et al. Atypical polypoid adenomyomas of the uterus. *Histopathology*. 1995;27:35–42.
3. Nejkovic L, Pazin V, Filimonovic D. Atypical polypoid adenomyoma mixed with endometrioid carcinoma: a case report. *Eur J Gynaecol Oncol*. 2013;34:101–103.
4. Zhang HK, Chen WD. Atypical polypoid adenomyomas progressed to endometrial endometrioid adenocarcinomas. *Arch Gynecol Obstet*. 2012;286:707–710.
5. Ota S, Catasus L, Matias-Guiu X, et al. Molecular pathology of atypical polypoid adenomyoma of the uterus. *Hum Pathol*. 2003;34:784–788.
6. Takahashi H, Yoshida T, Matsumoto T, et al. Frequent beta-catenin gene mutations in atypical polypoid adenomyoma of the uterus. *Hum Pathol*. 2014;45:33–40.
7. Kobel M, Reuss A, Du Bois A, et al. The biological and clinical value of p53 expression in pelvic high-grade serous carcinomas. *J Pathol*. 2010;222:191–198.
8. McCluggage WG, Soslow RA, Gilks CB. Patterns of p53 immunoreactivity in endometrial carcinomas: "all or nothing" staining is of importance. *Histopathology*. 2011;59:786–788.
9. Chen B, Zhu L, Lang JH. Atypical polypoid adenomyoma of the uterus: an analysis of 17 cases [In Chinese]. *Zhonghua Yi Xue Za Zhi*. 2012;92:1049–1051.
10. Longacre TA, Chung MH, Rouse RV, et al. Atypical polypoid adenomyofibromas (atypical polypoid adenomyomas) of the uterus. A clinicopathologic study of 55 cases. *Am J Surg Pathol*. 1996;20:1–20.
11. Matsumoto T, Hiura M, Baba T, et al. Clinical management of atypical polypoid adenomyoma of the uterus. A clinicopathological review of 29 cases. *Gynecol Oncol*. 2013;129:54–57.
12. Young RH, Treger T, Scully RE. Atypical polypoid adenomyoma of the uterus. A report of 27 cases. *Am J Clin Pathol*. 1986;86:139–145.
13. Heatley MK. Atypical polypoid adenomyoma: a systematic review of the English literature. *Histopathology*. 2006;48:609–610.
14. Jiang QY, Wang L, Wu RJ. A multiple perspectives on atypical polypoid adenomyoma of uterus. *Gynecol Endocrinol*. 2013;29:623–625.
15. Briggs S, Tomlinson I. Germline and somatic polymerase epsilon and delta mutations define a new class of hypermutated colorectal and endometrial cancers. *J Pathol*. 2013;230:148–153.
16. Byron SA, Gartside M, Powell MA, et al. FGFR2 point mutations in 466 endometrioid endometrial tumors: relationship with MSI, KRAS, PIK3CA, CTNNB1 mutations and clinicopathological features. *PLoS One*. 2012;7:e30801.
17. Cancer Genome Atlas Research Network. Kandoth C, Schultz N, Cherniack AD, et al. Integrated genomic characterization of endometrial carcinoma. *Nature*. 2013;497:67–73.
18. Chmelarova M, Kos S, Dvorakova E, et al. Importance of promoter methylation of GATA4 and TP53 genes in endometrioid carcinoma of endometrium. *Clin Chem Lab Med*. 2014;52:1229–1234.
19. Dvorakova E, Chmelarova M, Laco J, et al. Methylation analysis of tumor suppressor genes in endometroid carcinoma of endometrium using MS-MLPA. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2013;157:298–303.

20. Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. *J Clin Oncol*. 2006;24:4783–4791.
21. Lax SF, Kurman RJ. A dualistic model for endometrial carcinogenesis based on immunohistochemical and molecular genetic analyses. *Verh Dtsch Ges Pathol*. 1997;81:228–232.
22. Matias-Guiu X, Prat J. Molecular pathology of endometrial carcinoma. *Histopathology*. 2013;62:111–123.
23. Saegusa M, Hashimura M, Kuwata T, et al. Beta-catenin simultaneously induces activation of the p53-p21WAF1 pathway and overexpression of cyclin D1 during squamous differentiation of endometrial carcinoma cells. *Am J Pathol*. 2004;164:1739–1749.
24. Saegusa M, Okayasu I. Frequent nuclear beta-catenin accumulation and associated mutations in endometrioid-type endometrial and ovarian carcinomas with squamous differentiation. *J Pathol*. 2001;194:59–67.
25. Dobrzycka B, Terlikowski SJ, Mazurek A, et al. Mutations of the KRAS oncogene in endometrial hyperplasia and carcinoma. *Folia Histochem Cytobiol*. 2009;47:65–68.
26. Liu FS. Molecular carcinogenesis of endometrial cancer. *Taiwan J Obstet Gynecol*. 2007;46:26–32.
27. Feng YZ, Shiozawa T, Miyamoto T, et al. BRAF mutation in endometrial carcinoma and hyperplasia: correlation with KRAS and p53 mutations and mismatch repair protein expression. *Clin Cancer Res*. 2005;11:6133–6138.
28. Mutter GL, Lin MC, Fitzgerald JT, et al. Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *J Natl Cancer Inst*. 2000;92:924–930.
29. Lacey JV Jr, Mutter GL, Ronnett BM, et al. PTEN expression in endometrial biopsies as a marker of progression to endometrial carcinoma. *Cancer Res*. 2008;68:6014–6020.
30. Djordjevic B, Hennessy BT, Li J, et al. Clinical assessment of PTEN loss in endometrial carcinoma: immunohistochemistry outperforms gene sequencing. *Mod Pathol*. 2012;25:699–708.
31. Cheng H, Liu P, Zhang F, et al. A genetic mouse model of invasive endometrial cancer driven by concurrent loss of Pten and Lkb1 is highly responsive to mTOR inhibition. *Cancer Res*. 2014;74:15–23.
32. Ocana A, Vera-Badillo F, Al-Mubarak M, et al. Activation of the PI3K/mTOR/AKT pathway and survival in solid tumors: systematic review and meta-analysis. *PLoS One*. 2014;9:e95219.
33. Hamid AA, Mandai M, Konishi I, et al. Cyclical change of hMSH2 protein expression in normal endometrium during the menstrual cycle and its overexpression in endometrial hyperplasia and sporadic endometrial carcinoma. *Cancer*. 2002;94:997–1005.
34. Hardisson D, Moreno-Bueno G, Sanchez L, et al. Tissue microarray immunohistochemical expression analysis of mismatch repair (hMLH1 and hMSH2 genes) in endometrial carcinoma and atypical endometrial hyperplasia: relationship with microsatellite instability. *Mod Pathol*. 2003;16:1148–1158.
35. Nieminen TT, Gylling A, Abdel-Rahman WM, et al. Molecular analysis of endometrial tumorigenesis: importance of complex hyperplasia regardless of atypia. *Clin Cancer Res*. 2009;15:5772–5783.
36. Orbo A, Nilsen MN, Arnes MS, et al. Loss of expression of MLH1, MSH2, MSH6, and PTEN related to endometrial cancer in 68 patients with endometrial hyperplasia. *Int J Gynecol Pathol*. 2003;22:141–148.
37. Peiro G, Diebold J, Lohse P, et al. Microsatellite instability, loss of heterozygosity, and loss of hMLH1 and hMSH2 protein expression in endometrial carcinoma. *Hum Pathol*. 2002;33:347–354.
38. Berends MJ, Hollema H, Wu Y, et al. MLH1 and MSH2 protein expression as a pre-screening marker in hereditary and non-hereditary endometrial hyperplasia and cancer. *Int J Cancer*. 2001;92:398–403.
39. Ichikawa Y, Lemon SJ, Wang S, et al. Microsatellite instability and expression of MLH1 and MSH2 in normal and malignant endometrial and ovarian epithelium in hereditary nonpolyposis colorectal cancer family members. *Cancer Genet Cytogenet*. 1999;112:2–8.
40. Folkins AK, Longacre TA. Hereditary gynaecological malignancies: advances in screening and treatment. *Histopathology*. 2013;62:2–30.
41. Garg K, Leitao MM Jr, Kauff ND, et al. Selection of endometrial carcinomas for DNA mismatch repair protein immunohistochemistry using patient age and tumor morphology enhances detection of mismatch repair abnormalities. *Am J Surg Pathol*. 2009;33:925–933.
42. Garg K, Soslow RA. Lynch syndrome (hereditary non-polyposis colorectal cancer) and endometrial carcinoma. *J Clin Pathol*. 2009;62:679–684.
43. Tsuchiya A, Sakamoto M, Yasuda J, et al. Expression profiling in ovarian clear cell carcinoma: identification of hepatocyte nuclear factor-1 beta as a molecular marker and a possible molecular target for therapy of ovarian clear cell carcinoma. *Am J Pathol*. 2003;163:2503–2512.
44. Hoang LN, Han G, McConechy M, et al. Immunohistochemical characterization of prototypical endometrial clear cell carcinoma—diagnostic utility of HNF-1beta and oestrogen receptor. *Histopathology*. 2014;64:585–596.
45. Fadare O, Liang SX. Diagnostic utility of hepatocyte nuclear factor 1-beta immunoreactivity in endometrial carcinomas: lack of specificity for endometrial clear cell carcinoma. *Appl Immunohistochem Mol Morphol*. 2012;20:580–587.
46. Kato N, Sasou S, Motoyama T. Expression of hepatocyte nuclear factor-1beta (HNF-1beta) in clear cell tumors and endometriosis of the ovary. *Mod Pathol*. 2006;19:83–89.
47. Worley MJ, Welch WR, Berkowitz RS, et al. Endometriosis-associated ovarian cancer: a review of pathogenesis. *Int J Mol Sci*. 2013;14:5367–5379.
48. Wang BY, Kalir T, Sabo E, et al. Immunohistochemical staining of GLUT1 in benign, hyperplastic, and malignant endometrial epithelia. *Cancer*. 2000;88:2774–2781.
49. Ashton-Sager A, Paulino AF, Afify AM. GLUT-1 is preferentially expressed in atypical endometrial hyperplasia and endometrial adenocarcinoma. *Appl Immunohistochem Mol Morphol*. 2006;14:187–192.
50. Krzeslak A, Wojcik-Krowiranda K, Forma E, et al. Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. *Pathol Oncol Res*. 2012;18:721–728.

4.6. Expression, epigenetic and genetic changes of HNF1B in endometrial lesions.

Pathol. Oncol. Res.
DOI 10.1007/s12253-015-0037-2

ORIGINAL ARTICLE

Expression, Epigenetic and Genetic Changes of HNF1B in Endometrial Lesions

Kristýna Němejcová[1] · Ivana Tichá[1] · Petra Kleiblová[2,3] · Michaela Bártů[4] · David Cibula[5] · Kateřina Jirsová[6] · Pavel Dundr[1]

Received: 28 October 2015 / Accepted: 15 December 2015

Abstract Hepatocyte nuclear factor 1-beta (HNF-1-beta) is a transcription factor involved in cancerogenesis of various tumors, including endometrioid carcinoma. We performed comprehensive analysis of HNF-1-beta in lesions of the endometrium, including protein expression and genetic and epigenetic changes. Expression of HNF-1-beta was analyzed immunohistochemically in 320 cases including both tumor and non-tumor endometrial lesions. Promoter methylation and genetic variants were evaluated, using bisulphite and direct sequencing, in 30 (18 fresh frozen, 12 FFPE tumors) endometrioid carcinomas (ECs) and 15 ovarian clear cell carcinomas (OCCCs) as a control group. We detected expression of HNF-1-beta in 28 % of ECs (51/180 cases), 26 % of serous carcinoma (7/27 cases), 83 % of endometrial clear cell carcinoma (15/18 cases), 93 % of hyperplastic polyps with atypias (13/14 cases), 100 % of hyperplastic polyps without atypias (16/16 cases), 88 % of hyperplasias with atypias (14/16 cases), 91 % of hyperplasias without atypias (10/11 cases), and in ≥80 % of different normal endometrium samples. The control group of OCCCs showed HNF-1-beta expression in 95 % (18/19 cases). Methylation in promoter region was detected in 13.3 % (4/30) of ECs, but not in corresponding normal tissue where available, nor in OCCCs (0/15 cases). Mutation analysis revealed truncating variant c.454C > T (p.Gln152X) in one EC and missense variant c.848C > T (p.Ala283Val) was detected in one OCCC. In conclusion, expression of HNF-1-beta was detected in various extents in all types of lesions analyzed, nevertheless its strong expression was mostly limited to clear cell carcinomas. Biological significance of genetic and epigenetic changes needs further investigation.

Keywords Clear cell carcinoma · Endometrioid carcinoma · HNF-1-beta · Immunohistochemistry · Mutation analysis · Methylation

✉ Pavel Dundr
pdundr@seznam.cz

[1] Department of Pathology, First Faculty of Medicine and General University Hospital, Charles University in Prague, Studnickova 2, 2 12800 Prague, Czech Republic

[2] Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, General University Hospital, Charles University in Prague, Prague, Czech Republic

[3] Institute of Biology and Medical Genetics, First Faculty of Medicine, General University Hospital in Prague, Charles University in Prague, Prague, Czech Republic

[4] First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

[5] Gynecologic Oncology Center, Department of Obstetrics and Gynecology, First Faculty of Medicine and General University Hospital, Charles University in Prague, Studnickova 2, 2 12800 Prague, Czech Republic

[6] Bank of Biological Material, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Introduction

Hepatocyte nuclear factor 1-beta (HNF-1-beta protein is coded by the *HNF1B* gene, also known as *TCF2*) is a transcription factor that plays a crucial role during ontogenesis in the differentiation of visceral endoderm from primitive endoderm [1, 2]. The gene is located at chromosome 17q12, comprises 9 coding exons that spans around 60 kb (MIM#189,907) and encodes four protein isoforms (designated as A, B, C and 4) [3, 4]. The longest A isoform is 557 amino acid (aa) protein that contains a dimerization domain, aa 1–31, and a DNA binding domain,

Published online: 19 December 2015

aa 231–311 (UniProt P35680; http://www.uniprot.org). The B isoform, comparing to A, lacks aa 183–208. The C isoform differs in structure from A in aa 350–400, and in the C terminus aa 401–557 are missing [3, 4]. A and B isoforms are regarded as transcription activators, whereas the C isoform is probably a transcription repressor [3]. HNF-1-beta protein regulates expression of multiple genes involved in cell cycle modulation, susceptibility to apoptosis, and glucose metabolism [5–7]. Hereditary mutations in the *HNF1B* gene are associated with a number of diseases associated with defects in kidney development and a complex syndrome known as renal cysts and diabetes (RCAD) [8, 9]. Expression of the HNF-1-beta protein is altered in several tumor types, with both down- or up-regulations described based on the tumor type. There can also be changes in isoform expressions in benign and malignant conditions [10]. Moreover, genetic and epigenetic changes of *HNF1B* also play role in tumorigenesis. However, only few studies have analyzed epigenetic changes of *HNF1B* in female genital tract tumors and only 6 somatic genetic variants (2 silent, 3 missense and 1 frameshift) were described in a group of 514 endometrioid carcinomas as a part of the large study "Uterine Corpus Endometrioid Carcinoma (TCGA, US) import from ICGC" (Cosmic database; accessed October 2015; http://www.sanger.ac.uk/cosmic) [11, 12]. Immunohistochemical expression of HNF-1-beta was considered as a marker of clear cell carcinomas (CCCs) [5]. However, recent studies have shown that this marker is not entirely specific and its expression can be found in tumors of other histogenesis and also in some non-tumor lesions and normal endometrium [13–21]. In our study, we focused on comprehensive analysis of HNF-1-beta expression in various endometrial lesions and in normal endometrium. Moreover, we analyzed genetic variations of the *HNF1B* coding region and methylation of the promoter region in selected cases of endometrioid carcinoma and ovarian clear cell carcinoma (OCCC) and compared these findings with immunohistochemical results.

Material and Methods

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were obtained from the archive files of our department. Fresh frozen tissue samples were from Bank of Biological Material (BBM) of the First Faculty of Medicine, Charles University in Prague. Histologic review of the hematoxylin and eosin-stained slides was performed in all cases.

In total, 339 FFPE specimens was selected for immunohistochemical analysis, comprising of 320 endometrial lesions; 180 endometrioid carcinomas (77 well differentiated, 73 moderately differentiated, and 30 poorly differentiated), 27 serous carcinomas, 18 endometrial CCC, 14 hyperplastic polyps with atypias, 16 hyperplastic polyps without atypias, 16 hyperplasias with atypias, 11 hyperplasias without atypias, 38 samples from normal endometrium (18 proliferative endometrium, 15 secretory endometrium, and 5 atrophic endometrium); and as control 19 OCCC.

Promoter methylation and gene mutations were examined in 33 cases of endometrioid carcinoma (15 FFPE and 18 fresh frozen (FF) tumors), 15 FF corresponding normal non-tumor tissues and 19 FFPE samples of OCCC. We were able to isolate DNA of sufficient quality for molecular analysis from all fresh frozen tissues and from 12/15 (80 %) FFPE endometrioid carcinoma samples. Regarding OCCC FFPE samples, DNA of sufficient quality was obtained from 15/19 (79 %) cases for methylation analysis and 12/19 (63 %) cases for mutation analysis of whole coding region. Both, methylation and mutation analyses were successful in 10/15 endometrioid carcinomas and 12/19 OCCCs.

In compliance with the Helsinki Declaration, the project has been approved by Ethics Committee of General University Hospital in Prague.

Immunohistochemical Analysis

Immunohistochemical analysis was performed using the avidin-biotin complex method with antibody against the HNF-1-beta (polyclonal, dilution 1:500, Sigma-Aldrich, Prestige Antibodies, St. Louis, United States). Immunohistochemical results were assessed semi-quantitatively and graded on a four-tier scale based on the percentage of positive cells: 0 = <5 %; 1+ = 5–29 %; 2+ = 30–59 %; 3+ = >60 %. Only nuclear staining was regarded as positive. Moreover, the staining intensity of HNF-1-beta was assessed as weak, moderate or strong.

Statistical Analyses

Software R (https://www.r-project.org/) was used to perform Fisher exact test in order to compare different tumor groups based on the immunohistochemical staining intensity or the percentage of positive staining cells. All tests were two sided, and a *P*-value less than 0.05 was considered as significant.

Screening for Genetic and Epigenetic Alterations

Methylation status in promoter region and mutations in coding region and exon-intron boundaries of *HNF1B* were screened. DNA was extracted by using standard procedures implementing GeneRead™ DNA FFPE kit or QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). DNA was quantified fluorometrically by using dsDNA BR Assay Kit and Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). Bisulfite modification was analyzed by using EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions,

followed by sequencing. Experiments included two controls, non-methylated DNA and universally methylated DNA (Human HCT116 DKO Non-Methylated DNA and Human HCT116 DKO Methylated DNA; Zymo Research). Primers used for PCR amplification of promoter region and bisulphite sequencing of modified DNA (Table 1) were designed by using software http://www.zymoresearch.com/tools/bisulfite-primer-seeker. Primers for mutation analysis (Table 1) in the exons 1 to 9 and adjacent intron sequences were used for PCR reaction and direct Sanger sequencing. DNA regions of interest were amplified using 5× HOT FirePol EvaGreen HRM Mix NO ROX (Solis Biodyne, Tartu, Estonia) according to the manufacturer's instructions and analyzed by HRMA (High Resolution Melting Analysis). BigDye Terminator v3. 1 Ready Reaction Mix (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for sequencing reaction and separation was performed on ABI 3130 genetic analyzer (Applied Biosystems). Mutations were detected by both forward and reverse primer. Suspicious fragments were verified by another independent PCR amplification and sequence analysis.

NCBI Reference sequence of the *HNF1B* gene (alternatively TCF2) is NM_000458.3. Promoter region 95–695 bp upstream ATG (start codon) was defined as putative promoter region according RefSeq (Based on UCSC/NCBI Assembly, NCBI Build 35, UCSC Version 17). Nomenclature of variants follow recommendations by the Human Genome Variation Society (HGVS). Mutations which were not found until October 2015 in the literature, the Single Nucleotide Polymorphism Database (http://www.ncbi.nlm.nih.gov/SNP/), the 1000 Genomes Database (http://www.1000genomes.org/), in Exome Sequencing Project (ESP; http://varianttools.sourceforge.net/Annotation/EVS), or in the Catalogue of Somatic Mutations in Cancer (http://www.sanger.ac.uk/cosmic) were considered as novel [22]. To evaluate the predicted effects of missense variants on protein biological function and stability, in silico prediction tools were used: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), Mutation

Table 1 Primer pairs used for PCR amplification and sequence analysis of the *HNF1B* gene

Exon	Name	Primers 5′- > 3′	bp	Amplicon [bp]
Primers for methylation analysis in promoter region				
	HNF1B_mp2F	TTAGGTTGTAAGGTATTGGTTTAATAAGTTTAAAGG	36	160
	HNF1B_mp2R	AAACAAACCTAAAAAACAAAAAACCTAAACC	31	
Primers for mutation analysis of coding region (* long exons were divided in two overlapping amplicons)				
*1	HNF1B_01f	TGCTTGTGAAACTCCCCTCCAC	22	277
	HNF1B_02r	TGGTGAGAGTATGGAAGACCGGC	23	
1	HNF1B_03f	TTGCTGCCATCCCCGAACTTC	21	274
	HNF1B_04r	TCCCCTCCACCTCGCTCTG	19	
2	HNF1B_05f	CCCCAGATGTCTCCCACTAGTACC	24	287
	HNF1B_06r	CACTTCAGGTTGAGGCAGAGGC	22	
*3	HNF1B_07f	GGTGTCTTCGTCCGTTGTCTGTC	23	258
	HNF1B_08r	ATTTGAACCGGTTGCGGCG	19	
3	HNF1B_09f	GTCAACAGAGCCATGGGCCTG	21	234
	HNF1B_10r	TCCTGGGTCTGTGTACTTGCCC	22	
*4	HNF1B_11f	CTGCTGTGATTGTGTGTTTTTGGCC	25	245
	HNF1B_12r	AGTCTGGTTGGAGCTATAGGCGTC	24	
4	HNF1B_13f	TGCAAACCGCAGGAAGGAGG	22	270
	HNF1B_14r	GAGAGCGGCCCTAGGATCATCTC	23	
5	HNF1B_15f	CAGGACCCTGGTGGCACTAATG	22	246
	HNF1B_16r	AGCTCCAGAGCGACAATGGC	20	
6	HNF1B_17f	GGAAACTGCTCTTTGTGGTCCAAGTC	26	241
	HNF1B_18r	TCTTCTTCTCCCTGCCCCCAAG	22	
7	HNF1B_19f	CTCCTTATCCCAGGAGCTGTCTGTG	25	290
	HNF1B_20r	ACTGAGGGTCCTGAGTGCTCC	21	
8	HNF1B_21f	GCCTGTGTATGCACCTTGATTCTG	24	226
	HNF1B_22r	CACATCCATGGCCTTATCACACCC	24	
9	HNF1B_23f	AATGACACAGCTGAGCACCCTC	22	172
	HNF1B_24r	CTCGCAGGTGCTGGTCAGG	19	

Taster (http://www.mutationtaster.org), and MUpro (http://mupro.proteomics.ics.uci.edu).

Results

Immunohistochemical Findings

All the results are summarized in Table 2. Figure 1 shows representative examples of the HNF-1-beta expression in various endometrial lesions.

The group of all CCCs in comparison with the tumors of different histogenesis showed significant difference in the HNF-1-beta expression based on the intensity ($p < 0.001$) as well as the extension of staining ($p < 0.001$) when comparing CCCs with serous carcinomas and comparing CCC with endometrioid carcinomas (EC).

Expression of HNF-1-beta was found in 33/37 (89 %) of all CCCs (18/19 [95 %] ovarian and 15/18 [83 %] endometrial). Fourteen cases OCCCs were 3+ positive, four cases showed 2+ positivity, and only one case was negative. From the group of endometrial CCCs there were eleven cases 3+ positive, two were 2+ positive, two were positive only focally 1+, and three were negative. The intensity of staining was in all HNF-1-beta positive CCCs strong, with the exception of 1 case of endometrial tumor, which showed weak staining in about 7 % of the tumor cells (1+).

ECs showed positivity of HNF-1-beta in 25/77 (32 %) of well differentiated tumors, 24/73 (33 %) of moderately differentiated tumors, and 2/30 (7 %) of poorly differentiated tumors. In total, 51/180 (28 %) ECs were HNF-1-beta positive. Nine ECs were 3+, 14 were 2+, and 28 were 1+ positive. The staining intensity was usually weak, only five cases showed moderate intensity. No EC demonstrated strong HNF-1-beta expression.

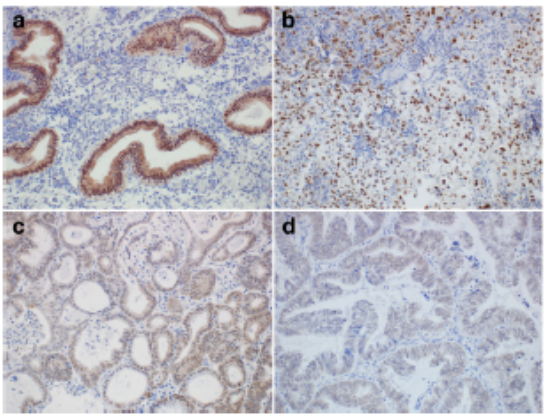


Fig. 1 HNF-1 beta expression in endometrial lesions. Strong HNF-1 beta expression in normal secretory endometrium (1a, 400×) and clear cell carcinoma (1b, 200×). Staining of moderate intensity in grade 1 endometrioid carcinoma (1c, 400×). Weak nuclear staining in grade 1 endometrioid carcinoma (1d, 400×). Note the nonspecific cytoplasmic staining in some cases

Serous carcinomas were HNF-1-beta positive in 7/27 cases (26 %). 2 cases were 1+ positive, 5 cases showed 3+ positivity. The intensity of staining was weak in three cases, moderate in three cases and strong in one case.

All 16 hyperplastic polyps without atypias and 13/14 (93 %) hyperplastic polyps with atypias were HNF-1-beta positive: 4 cases 2+, 12 cases 3+; and 3 cases 1+, 5 cases 2+, 5 cases 3+, respectively. The intensity of staining was mostly weak or moderate.

Weak or moderate intensity of HNF-1-beta was found in 10/11 (91 %) hyperplasias without atypias (2 cases 1+, 3 cases 2+, 5 cases 3+) and in 14/16 (88 %) of hyperplasias with atypias (8 cases 1+, 2 cases 2+, 4 cases 3+).

Samples from normal endometrium showed positivity of HNF-1-beta in 16/18 (89 %) of proliferative endometrium

Table 2 Summary of immunohistochemical results

HNF1B	OCCC	CCCE	SC	EC			HPA	HP	HA	H	Normal endom.		
				G1	G2	G3					Sec.	Pro.	Atr.
Total	19	18	27	77	73	30	14	16	16	11	15	18	5
Positive	18	15	7	25	24	2	13	16	14	10	15	16	4
3+	14	11	5	3	4	2	5	12	4	5	13	11	4
2+	4	2	0	7	7	0	5	4	2	3	1	4	0
1+	0	2	2	15	13	0	3	0	8	2	1	1	0
Negative	1	3	20	52	49	28	1	0	2	1	0	2	1

OCCC Ovarian clear cell carcinomas, *CCCE* Endometrial clear cell carcinomas, *SC* Serous carcinomas of endometrium, *EC* Endometrioid carcinomas, *HPA* Hyperplastic polyps with atypias, *HP* Hyperplastic polyps without atypias, *HA* Hyperplasias with atypias, *H* Hyperplasias without atypias, *Normal endom.* Normal endometrium, *Sec.* Secretory, *Pro.* Proliferative, *Atr.* Atrophic, *G1* Well differentiated, *G2* Moderately differentiated, *G3* Poorly differentiated carcinomas. Immunohistochemical results were assessed semi-quantitatively and graded on a four-tier scale based on the percentage of positive cells: 0 = <5 %; 1+ = 5–29 %; 2+ = 30–59 %; 3+ = >60 %

(1 case 1+, 4 cases 2+, 11 cases 3+), in all 15 cases of secretory endometrium (1 case 1+, 1 case 2+, 13 cases 3+), and in 4/5 (80 %) of atrophic endometrium (4 cases 3+). Intensity of staining in proliferative endometrium was weak in 10 cases and moderate in 8 cases. In secretory endometrium the intensity of staining was mostly moderate or strong, except of two cases which showed only weak intensity. In all cases of atrophic endometrium the intensity of staining was weak.

Genetic and Epigenetic Changes of the *HNF1B* Gene

Epigenetic Analysis

Gold standard method employing bisulphite modification of DNA followed by PCR amplification and direct sequencing of target area was used for the analysis of DNA methylation. We assessed DNA methylation in a part of the promoter region that spans 3 CpG islands (Fig. 2). Methylation in promoter region was detected in 4/30 (13.3 %) ECs, but not in 3 available corresponding normal tissues. Compared to EC, no case of promoter methylation was found in 15 cases of OCCC examined.

Mutation Analysis

Direct DNA sequence analysis of the *HNF1B* gene demonstrates 4 different single nucleotide variants in 4 ECs (one nonsense variant in exon 2, c.454C > T, p.Gln152X; Fig. 3; one silent variant in exon 3, c.585C > T, p.Asp195; two noncoding variants in intron 2 and intron 3, flanking exon 3: c.545-25C > T and c.809 + 27C > T), and one missense variant in exon 4 (c.848C > T, p.Ala283Val) in OCCC. All 3 exon variants were heterozygous transitions C > T, except mutation p.Gln152X where the major variant was mutant allele T. All variants have been reported for the first time. Non-tumor tissue was not available to confirm somatic status of the variant. Truncating mutation p.Gln152X leads to a shorter dysfunctional protein and is generally considered as pathogenic, and p.Ala283Val was evaluated by in silico model as deleterious (Table 3).

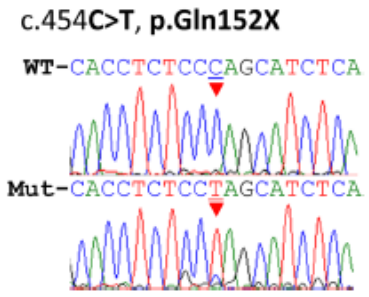


Fig. 2 Bisulfite sequence electropherograms. Fragments show methylation status in three CpG sites from HNF1B promoter. Representative electropherograms of (**a**) methylated, and (**b**) non-methylated are shown. Sodium bisulfite deaminates cytosine into uracil but does not affect 5-methylcytosine. Uracils are detected as thymines after PCR and sequencing reaction. *Triangles* point cytosins in CpG islands

Discussion

HNF-1-beta is a transcription factor which plays an essential role during ontogenesis and seems to be involved in cancerogenesis of various tumors, including endometrioid carcinoma [1, 2, 23–25]. As indicated by recent genome-wide association studies, common genetic variants of this gene are associated with risk of type II diabetes and either increased or decreased risk of several cancers including endometrioid endometrial carcinoma and serous ovarian carcinoma [7, 11, 26–29]. However, the exact mechanism by which *HNF1B* participates in the process of cancerogenesis is unknown and probably differs in various types of tumors.

In one study, knock-down of *HNF1B* in OCCC led to the induction of apoptosis [5]. This correlates with results of a recent study which has shown that upregulation of *HNF1B* by inflammatory cytokine NF-κB/p65 decreases OCCC susceptibility to apoptosis [6].

Regarding the fact that *HNF1B* can be either down-regulated or up-regulated, *HNF1B* can probably serve as either a tumor suppressor gene or an oncogene in different cancers. It has been shown that down-regulation of *HNF1B* in clear-cell renal cell carcinoma and prostate carcinoma is associated with tumor progression and poor prognosis [24, 30]. A different situation is present in OCCC. The promotor of *HNF1B* in OCCC is typically unmethylated and gene expression is increased compared with other ovarian cancer types [5, 11]. This corresponds to our data. We have not identified any methylated case in the control group of 15 OCCCs. On the contrary, methylation of *HNF1B* gene promoter was detected in 4/30 EC samples (in 1/12 FFPE and 3/18 fresh frozen tumors). The corresponding non-tumor tissue available in 15/18 fresh frozen patients was unmethylated. Methylation of the *HNF1B* gene promoter was found in some cancer cell lines derived from pancreatic, colorectal, gastric, and ovarian tumors [12]. DNA methylation is typically associated with the silencing of the gene expression. In our study, it was not possible to correlate *HNF1B* genetic and epigenetic variations with expression pattern of HNF-1-beta because of a small sample set. Nevertheless, we observed in all 4 cases weak or negative HNF-1-beta staining with methylated promoter, while stronger expression was detected in the group with unmethylated promoter.

Surprisingly, despite the growing knowledge about the importance of single nucleotide polymorphisms (SNPs) of *HNF1B* in several cancer types, little is known about mutations of this gene in various tumors, except for some germline mutations associated with kidney cancer [31]. In our study, screening of genetic variants in the whole coding region

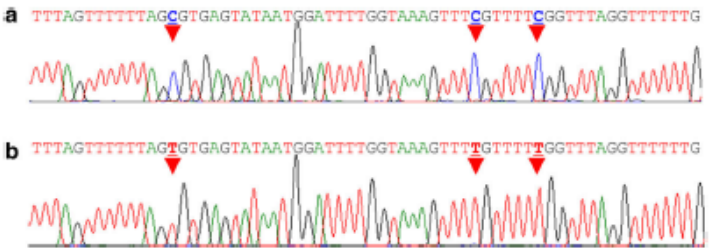


Fig. 3 Novel frame-shift mutation of *HNF1B*. Comparison between wild type (WT) and mutated (Mut) sequence. Changed nucleotide is indicated by a *triangle*

revealed 4 sequence variants in 30 ECs and one missense variant among 12 OCCCs. Nonsense mutation (p.Gln152X) identified in one EC leads to premature translation termination resulting to the aberrant protein formation. This truncated protein has retained epitope detected by our antibody which can explain detection of HNF-1-beta expression. Mutation detected in one OCCC case was missense variant leading to exchange of amino acid Alanine to Valine at the position 283 (p.Ala283Val). This variant affects DNA binding domain (amino acids 231–311) which suggests that this variant affects crucial function of the protein. High probability of damaging effect of this mutation was suggested also by in silico predictive model.

Regarding protein expression, HNF-1-beta was identified in 2003 as the first positive relatively specific immunohistochemical marker of CCC [5]. Since that, expression of HNF-1-beta in non-neoplastic tissue and neoplasms of the female genital tract was analyzed in few studies [13–18]. These studies have analyzed HNF-1-beta expression in endometriosis, normal endometrium, lesions and tumors of cervix and ovary. Most of the early studies found that expression of HNF-1-beta is mostly restricted to CCC [5, 14–16]. However, more recent studies have described HNF-1-beta expression not only in CCC but also in other tumor types including serous, endometrioid, and mucinous carcinomas and most types of borderline tumors [17, 19–21]. We and others have recently described expression of HNF-1-beta in cervical adenocarcinomas and we have also described its expression in atypical polypoid adenomyomas of the uterus [32, 33]. Other recent studies have found expression of HNF-1-beta in some cases of endometriosis and in normal endometrium, especially in the secretory phase or gestational state [13, 14]. The results of our study are in concordance with other recent studies, which have shown that HNF-1-beta is not a specific marker of CCC and can be commonly found in other non-tumor and tumor lesions. However, among tumors, strong expression of HNF-1-beta was in our study found only in CCC, with exception of one case of endometrial serous carcinoma. Moreover, we observed different HNF-1-beta expression in ECs depending on differentiation of tumor. ECs with poor differentiation seldom express HNF-1-beta compared with ECs with well or moderate differentiation.

Despite the fact that HNF-1-beta is a relatively sensitive marker of CCC, specificity is lower than previously thought. For example, in a recent study of ovarian and uterine ECs, ECs with clear cell change and CCC, the authors found sensitivity of HNF-1-beta similar as for Napsin A, but the specificity was lower (55.9 % vs. 93 %) [34]. Another recent study analyzed expression of HNF-1-beta, Napsin A, and AMACR in 279 ovarian carcinomas [35]. HNF-1-beta and Napsin A were expressed in 92 % and 82 % of OCCC, 7 % and 1 % of serous carcinoma, 37 % and 5.3 % of EC, 60 % and 0 % of mucinous tumors, and 100 % and 0 % of yolk sac tumors, respectively. HNF-1-beta expression in 45 endometrial carcinomas has also been assessed in another study analyzing 15 cases each from CCC, serous carcinoma and EC, respectively [25]. In their study, strong expression of HNF-1-beta was found in 100 % of CCC. Three out of 15 cases of EC (grade 3) showed expression in >25 % of tumor cells (50 % tumor cells, weak to moderate intensity). Five serous carcinomas were completely HNF-1-beta negative, 4 showed focal positivity in <25 % of tumor cells, and 6 cases showed strong positivity.

In conclusion, we performed comprehensive analysis of HNF-1-beta in normal endometrium and in various endometrial lesions, including its immunohistochemical expression

Table 3 *In silico* prediction of pathogenicity. Prediction analysis of identified novel variants in the *HNF1B* gene

Exon	Mutation designation*	Predicted effect on protein	Mutation Taster	PolyPhen-2	MUpro
2	c.454C > T	p.Q152X	Disease causing	NA	NA
4	c.848C > T	p.A283V	Disease causing	Probably damaging	Decrease stability

* GenBank Reference Sequence: NM_000458.3: +1 corresponds to the A of the ATG translation initiation codon. NA – in silico prediction tool is not applicable for nonsense mutations

and analysis of epigenetic and genetic changes. Immunohistochemical analysis of HNF-1-beta expression can be of use in the differential diagnosis of female genital tract tumors. We should be aware of some limitations, especially its low specificity for CCC. However, if we consider the character of staining, strong expression of HNF-1-beta was found in our study, with one exception, only in a group of CCCs. Further, molecular analysis revealed relatively common (13.3 %) methylation of the *HNF1B* promotor in EC compared to the unmethylated *HNF1B* promotor in OCCC. We have also found nonsense heterozygous mutations of *HNF1B* in 1 case of EC. Four other single nucleotide variants were identified, 3 of them in ECs (one silent variant in exon 3, and two noncoding variants in intron 2 and intron 3) and one in OCCC (missense variant in exon 4). However, regarding genetic and epigenetic changes, we are well aware of the limitations of our study because of small cohort of cases. The precise role of *HNF1B* in carcinogenesis as well as the importance of molecular targeting of the HNF-1-beta protein for therapeutic purposes remain unknown and require additional studies.

Acknowledgments This work was supported by Ministry of Health, Czech Republic (IGA MZ CR project NT14001-3/2013 and RVO-VFN 64165), by Charles University in Prague (Project PRVOUK-P27/LF1/1, UNCE 204024, and SVV UK 260148/2015), by Bank of Biological Material project BBM LM2010004, and by OPPK (Research Laboratory of Tumor Diseases, CZ.2.16/3.1.00/24509).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Barbacci E, Chalkiadaki A, Masdeu C, et al. (2004) HNF1β/TCF2 mutations impair transactivation potential through altered co-regulator recruitment. Hum Mol Genet 13:3139–3149
2. Cereghini S (1996) Liver-enriched transcription factors and hepatocyte differentiation. FASEB J 10:267–282
3. Bach I, Yaniv M (1993) More potent transcriptional activators or a transdominant inhibitor of the HNF1 homeoprotein family are generated by alternative RNA processing. EMBO J 12:4229–4242 Erratum in: EMBO J 1994 13:492
4. Horikawa Y, Iwasaki N, Hara M, et al. (1997) Mutation in hepatocyte nuclear factor-1-beta gene (TCF2) associated with MODY. Nat Genet 17:384–385
5. Tsuchiya A, Sakamoto M, Yasuda J, et al. (2003) Expression profiling in ovarian clear cell carcinoma: identification of hepatocyte nuclear factor-1 β as a molecular marker and a possible molecular target for therapy of ovarian clear cell carcinoma. Am J Pathol 163: 2503–2512
6. Suzuki E, Kajita S, Takahashi H, Matsumoto T, Tsuruta T, Saegusa M (2015) Transcriptional upregulation of HNF-1β by NF-κB in ovarian clear cell carcinoma modulates susceptibility to apoptosis through alteration in bcl-2 expression. Lab Investig 95:962–972. doi:10.1038/labinvest.2015.73
7. Pontoglio M (2000) Hepatocyte nuclear factor 1, a transcription factor at the crossroads of glucose homeostasis. J Am Soc Nephrol 16:140–143
8. Bellanné-Chantelot C, Chauveau D, Gautier JF, et al. (2004) Clinical spectrum associated with hepatocyte nuclear factor-1β mutations. Ann Intern Med 140:510–517
9. Lokmane L, Haumaitre C, Garcia-Villalba P, Anselme I, Schneider-Maunoury S, Cereghini S (2008) Crucial role of vHNF1 in vertebrate hepatic specification. Development 135:2777–2786. doi:10.1242/dev.023010
10. Harries LW, Perry JR, McCullagh P, Crundwell M (2010) Alterations in LMTK2, MSMB and HNF1B gene expression are associated with the development of prostate cancer. BMC Cancer 10:315. doi:10.1186/1471-2407-10-315
11. Shen H, Fridley BL, Song H, et al. (2013) Epigenetic analysis leads to identification of HNF1B as a subtype-specific susceptibility gene for ovarian cancer. Nat Commun 4:1628. doi:10.1038/ncomms2629
12. Terasawa K, Toyota M, Sagae S, et al. (2006) Epigenetic inactivation of TCF2 in ovarian cancer and various cancer cell lines. Br J Cancer 94:914–921
13. Kato N, Motoyama T (2009) Hepatocyte nuclear factor-1beta(HNF-1beta) in human urogenital organs: its expression and role in embryogenesis and tumorigenesis. Histol Histopathol 24:1479–1486
14. Yamamoto S, Tsuda H, Aida S, Shimazaki H, Tamai S, Matsubara O (2007) Immunohistochemical detection of hepatocyte nuclear factor 1β in ovarian and endometrial clear-cell adenocarcinomas and nonneoplastic endometrium. Hum Pathol 38:1074–1080
15. Kato N, Sasou S, Motoyama T (2006) Expression of hepatocyte nuclear factor-1β (HNF-1β) in clear cell tumors and endometriosis of the ovary. Mod Pathol 19:83–89
16. Kato N, Tamura G, Motoyama T (2008) Hypomethylation of hepatocyte nuclear factor-1β (HNF-1β) CpG island in clear cell carcinoma of the ovary. Virchows Arch 452:175–180
17. Park KJ, Kiyokawa T, Soslow RA, Lamb CA, Oliva E, Zivanovic O, Juretzka MM, Pirog EC (2011) Unusual endocervical adenocarcinomas: an immunohistochemical analysis with molecular detection of human papillomavirus. Am J Surg Pathol 35:633–646. doi:10.1097/PAS.0b013e31821534b9
18. Kenny SL, McBride HA, Jamison J, McCluggage WG (2012) Mesonephric adenocarcinomas of the uterine cervix and corpus: HPV-negative neoplasms that are commonly PAX8, CA125, and HMGA2 positive and that may be immunoreactive with TTF1 and hepatocyte nuclear factor 1-β. Am J Surg Pathol 36:799–807. doi:10.1097/PAS.0b013e31824a72c6
19. Fadare O, Liang SX (2012) Diagnostic utility of hepatocyte nuclear factor 1-beta immunoreactivity in endometrial carcinomas: lack of specificity for endometrial clear cell carcinoma. Appl Immunohistochem Mol Morphol 20:580–587. doi:10.1097/PAI.0b013e31824973d1
20. Kalloger SE, Köbel M, Leung S, et al. (2011) Calculator for ovarian carcinoma subtype prediction. Mod Pathol 24:512–521. doi:10.1038/modpathol.2010.215
21. Tomassetti A, De Santis G, Castellano G, Miotti S, Mazzi M, Tomasoni D, Van Roy F, Carcangiu ML, Canevari S (2008) Variant HNF1 modulates epithelial plasticity of normal and transformed ovary cells. Neoplasia 10:1481–1492
22. Forbes SA, Bindal N, Bamford S, et al. (2011) COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res 39(Database issue):D945–D950. doi:10.1093/nar/gkq929
23. Kim L, Liao J, Zhang M, Talamonti M, Bentrem D, Rao S, Yang GY (2008) Clear cell carcinoma of the pancreas: histopathologic features and a unique biomarker: hepatocyte nuclear factor-1beta. Mod Pathol 21:1075–1083. doi:10.1038/modpathol.2008.95

24. Buchner A, Castro M, Hennig A, Popp T, Assmann G, Stief CG, Zimmermann W (2010) Downregulation of HNF-1B in renal cell carcinoma is associated with tumor progression and poor prognosis. Urology 76:507e6–50711. doi:10.1016/j.urology.2010.03.042
25. Hoang LN, Han G, McConechy M, et al. (2014) Immunohistochemical characterization of prototypical endometrial clear cell carcinoma–diagnostic utility of HNF-1β and oestrogen receptor. Histopathology 64: 585–596. doi:10.1111/his.12286
26. Painter JN, O'Mara TA, Batra J, et al. (2015) Fine-mapping of the HNF1B multicancer locus identifies candidate variants that mediate endometrial cancer risk. Hum Mol Genet 24:1478–1492. doi:10.1093/hmg/ddu552
27. Spurdle AB, Thompson DJ, Ahmed S, et al. (2011) Genome-wide association study identifies a common variant associated with risk of endometrial cancer. Nat Genet 43:451–454. doi:10.1038/ng.812
28. Mandato VD, Farnetti E, Torricelli F, et al. (2015) HNF1B polymorphism influence the prognosis of endometrial cancer patients: a cohort study. BMC Cancer 15:229. doi:10.1186/s12885-015-1246-5
29. Pharoah PD, Tsai YY, Ramus SJ, et al. (2013) GWAS meta-analysis and replication identifies three new susceptibility loci for ovarian cancer. Nat Genet 45:362–370. doi:10.1038/ng.2564
30. Noto H, Osame K, Sasazuki T, Noda M (2010) Substantially increased risk of cancer in patients with diabetes mellitus: a systematic review and meta-analysis of epidemiologic evidence in Japan. J Diabetes Complicat 24:345–353. doi:10.1016/j.jdiacomp.2010.06.004
31. Rebouissou S, Vasiliu V, Thomas C, et al. (2005) Germline hepatocyte nuclear factor 1alpha and 1beta mutations in renal cell carcinomas. Hum Mol Genet 14:603–614
32. Němejcová K, Cibula D, Dundr P (2015) Expression of HNF-1β in cervical carcinomas: an immunohistochemical study of 155 cases. Diagn Pathol 10:8. doi:10.1186/s13000-015-0245-9
33. Němejcová K, Kenny SL, Laco J, et al. (2015) Atypical Polypoid Adenomyoma of the Uterus: An Immunohistochemical and Molecular Study of 21 Cases. Am J Surg Pathol 39:1148–1155. doi:10.1097/PAS.0000000000000428
34. Lim D, Ip PP, Cheung AN, Kiyokawa T, Oliva E (2015) Immunohistochemical Comparison of Ovarian and Uterine Endometrioid Carcinoma, Endometrioid Carcinoma With Clear Cell Change, and Clear Cell Carcinoma. Am J Surg Pathol 39: 1061–1069. doi:10.1097/PAS.0000000000000436
35. Fadare O, Zhao C, Khabele D, et al. (2015) Comparative analysis of Napsin A, alpha-methylacyl-coenzyme A racemase (AMACR, P504S), and hepatocyte nuclear factor 1 beta as diagnostic markers of ovarian clear cell carcinoma: an immunohistochemical study of 279 ovarian tumours. Pathology 47:105–111. doi:10.1097/PAT.0000000000000223

4.7. Clear cell sarcoma of vulva. A case report.

ORIGINAL ARTICLE

Clear cell sarcoma of vulva. A case report

Kristýna Němejcová[1], Pavel Dundr[1], Ivana Krajsová[2]

[1]*Department of Pathology, First Faculty of Medicine and General University Hospital, Charles University in Prague, Czech Republic*
[2]*Department of Dermatovenereology, First Faculty of Medicine and General University Hospital, Charles University in Prague, Czech Republic*

SUMMARY

We report the case of a 67-year-old female with clear cell sarcoma (CCS) of the vulva.
Grossly, the tumor was a partly exophytical vulvar mass, measuring 20 x 15 cm. At the time of presentation, the patient showed metastases to the lung, inguinal and pelvic lymph nodes. Histologically, the tumor consisted of oval or spindle cells with only mild nuclear pleomorphism and rare mitoses (up to 2/10 HPF). The cytoplasm was pale eosinophilic or clear. The tumor cells were arranged in confluent sheets. There were large areas of necrosis and surface ulceration. Immunohistochemically, the tumor cells showed expression of S-100 protein and focal melan A and HMB45 expression. Fluorescent in situ hybridization analysis revealed rearrangement of the EWSR1 gene. To the best of our knowledge, this is the first report of CCS arising in the vulva.

Keywords: clear cell sarcoma – EWSR1 gene – melanoma of soft parts – vulva

Světlobuněčný sarkom vulvy. Kazuistika

SOUHRN

Prezentujeme případ 67 leté ženy se světlobuněčným sarkomem vulvy. Nádor byl zčásti exofytický a dosáhl velikosti 20 x 15 cm. V době klinické prezentace byly prokázány metastázy v plicích a v ingvinálních a pánevních lymfatických uzlinách. Mikroskopicky byl nádor tvořený oválnými nebo vřetenitými buňkami s pouze mírným jaderným pleomorfismem. Mitózy byly zastiženy pouze řídce (nejvýše 2/10 HPF). Nádorové buňky měly světle eosinofilní či vodojasnou cytoplasmu a byly uspořádány ve splývající plochy. Nádor byl povrchově ulcerovaný, s rozsáhlými ložisky nekróz. Imunohistochemicky byl v nádorových buňkách pozitivní průkaz S-100 proteinu a fokálně průkaz Melanu A a HMB45. Fluorescenční in situ hybridizací jsme prokázali přestavbu genu EWSR1. Prezentujeme první případ primárního světlobuněčného sarkomu vulvy

Klíčova slova: světlobuněčný sarkom – gen EWSR1 – melanom měkkých částí – vulva

Cesk Patol 2016; 52(4): 215 –217

Clear cell sarcoma (CCS), also known as a melanoma of soft parts, is a malignant neoplasm first described by Enzinger in 1965 (1). This tumor is rare and accounts for 1 % of all soft tissue tumors. CCS typically involves deep soft tissues of the extremities, in close proximity to tendons and aponeurotic structures, especially in young adults (2). However, it can arise in other locations including the head and neck or trunk region and retroperitoneum. We report the case of a 67-year-old female with clear cell sarcoma of the vulva. To the best of our knowledge, our case represents the first report of CCS arising in the vulva.

CASE REPORT

Clinical history

A 67-year-old female presented with a one-year history of a slowly growing vulvar mass, finally measuring 20 x 15 cm at its largest size (Fig. 1). At the time of presentation, the patient showed metastases to the lung, inguinal and pelvic lymph nodes. A biopsy of the vulvar tumor was performed.

✉ ***Correspondence address:***
Kristýna Němejcová, M.D., Ph.D.
First Faculty of Medicine and General University Hospital,
Charles University in Prague, Studničkova 2, Prague 2, 12800,
Czech Republic
phone: +420224968632
e-mail: kristyna.nemejcova@vfn.cz

MATERIALS AND METHODS

Immunohistochemical analysis. Sections from formalin-fixed, paraffin-embedded tissue blocks were stained with hematoxylin-eosin. Selected sections were analysed immunohistochemically using the avidin-biotin complex method with antibody directed against following antigens: S-100 protein (1:600, Dako, Glostrup, Denmark), Melan A (clone A103, 1:25, Novocastra, Newcastle, UK), HMB45 (1:50, Dako) and Ki-67 (clone MIB-1, 1:50, Dako), cytokeratin AE1/AE3 (1:50, Dako, Glostrup, Denmark), cytokeratin high molecular weight (clone 34betaE12, 1: 200, Dako), estrogen receptors (ER, clone 6F11, 1:50, Novocastra), progesterone receptors (PR, clone 16, dilution 1:200, Novocastra), desmin (clone D33, 1:200, Dako), actin (clone HHF 35, 1:400 Dako), CD34 (clone QBEND 10, 1:50, Dako), CD99 (MIC2, clone 12E7, 1:50, Dako), synaptophysin (clone SY38, 1:25, Dako), chromogranin A (1:50, Dako) and p53 (clone DO-1, 1: 50, Bio, Genex).

Fluorescence in situ hybridization. The rearrangement of the EWSR1 gene (22q12) was analyzed using the Fluorescent in situ hybridization method with probe (Dual Color, Break Apart Rearrangement Probe from Abbott Vysis, Downers Grove, IL, USA). The assay procedure was conducted according to the manufacturer's instructions.

Isolation of nucleic acid. Sections of formalin-fixed, paraffin-embedded tissue were used for DNA isolation using standard procedures. Approximately seven 10-mm-thick sections from each sample were deparaffinized in xylene. The DNA was then extracted using the QIAamp DNA mini kit (Qiagen, Hamburg, Germany).

Analysis of the BRAF Gene. BRAF 600/601 mutations were detected by polymerase chain reaction (PCR) or reverse-hybridi-

Fig. 1. Clear cell sarcoma of vulva growing as partly exophytical tumorous mass.

zation (StripAssay, ViennaLab, Austria) according to the manufacturer's instructions.

RESULTS

Five incisional biopsies from the vulva of a 67-year-old female measured 2 mm to 7 x 5 x 3 mm. Histologically, the tumor consisted of oval cells arranged in confluent sheets mostly with only mild nuclear pleomorphism and rare mitoses (up to 2/10 HPF) (Fig. 2). The cytoplasm was pale eosinophilic or clear. There were large areas of necrosis and surface ulceration. Immunohistochemically, the tumor cells showed expression of S-100 protein and focal expression of melan A and HMB45. Ki-67 showed positivity in approximately 10 % of tumor cells (Fig. 3). Other markers examined were negative, including cytokeratin AE1/AE3, cytokeratin high molecular weight, estrogen receptors, progesterone receptors, desmin, actin, CD34, CD99, synaptophysin, chromogranin A and p53. Fluorescent in situ hybridization analysis revealed rearrangement of the EWSR1 gene (22q12). BRAF 600/601 mutations were not detected by polymerase chain reaction (PCR) or reverse-hybridization.

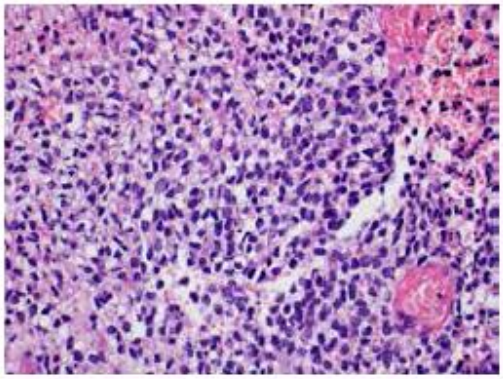


Fig. 2. CCS shows a proliferation of oval cells with pale eosinophilic or clear cytoplasm. (H&E, original magnification 400x).

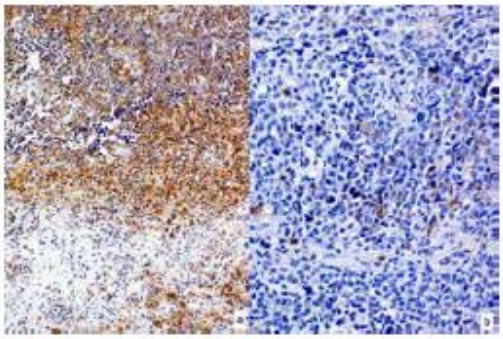


Fig. 3. Immunohistochemical expression of a) S100 protein (original magnification 200x), b) HMB45 (original magnification 400x) in tumor cells.

DISCUSSION

CCS is a rare tumor that usually presents as a slowly growing mass. Recurrences occur in 14 - 39 % of patients, whereas metastases develop in approximately 50 % of patients. The prognosis of metastasizing CCS is poor with a mean survival of 18.4 months (3). In non-metastasizing cases, unfavorable prognostic factors include tumor size > 5 cm, histological detection of necrosis, and local recurrence (4). Besides soft tissues, CCS can also arise in the gastrointestinal tract. However, it has been a matter of dispute whether CCS of the gastrointestinal tract represents an entity distinct from soft tissue lesions. Recently, a series of 16 cases was published under the term malignant gastrointestinal neuroectodermal tumor (5). CCS shows some phenotypic and immunohistochemical features overlapping with malignant melanoma (MM), which is the most important differential diagnostic consideration (6). However, in contrast to MM, CCS usually arises in deep soft tissues without involvement of the skin. Moreover, neither nuclear pleomorphism nor high mitotic activity are typical features of CCS. However, pleomorphic cases can occur. Immunohistochemically, CCS and MM show the expression of S-100 protein, HMB-45, Melan A and microphthalmia transcription factor (MiTF). In contrast to MM, CCS is characterized by a rearrangement of the Ewing's sarcoma (EWS) gene. The most common rearrangement is a balanced translocation (12;22)(q13;q12). This results in the fusion of the EWS gene at 22q12 to the activating transcription factor 1 (ATF1) gene localized at 12q13 (7,8). A translocation (2;22)(q34;q12) resulting in fusion of EWSR1/CREB1 occurs less frequently (9). These changes have not been identified in MM. Moreover, BRAF and NRAS mutations that are commonly found in MM have not been described in CCS (10).

In conclusion, we have described the first reported case of CCS arising in the vulva. The clinician should be aware of the possibility of this unusual primary location of CCS to avoid confusion with other tumors, especially malignant melanoma. In doubtful cases, molecular analysis of the EWSR1 gene will distinguish between these tumors.

ACKNOWLEDGEMENTS

This work was supported by Charles University in Prague (Project PRVOUK-P27/LF1/1, UNCE 204024, Ministry of health, Czech Republic - conceptual development of research organisation 64 165, General University Hospital in Prague, Czech Republic, and by OPPK (Research Laboratory of Tumor Diseases, CZ.2.16/3.1.00/24509).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

1. **Enzinger FM.** Clear-cell sarcoma of tendons and aponeuroses. An analysis of 21 cases. *Cancer* 1965; 18: 1163–1174.
2. **Weiss SW, Goldblum JR, Folpe AL.** Malignant tumors of the peripheral nerves. In: **Weiss SW, Goldblum JR,** eds. Enzinger and Weiss's Soft Tissue Tumors (5th ed). Philadelphia, Pennsylvania: Elsevier Mosby; 2008: 903–944.
3. **Ipach I, Mittag F, Kopp HG, Kunze B, Wolf P, Kluba T.** Clear-cell sarcoma of the soft tissue – a rare diagnosis with a fatal outcome. *Eur J Cancer Care* 2012; 21: 412–420.
4. **Sara AS, Evans HL, Benjamin RS.** Malignant melanoma of soft parts (clear cell sarcoma). A study of 17 cases, with emphasis on prognostic factors. *Cancer* 1990; 65: 367–374.
5. **Stockman DL, Miettinen M, Suster S et al.** Malignant gastrointestinal neuroectodermal tumor: clinicopathologic, immunohistochemical, ultrastructural, and molecular analysis of 16 cases with a reappraisal of clear cell sarcoma-like tumors of the gastrointestinal tract. *Am J Surg Pathol* 2012; 36: 857-868.
6. **Bearman RM, Noe J, Kempson RL.** Clear cell sarcoma with melanin pigment. *Cancer* 1975; 36: 977-984.
7. **Bridge JA, Sreekantaiah C, Neff JR, Sandberg AA.** Cytogenetic findings in clear cell sarcoma of tendons and aponeuroses. Malignant melanoma of soft parts. *Cancer Genet Cytogenet* 1991; 52: 101–106.
8. **Sandberg AA, Bridge JA.** Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: clear cell sarcoma (malignant melanoma of soft parts). *Cancer Genet Cytogenet* 2001; 130: 1–7.
9. **Hisaoka M, Ishida T, Kuo TT et al.** Clear cell sarcoma of soft tissue: a clinicopathologic, immunohistochemical, and molecular analysis of 33 cases. *Am J Surg Pathol* 2008; 32: 452–460.
10. **Yang L, Chen Y, Cui T et al.** Identification of biomarkers to distinguish clear cell sarcoma from malignant melanoma. *Hum Pathol* 2012; 43: 1463–1470.

MONITOR
aneb nemělo by vám uniknout, že ...

PATOLOGIE GIT

... byl popsán další typ smíšeného neuroendokrinního nádoru GIT: primární NEC a dlaždicobuněčný karcinom

Nebývá zvykem monitorovat kazuistické sdělení, ale v dubnovém čísle Histopathology skupina autorů z Bristolu ve Velké Británii přinesla popis zajímavého nádoru céka u 67letého pacienta. Jednalo se o kombinaci malobuněčného neuroendokrinního karcinomu (NEC G3) a dlaždicobuněčného nerohovějícího karcinomu. Přes cílené pátrání nenalezli autoři v nádoru jakákoli ložiska struktur adenokarcinomu (včetně popsaného negativního výsledku histochemického průkazu hlenu v nádoru). V předloženém sdělení popisují autoři detailně imunoprofil obou komponent nádoru: pozitivita CK 5/6 a fokální p63 u dlaždicové komponenty, při negativním barvení neuroendokrinních znaků a Cam5.2 a Ki-67 indexu pod 5 %; „inverzní" imunoprofil u NEC (pozitivita CD56, synaptofyzinu a Cam5.2, negativita CK 5/6, fokální pozitivita p63, Ki-67 index více než 40 %). Obě komponenty nádoru byly negativní v průkazu chromograninu A, CEA a TTF-1; NEC dále vykazoval silnou jadernou pozitivitu CDX-2. Při genetické analýze vykazoval nádor bodovou mutaci *KRAS* genu v exonu 2: c.35G>A p.(Gly12Asp); analýza kodonů 600 a 601 genu *BRAF* nevykazovala přítomnost mutací.

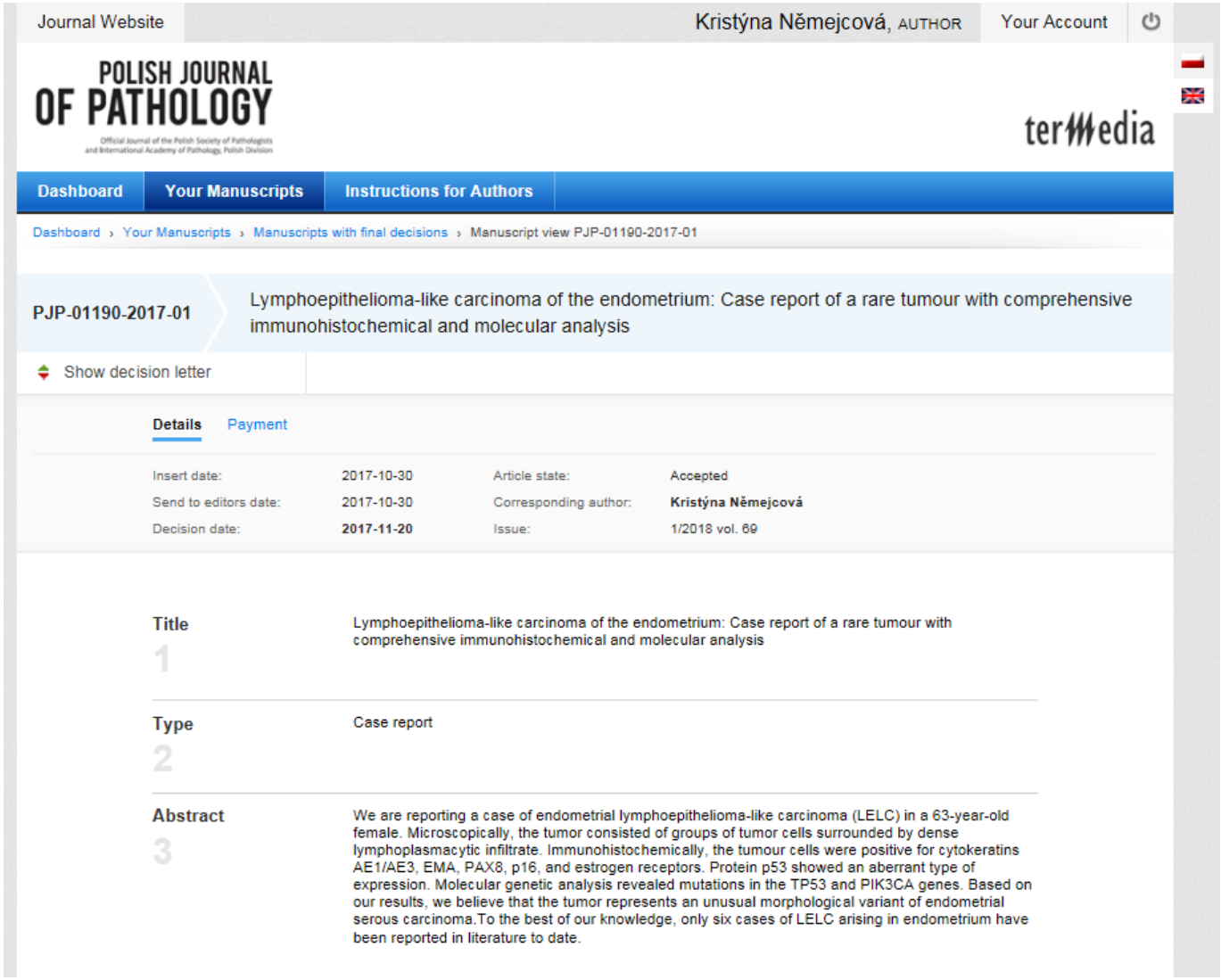
Klinicky se jednalo o objemný (největší rozměr 60 mm), střevo obturující nádor, s metastázami v 17 z 22 vyšetřených lymfatických uzlin; část metastáz vykazovala morfologii čistého NEC, část pak smíšenou morfologii. Pacient zemřel při progresi nádoru 9 měsíců od operace.

Předložený nádor je raritní a ukazuje, že neuroendokrinní nádory nemusí být smíšeny pouze s adenomy/adenokarcinomy (MANEC), ale vzácně i s jinými histologickými vidy v nádorové populaci. Na základě zjištěné mutace genu *KRAS* uvažují autoři o možnosti vzniku takového nádoru z pluripotentní kmenové buňky, analogicky s běžnými adenokarcinomy v této oblasti. Určitou slabinu práce spatřuji v málo přesném odhadu proporcí obou nádorových komponent, kdy autoři uvádějí, že převažující komponentou nádoru byl NEC a záchyt dlaždicového karcinomu byl v nádoru „occasional". Analogicky v MANEC má každá z komponent představovat alespoň 30 % buněčné populace nádoru.

Zdroj:
Hassan U et al. Primary combined neuroendocrine carcinoma (small-cell type) and sqamous cell carcinoma of the colon. ***Histopathology*** 2016; 68: 755-766.

- T. Jirásek -

4.8. Lymphoepithelioma-like carcinoma of the endometrium: Case report of rare tumour with comprehensive immunohistochemical and molecular analysis.



Journal Website | Kristýna Němejcová, AUTHOR | Your Account

POLISH JOURNAL OF PATHOLOGY

Official Journal of the Polish Society of Pathologists and International Academy of Pathology, Polish Division

termedia

Dashboard | Your Manuscripts | Instructions for Authors

Dashboard › Your Manuscripts › Manuscripts with final decisions › Manuscript view PJP-01190-2017-01

PJP-01190-2017-01

Lymphoepithelioma-like carcinoma of the endometrium: Case report of a rare tumour with comprehensive immunohistochemical and molecular analysis

Show decision letter

Details | Payment

Insert date:	2017-10-30	Article state:	Accepted
Send to editors date:	2017-10-30	Corresponding author:	**Kristýna Němejcová**
Decision date:	**2017-11-20**	Issue:	1/2018 vol. 69

Title 1

Lymphoepithelioma-like carcinoma of the endometrium: Case report of a rare tumour with comprehensive immunohistochemical and molecular analysis

Type 2

Case report

Abstract 3

We are reporting a case of endometrial lymphoepithelioma-like carcinoma (LELC) in a 63-year-old female. Microscopically, the tumor consisted of groups of tumor cells surrounded by dense lymphoplasmacytic infiltrate. Immunohistochemically, the tumour cells were positive for cytokeratins AE1/AE3, EMA, PAX8, p16, and estrogen receptors. Protein p53 showed an aberrant type of expression. Molecular genetic analysis revealed mutations in the TP53 and PIK3CA genes. Based on our results, we believe that the tumor represents an unusual morphological variant of endometrial serous carcinoma.To the best of our knowledge, only six cases of LELC arising in endometrium have been reported in literature to date.

PJP-01190-2017-01

POLISH JOURNAL OF PATHOLOGY

Authors:
Kristýna Němejcová, Nikola Hájková, Ivana Tichá, Michaela Bártů, Dagmar Dolínská, Vladimír Kalist, Pavel Dundr

Decision letter:
November 20, 2017
PJP-01190-2017-01
Lymphoepithelioma-like carcinoma of the endometrium: Case report of a rare tumour with comprehensive immunohistochemical and molecular analysis

Dear Dr. Němejcová,

I am pleased to inform you that your manuscript, entitled: Lymphoepithelioma-like carcinoma of the endometrium: Case report of a rare tumour with comprehensive immunohistochemical and molecular analysis, has been finally accepted for publication in our journal.

We would like to inform that your paper will be published after we receive the publishing fee. Please complete the form including data for invoicing, which is available in the invoice bookmark (your Tax ID / your institution VAT number may be required). You will receive the invoice 30 days before publishing your paper.
IMPORTANT ! Please wait with your payment until you receive the invoice.

Please log in to your account:
https://www.editorialsystem.com/pjpen/article/106495/view/#payment
to complete data for invoicing.

Thank you for submitting your work to us.

Kindest regards,

on behalf of Editor-in-chief, prof. Janusz Ryś,
Krzysztof Okoń, FEBP, MD PhD
Polish Journal of Pathology

Review 1:
This is a very nice paper describing a case of Lymphoepithelioma-like carcinoma of the endometrium, which is a very rare entity. The description of the morphology, the immunohistochemical profile and the add of molecular genetic analysis help the reader to better understand this tumor.

Lymphoepithelioma-like carcinoma of the endometrium: Case report of a rare tumour with comprehensive immunohistochemical and molecular analysis

Kristýna Němejcová[1], Nikola Hájková[1], Ivana Tichá[1], Michaela Bártů[1], Dagmar Dolinská[2], Vladimír Kalist[3], Pavel Dundr[1].

[1]Institute of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic.

[2]Department of Pathology, Baťa Regional Hospital in Zlín, Czech Republic.

[3]Department of Gynecology and Obstetrics, Baťa Regional Hospital in Zlín, Czech Republic.

Address for Correspondence

Kristýna Němejcová

Institute of Pathology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Studnickova 2, Prague 2 12800, Czech Republic

e-mail: kristyna.nemejcova@vfn.cz

Phone: +420224968632

Fax: +420224911715

Abstract
We are reporting a case of endometrial lymphoepithelioma-like carcinoma (LELC) in a 63-year-old female. Microscopically, the tumor consisted of groups of tumor cells surrounded by dense lymphoplasmacytic infiltrate. Immunohistochemically, the tumour cells were positive for cytokeratins AE1/AE3, EMA, PAX8, p16, and estrogen receptors. Protein p53 showed an aberrant type of expression. Molecular genetic analysis revealed mutations in the *TP53* and *PIK3CA* genes. Based on our results, we believe that this type of tumor represents an unusual morphological variant of endometrial serous carcinoma.To the best of our knowledge, only six cases of LELC arising in the endometrium have been reported in literature to date.

Key Words: Endometrial serous carcinoma - Lymphoepithelial carcinoma - Lymphoepithelioma-like carcinoma - Endometrium

Introduction

Lymphoepithelial carcinoma (lymphoepithelioma) is an undifferentiated
type of carcinoma with distinctive morphological features and common association with Epstein-Barr virus (EBV), first described in the nasopharynx [61]. This tumour is characterized by the proliferation of undifferentiated neoplastic epithelial cells, with a prominent reactive inflammatory cell infiltrate. Carcinomas with morphologically similar features occurring outside of the nasopharynx are termed "lymphoepithelioma-like carcinoma" (LELC). These tumors have been reported in a number of various organs. The association with EBV infection in LELC is different in various locations and also in different geographic regions. EBV positivity has been described in tumours of lung, stomach, thymus, and salivary gland [63, 71-73], but it hasn´t been demonstrated in cases of LELC occurring in the urinary bladder, renal pelvis, breast, and in female genital tract [74-78]. It has been suggested that EBV positivity is related to a direct organ exposure to the external environment, and EBV negativity is more common in internal organs [62]. However, in LELC of uterine cervix the association with EBV has been observed only in a few Asian patients, but not in western countries [64, 66]. In the female genital tract LELC mostly occurs in the cervix, where it is regarded as a variant of squamous cell carcinoma, but cases involving the vulva, vagina, endometrium, and ovary have also been reported [64-70, 77-79]. To the best of our knowledge, only 6 cases of LELC arising in the endometrium have been reported to date, however, none of these were accompanied by a molecular genetic analysis, with the exception of molecular testing for microsatellite instability (MSI) in one of the cases [65, 67-70]. We report the seventh case of endometrial LELC with comprehensive immunohistochemical and molecular genetic analysis [65, 67, 68, 70].

Clinical History

A 63-year-old woman presented with postmenopausal bleeding. Her gynecologic history includes menarche at the age of 14, with irregular menstrual cycles, and menopause at the age of 51. She had had two vaginal spontaneous deliveries. Physical examination was unremarkable, and laboratory data were within normal limits. A transvaginal ultrasound showed irregular endometrial thickening, and the patient underwent endometrial curettage. Biopsy examination showed high grade carcinoma with LELC features. Based on this diagnosis, the patient underwent radical hysterectomy with bilateral adnexectomy and pelvic and para-aortic lymphadenectomy. Biopsy examination of the resection specimen showed only residual tumour structures limited to the endometrium. The patient was staged as FIGO IA and received adjuvant vaginal brachytherapy. Six months after the surgery she is free of any signs of disease.

MATERIALS AND METHODS

Immunohistochemistry

Sections from formalin-fixed paraffin-embedded tissue blocks were stained with hematoxylin-eosin. Selected sections were analyzed immunohistochemically, using the avidin-biotin complex method with antibodies directed against the antigens listed in Table I.

In situ hybridization (ISH)

ISH staining for EBV encoded RNA (EBER) transcript was performed on deparaffinized tissue sections. The Epstein–Barr Virus (EBER) PNA Probe/Flourescein kit was used (Dako, code Y5200) and visualized with the Dako PNA ISH Detection Kit (Dako, code K5201). For negative controls, the EBER probe was omitted.

Molecular analysis

DNA was isolated from formaline-fixed paraffin-embedded (FFPE) tissue by cobas® DNA Sample Preparation kit (Roche; Germany). Macrodissection was performed in order to enrich the tumor content. Sequence capture next-generation sequencing (NGS) was performed with 300 ng of gDNA (40% of neoplastic cells). A library was constructed using KAPA HyperPlus kit, and the target area (219 kbp, 73 genes) was enriched using custom-designed hybridization probes (Nimblegen, Roche) and pair-end sequenced using Miseq instrument (Illumina).

Reads were aligned to the human genome (GRCh37/hg19), with an average coverage of 550.7 (median 660.3; range 2-1174). Nonsynonymous variants in exons and adjacent intronic regions with minimal coverage 100x and frequency of >5% were evaluated, and manually controlled using Integrative Genomic Viewer (Broad Institute). Copy number variations were not evaluated due to the low quality of FFPE sample. The list of investigated genes and characteristics of detected variants is provided in Table II.

For the evaluation of functional or clinical importance of detected missense variants a widely used *in silico* prediction tools were utilized (part of NextGENe software: dbNSFP v 2.9 – set of algorithms for predicting the impact of the variant on protein function, according to the sequence conservation and population frequency; ClinVar; dbscSNV for evaluating the impact of splice variants). Further, IARC TP53 database (http://p53.iarc.fr) and Catalogue of somatic mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic/, accessed on October 20, 2017) were employed [80].

MSI was analyzed using fragment analysis on ABI 3500, examining five quasimonomorphic mononucleotide microsatellite markers (MSI) BAT-26, BAT-25, NR-21, NR-22, NR-24.

Ethics Statement

In compliance with the Helsinki Declaration, the project has been approved by Ethics Committee of General University Hospital in Prague.

Results

Material from endometrial curettage consisted of small fragments of poorly differentiated high-grade carcinoma. The tumour was composed of irregular sheets, poorly defined nests or individual tumour cells, surrounded by dense lymphoplasmacytic infiltrate (Fig. 1). The tumour cells had indistinct margins, with round to oval vesicular nuclei with prominent nucleoli, and eosinophilic to amphophilic cytoplasm (Fig. 2). The mitotic index was up to 7 mitoses per 10 high-power fields.

Immunohistochemically, the tumour cells were strongly and diffusely positive for cytokeratins AE1/AE3, EMA, PAX8, p16, and some of them were positive for vimentin and S100 protein (Fig. 3). Protein p53 showed aberrant type of expression (positivity in more than 75% of tumor cells), which is in concordance with the detected pathogenic mutation in the *TP53* gene (Fig. 4, Table II). Estrogen receptors were positive in 75 % of tumour cells. The proliferative (Ki-67) index was about 80%. Nuclear staining of the mismatch repair (MMR) proteins (MLH-1, MSH-2, MSH-6, and PMS-2) was positive, and the results of fragment analysis confirmed microsatellite stable tumor. Progesterone receptors, chromogranin A, synaptophysin, HNF1β, and napsin A were negative. EBER in situ hybridization was negative. The lymphoid stromal

infiltrate consisted of a mixture of CD20 positive B-lymphocytes and CD3 positive T-lymphocytes.
Mutation analysis was performed in 59/73 genes with sufficient coverage (14 genes in this panel were poorly covered due to the insufficient quality of DNA). Non-synonymous variants were detected in the *TP53* (c.584T>C (p.Ile195Thr) located in the DNA binding domain, *PIK3CA* genes (c.323G>A (p.Arg108His) in exon 2, and c.1048G>A (p.Asp350Asn) in exon 5) (Table II). All of these variants were previously described in COSMIC database (http://cancer.sanger.ac.uk/cosmic/) and were considered pathogenic. No pathogenic mutations were found in genes such as *PTEN*, *ARID1A*, *KRAS*, *POLE*, *HNF1B* or *BRCA2*, which are frequently mutated in endometrial carcinomas.
Additionally, heterozygous polymorphism was detected in *BRCA2*, NM_00059.3: c.1114A>C (p.Asn372His, rs144848;38.2% MAF), although this finding is considered to be tolerated or neutral by *in silico* predictive tools.
In a subsequent hysterectomy the uterus measured 46×27×40 mm, with endometrial thickness of 3 mm. There were some regressive changes with only residual tumour structures in the fundus, limited to the endometrium. The cervix and both adnexa were unremarkable without any signs of tumour involvement. All 35 sampled and examined lymph nodes were without metastases.

Discussion

Endometrial carcinomas can be classified into four main histological types encompassing endometrioid, serous, mucinous and clear cell carcinoma. These tumors can usually be distinguished based on their morphology, however in doubtful cases ancillary methods are needed. In most cases, immunohistochemistry can be helpful but some equivocal cases with overlapping morphological and immunohistochemical features still continue to be a diagnostic challenge. In these cases, molecular genetic analysis can be used, which allow us to classify most tumors into the correct category. Especially problematic can be the distinction between endometrioid carcinoma (EC) and endometrial serous carcinoma. Immunohistochemically, analysis of p53, p16, MMR proteins and PTEN expression can be very helpful in this case. EC is characterized by the loss of PTEN expression, non-diffuse p16 staining, wild type p53 in most cases and MSI. On the contrary, endometrial serous carcinomas are in most cases p16 diffusely positive, show abnormal expression of p53, retained expression of PTEN and are MSS.
Regarding their genotype, endometrioid carcinomas are characterized by: i) concurrent *PTEN* and *ARID1A* mutation; or ii) *PTEN* or *ARID1A* mutations in the absence of concurrent *TP53* (and *PPP2R1A*) mutations. Serous genotype is characterized by *TP53* mutation in the absence of *PTEN* or *ARID1A* mutations. *PIK3CA* mutation can occur in a subset of both endometrial and serous carcinomas and is not helpful in discriminating between these tumors [81].
Recent approaches classify endometrial carcinomas based on their molecular characteristics into 4 groups: *POLE* ultramutated (POLE), microsatellite instability-high, copy number-low (CN-L), and copy number-high (CN-H) [82, 83].The TCGA described the differences between high-grade endometrioid carcinoma and serous carcinoma based on frequent mutations in *PTEN*, *ARID1A*, mismatch repair genes, and *KRAS*. While these are common in endometrioid carcinoma, serous carcinoma however carries frequent mutations in *TP53* and *PPP2R1A*, and mutations in *PTEN*, *ARID1A*, mismatch repair genes, and *KRAS* are not frequent in this carcinoma. Generally, all serous carcinomas belong to the category CN-H, which is characterized by *TP53* mutation. However, high grade endometrioid carcinomas show substantial genetic heterogeneity and a certain proportion of them fall under in the same category as serous carcinoma (CN-H tumours). Nevertheless, these cases can be distinguished by analysis of the above mentioned genes, *ARID1A* and *PTEN*. Further studies are needed to show whether CN-H grade 3 endometrioid carcinomas are clinically different from endometrial serous carcinoma.

In our case, despite its unusual morphology, the tumor showed the typical immunophenotype (diffuse p16 staining, aberrant expression of p53) and genotype (mutation of *TP53*, absence of mutations of *ARID1A* or *PTEN*) of endometrial serous carcinoma. Expression of PAX8 and estrogen receptors can be seen in both tumor types and as such is not particularly helpful in the differential diagnosis, but it is suggestive of Müllerian origin of the tumor. Based on these features, we believe that our case represents an unusual morphological variant of endometrial serous carcinoma with LELC features due to the heavy inflammatory infiltration of tumor stroma.

Endometrial LELCs are rare tumours, only 7 cases including ours have been reported to date. All of the reviewed cases were EBV negative with only one exception, and even then only a few cells showed positivity for EBV LMP1 [69]. MSI testing was performed only in one previous case (evaluated by PCR using capillary electrophoresis), and identically to our case the tumor was microsatellite stable [68]. So far, MSI was found only in a subset of gastric LELCs [84]. Regarding immunohistochemical results, all the reported endometrial LELCs showed positivity for epithelial markers in tumour cells, and CD3 and CD20 positivity in stromal cells, but other results are equivocal [65, 67-70]. Positivity for estrogen and progesterone receptors was found in 1of 3 cases [65, 67, 68]. The aberrant type of p53 expression was found in 2 of the 3 investigated cases, in accordance with our results [67, 68]. One case of the 3 reported was negative [65], but the authors did not specify whether the 'negativity' meant a complete loss of p53 expression, as this finding may also in some cases be regarded as aberrant expression. The expression of p16 was investigated only in one previous case, with a positive result, which is also in accordance with our reported case [68]. The aberrant expression of p53 and overexpression of p16 are likely signs of manifestation of cell-cycle dysregulation, which is quite a common finding in high grade carcinomas [68].

Differential diagnosis may include lymphoproliferative lesions/tumours, which can however be easily excluded by immunohistochemical recognition of their epithelial component. Moreover, large cell neuroendocrine carcinoma with LELC features was rarely described, but this entity could be ruled out based on the absence of neuroendocrine differentiation markers [85]. The most significant differential diagnostic consideration is an undifferentiated carcinoma (UC). This differential diagnosis may have certain clinical implications, as some authors suggested that the prognosis of LELC is unclear or possibly favourable, whereas the prognosis of UC tends to be poor [68]. Regarding this particular differential diagnosis, the evaluation of immunohistochemical expression of epithelial markers can be helpful. LELC typically show almost diffuse positivity, in contrast to the focal or patchy expression found in UC [86].

In conclusion, endometrial LELCs are rare carcinomas that are not associated with EBV. The histogenesis of endometrial LELC is unknown, however, the immunohistochemical and molecular genetic profile in our case suggests that this tumor could represent an unusual morphological variant of endometrial serous carcinoma. Nevertheless, a comprehensive analysis of more cases is needed to fully understand the histogenesis, biologic behaviour, and prognosis of these tumors. This could, however, be problematic due to their rarity.

Acknowledgments

Sources of support and grant numbers:

This work was supported by Ministry of Health, Czech Republic (Conceptual development of research organization 64165, General University Hospital in Prague and Project AZV 17-28404A), by Charles University (Project Progres Q28/LF1 and SVV 260367), and by OPPK (Research Laboratory of Tumor Diseases, CZ.2.16/3.1.00/24509).

The authors declare no conflicts of interest.

The authors extend their thanks to Zachary H.K. Kendall, B.A. for English language corrections.

References:

1. Ewing J. Lymphoepithelioma. Am J Pathol 1929; 5: 99-108.
2. Ramos M, Pereira MA, Dias AR, et al. Lymphoepithelioma-like gastric carcinoma: clinicopathological characteristics and infection status. J Surg Res 2017; 210: 159-168.
3. Jafarian AH, Omidi-Ashrafi A, Mohamadian-Roshan N, et al. Association of Epstein Barr virus deoxyribonucleic acid with lung carcinoma. Indian J PatholMicrobiol 2013; 56: 359-364.
4. Chen PC, Pan CC, Yang AH, et al. Detection of Epstein-Barr virus genome within thymic epithelial tumours in Taiwanese patients by nested PCR, PCR in situ hybridization, and RNA in situ hybridization. J Pathol 2002; 197: 684-688.
5. Zhao W, Deng N, Gao X, et al. Primary lymphoepithelioma-like carcinoma of salivary glands: a clinicopathological study of 21 cases. Int J ClinExpPathol 2014; 7: 7951-7956.
6. Yang AW, Pooli A, Lele SM, et al. Lymphoepithelioma-like, a variant of urothelial carcinoma of the urinary bladder: a case report and systematic review for optimal treatment modality for disease-free survival. BMC Urol 2017; 17: 34.
7. Ahn H, Sim J, Kim H, et al. Lymphoepithelioma-like Carcinoma of the Renal Pelvis: A Case Report and Review of the Literature. Korean J Pathol 2014; 48: 458-461.
8. Abouelfad DM, Yassen NN, Amin HAA, et al. Lymphoepithelioma-Like Carcinoma of the Breast Mimicking Granulomatous Mastitis- Case Report and Review of the Literature. Asian Pac J Cancer Prev 2017; 18: 1737-1741.
9. McCluggage WG. Lymphoepithelioma-like carcinoma of the vagina. J ClinPathol 2001; 54: 964-965.
10. Bosmuller H, Haitchi-Petnehazy S, Gruber C, et al. Lymphoepithelioma-like carcinoma of the vulva, an underrecognized entity? Case report with a single inguinal micrometastasis detected by sentinel node technique. DiagnPathol 2011; 6: 4.
11. Haga K, Aoyagi T, Kashiwagi A, et al. Lymphoepithelioma-like carcinoma of the renal pelvis. Int J Urol 2007; 14: 851-853.
12. Tseng CJ, Pao CC, Tseng LH, et al. Lymphoepithelioma-like carcinoma of the uterine cervix: association with Epstein-Barr virus and human papillomavirus. Cancer 1997; 80: 91-97.
13. Chao A, Tsai CN, Hsueh S, et al. Does Epstein-Barr virus play a role in lymphoepithelioma-like carcinoma of the uterine cervix? Int J GynecolPathol 2009; 28: 279-285.
14. Ambrosio MR, Rocca BJ, Mourmouras V, et al. Lymphoepithelioma-like carcinoma of the endometrium. Pathologica 2010; 102: 57-61.
15. Jones H, Gold MA, Giannico G, et al. Lymphoepithelioma-like carcinoma of the endometrium: immunophenotypic characterization of a rare tumor with microsatellite instability testing. Int J GynecolPathol 2014; 33: 64-73.
16. Makannavar JH, KishanPrasadHL,Shetty JK. Lymphoepithelioma-like Carcinoma of Endometrium; A Rare Case Report. Indian J SurgOncol 2015; 6: 130-134.
17. Rahimi S, Lena A,Vittori G. Endometrial lymphoepitheliomalike carcinoma: absence of Epstein-Barr virus genomes. Int J Gynecol Cancer 2007; 17: 532-535.
18. Vargas MP,Merino MJ. Lymphoepitheliomalike carcinoma: an unusual variant of endometrial cancer. A report of two cases. Int J GynecolPathol 1998; 17: 272-276.
19. Lee S, Park SY, Hong EK, et al. Lymphoepithelioma-like carcinoma of the ovary: a case report and review of the literature. Arch Pathol Lab Med 2007; 131: 1715-1718.
20. Forbes SA, Bindal N, Bamford S, et al. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res 2011; 39: 945-950.
21. Hoang LN, McConechy MK, Kobel M, et al. Histotype-genotype correlation in 36 high-grade endometrial carcinomas. Am J SurgPathol 2013; 37: 1421-1432.
22. Hussein YR, Broaddus R, Weigelt B, et al. The Genomic Heterogeneity of FIGO Grade 3 Endometrioid Carcinoma Impacts Diagnostic Accuracy and Reproducibility. Int J GynecolPathol 2016; 35: 16-24.
23. Hussein YR, Weigelt B, Levine DA, et al. Clinicopathological analysis of endometrial carcinomas harboring somatic POLE exonuclease domain mutations. Mod Pathol 2015; 28: 505-514.
24. Herath CH,Chetty R. Epstein-Barr virus-associated lymphoepithelioma-like gastric carcinoma. Arch Pathol Lab Med 2008; 132: 706-709.
25. Dundr P, Pešl M, Povýšil C, et al. Large cell neuroendocrine carcinoma of the urinary bladder with lymphoepithelioma-like features. Pathol Res Pract 2003; 199: 559-563.
26. Tafe LJ, Garg K, Chew I, et al. Endometrial and ovarian carcinomas with undifferentiated components: clinically aggressive and frequently underrecognized neoplasms. Mod Pathol 2010; 23: 781-789.

Fig. 1

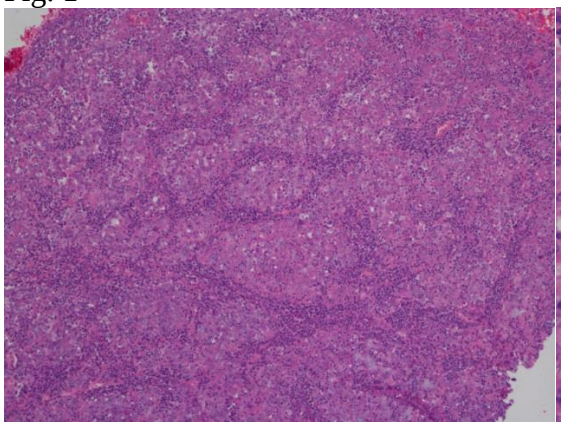


Fig. 2

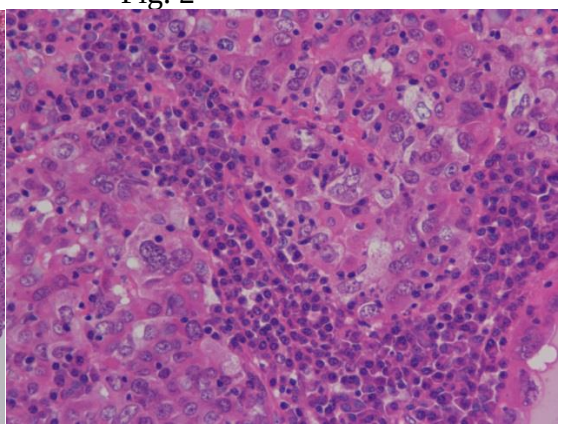


Fig. 3

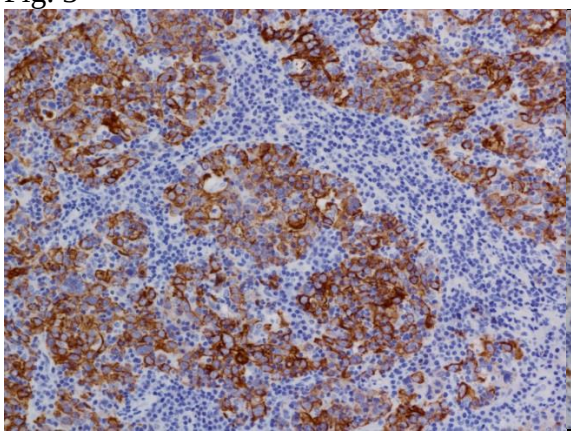


Fig. 4

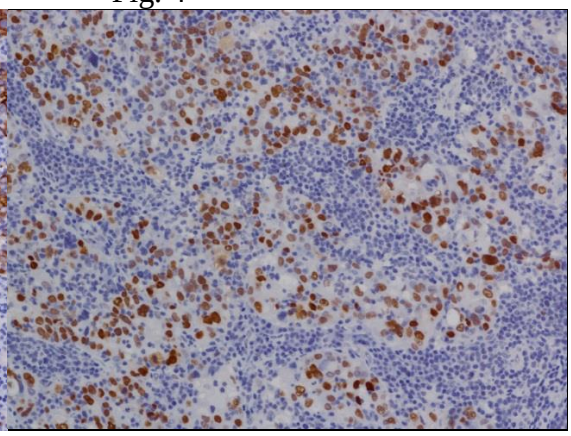


Figure legends

Fig. 1. Proliferation of epithelial tumour cells surrounded by an intense lymphoplasmacytic infiltrate (haematoxylin and eosin, magnification 100×).

Fig. 2. Poorly defined aggregates of syncytial tumor cells, round nuclei with prominent nucleoli (haematoxylin and eosin, magnification 400×).

Fig. 3. Diffuse positivity for CK AE1/AE3 in epithelial component (magnification 200×).

Fig. 4. Aberant type expression of p53 in epithelial

Table I. Antibodies used for the immunohistochemical analysis

Antibody	Clone	Source	Dilution	Result
CK AE1/AE3	AE1/AE3	Dako, Glostrup, Denmark	1:50	Diffusely positive
EMA	E29	Dako, Glostrup, Denmark	1:100	Diffusely positive
ER	SP1	Zytomed Systems, Berlin, Germany	1:50	Positive in 75 %
PR	16	Novocastra, Newcastle, UK	1:200	Negative
HNF1β	Polyclonal	Sigma-Aldrich, Prestige antibodies, St. Louis, United States	1:600	Negative
Ki-67	MIB-1	Dako, Glostrup, Denmark	1:50	Positive in 80 %
napsin A	IP64	Novocastra, Newcastle, UK	1:200	Negative
p53	BP 53-12	Zytomed Systems, Berlin, Germany	1: 200	Diffusely positive
synaptophysin	DAK-SYNAP	Dako, Glostrup, Denmark	1:50	Negative
chromogranin A	LK2H10	Zytomed Systems, Berlin, Germany	1:400	Negative
S-100 protein	Polyclonal	Dako, Glostrup, Denmark	1:1600	Focally positive
vimentin	V9	Dako, Glostrup, Denmark	1:50	Focally positive
PAX 8	Polyclonal	Cell Marque, Rocklin, California	1:50	Diffusely positive
P16	E6H4™	Roche mtm Laboratories AG, Manheim, Germany	RTU	Diffusely positive
MLH-1	G168-15	Spring Bioscience, Pleasanton, CA	1:200	Positive
MSH-2	FE 11	Zytomed Systems, Berlin, Germany	1:50	Positive
MSH-6	44	Zytomed Systems, Berlin, Germany	1:50	Positive
PMS-2	EPR3947	Zytomed Systems, Berlin, Germany	RTU	Positive
CD3	SP7	Spring Bioscience, Pleasanton, CA	1:200	Positive in T cells
CD20	L26	Dako, Glostrup, Denmark	1:100	Positive in B cells

CK - cytokeratin, ER- estrogen receptors, PR- progesterone receptors, RTU – ready-to-use

Table II. Somatic variants detected in the lymphoepitheloid-like carcinoma of uterus

Gene*	exon	Ref. seq. mutation	Predicted effect	Mutant allele frequency %	Predicted impact - *in silico / databases and literature*
TP53	6	NM_001126112.2 c.584T>C rs760043106	p.Ile195Thr	18	Pathogenic / pathogenic
PIK3CA	2	NM_006218.2 c.323G>A	p.Arg108His	24	Pathogenic / pathogenic
	5	c.1048G>A	p.Asp350Asn	18	Pathogenic / pathogenic

* genes evaluated by NGS:*AKT3, ARID1A, ARID2, ATM, BAP1, BARD1, BRAF (ex11, 15) , BRCA1, BRCA2, BRIP1, CCND2, CCND3, CDH1, CDK4, , CYP19A1, ERBB2, ERCC3, ESR1, ESR2, F11R, GNAQ, HNF1B, IDH1, JAM2, JAM3, KDR, KIT, KRAS, MAP2K1, MAPK1, MDM2, MET, MITF, MLH1, MLH3, MSH2, MSH6, MYC, NBN, NRAS, PALB2, PARD3, PDGFRA, PIK3CA, POLE, POT1, PPM1D, PPP6C, PTEN, RAD51C, RAD51D, RB1, SF3B1, SNAI2, TJP1, TP53, TWIST1, ZEB1, ZEB2; AKT1, BIRC5, FOXL2, GNA11, HRAS, MAP2K2, SNAI3, TWIST2I;*partially evaluated because of low coverage in part of the genes: *CDKN2A, MAPK3, SMARCA4, SMARCB1, SNAI1;* impossible to evaluate because of low coverage: *AKT1, BIRC5, FOXL2, GNA11, HRAS, MAP2K2, SNAI3, TWIST2*

5. LITERATURA

1. Dundr, P., et al., *Anastomosing Hemangioma of the Ovary: A Clinicopathological Study of Six Cases with Stromal Luteinization.* Pathol Oncol Res, 2017. **23**(4): p. 717-722.
2. Nemejcova, K., et al., *Expression of Glut-1 in Normal Endometrium and Endometrial Lesions: Analysis of 336 Cases.* Int J Surg Pathol, 2017. **25**(5): p. 389-396.
3. Nemejcova, K., D. Cibula, and P. Dundr, *Expression of HNF-1beta in cervical carcinomas: an immunohistochemical study of 155 cases.* Diagn Pathol, 2015. **10**: p. 8.
4. Nemejcova, K., et al., *Atypical Polypoid Adenomyoma of the Uterus: An Immunohistochemical and Molecular Study of 21 Cases.* Am J Surg Pathol, 2015. **39**(8): p. 1148-55.
5. Nemejcova, K., et al., *Expression, Epigenetic and Genetic Changes of HNF1B in Endometrial Lesions.* Pathol Oncol Res, 2016. **22**(3): p. 523-30.
6. Němejcová Kristýna, H.N., Tichá Ivana, Bártů Michaela, Dolinská Dagmar, Kalist Vladimír, Dundr Pavel *Lymphoepithelioma-like carcinoma of the endometrium: Case report of rare tumour with comprehensive immunohistochemical and molecular analysis.* Polish Journal of Pathology, 2018.
7. Cibula, D., et al., *Risk of micrometastases in non-sentinel pelvic lymph nodes in cervical cancer.* Gynecol Oncol, 2016. **143**(1): p. 83-86.
8. Nemejcova, K., P. Dundr, and I. Krajsova, *Clear cell sarcoma of vulva. A case report.* Cesk Patol, 2016. **52**(4): p. 215-217.
9. Dumoff, K.L., et al., *Low D2-40 immunoreactivity correlates with lymphatic invasion and nodal metastasis in early-stage squamous cell carcinoma of the uterine cervix.* Mod Pathol, 2005. **18**(1): p. 97-104.
10. Chuang, W.Y., et al., *Tumor cell expression of podoplanin correlates with nodal metastasis in esophageal squamous cell carcinoma.* Histol Histopathol, 2009. **24**(8): p. 1021-7.
11. Yuan, P., et al., *Overexpression of podoplanin in oral cancer and its association with poor clinical outcome.* Cancer, 2006. **107**(3): p. 563-9.
12. Fadare, O. and S.X. Liang, *Diagnostic utility of hepatocyte nuclear factor 1-beta immunoreactivity in endometrial carcinomas: lack of specificity for endometrial clear cell carcinoma.* Appl Immunohistochem Mol Morphol, 2012. **20**(6): p. 580-7.
13. Kalloger, S.E., et al., *Calculator for ovarian carcinoma subtype prediction.* Mod Pathol, 2011. **24**(4): p. 512-21.
14. Tomassetti, A., et al., *Variant HNF1 modulates epithelial plasticity of normal and transformed ovary cells.* Neoplasia, 2008. **10**(12): p. 1481-92, 3p following 1492.
15. Kenny, S.L., et al., *Mesonephric adenocarcinomas of the uterine cervix and corpus: HPV-negative neoplasms that are commonly PAX8, CA125, and HMGA2 positive and that may be immunoreactive with TTF1 and hepatocyte nuclear factor 1-beta.* Am J Surg Pathol, 2012. **36**(6): p. 799-807.
16. Park, K.J., et al., *Unusual endocervical adenocarcinomas: an immunohistochemical analysis with molecular detection of human papillomavirus.* Am J Surg Pathol, 2011. **35**(5): p. 633-46.
17. Barbacci, E., et al., *HNF1beta/TCF2 mutations impair transactivation potential through altered co-regulator recruitment.* Hum Mol Genet, 2004. **13**(24): p. 3139-49.
18. Hoang, L.N., et al., *Immunohistochemical characterization of prototypical endometrial clear cell carcinoma--diagnostic utility of HNF-1beta and oestrogen receptor.* Histopathology, 2014. **64**(4): p. 585-96.
19. Buchner, A., et al., *Downregulation of HNF-1B in renal cell carcinoma is associated with tumor progression and poor prognosis.* Urology, 2010. **76**(2): p. 507 e6-11.

20. Kim, L., et al., *Clear cell carcinoma of the pancreas: histopathologic features and a unique biomarker: hepatocyte nuclear factor-1beta.* Mod Pathol, 2008. **21**(9): p. 1075-83.
21. Suzuki, E., et al., *Transcriptional upregulation of HNF-1beta by NF-kappaB in ovarian clear cell carcinoma modulates susceptibility to apoptosis through alteration in bcl-2 expression.* Lab Invest, 2015. **95**(8): p. 962-72.
22. Tsuchiya, A., et al., *Expression profiling in ovarian clear cell carcinoma: identification of hepatocyte nuclear factor-1 beta as a molecular marker and a possible molecular target for therapy of ovarian clear cell carcinoma.* Am J Pathol, 2003. **163**(6): p. 2503-12.
23. Kato, N., S. Sasou, and T. Motoyama, *Expression of hepatocyte nuclear factor-1beta (HNF-1beta) in clear cell tumors and endometriosis of the ovary.* Mod Pathol, 2006. **19**(1): p. 83-9.
24. Yamamoto, S., et al., *Immunohistochemical detection of hepatocyte nuclear factor 1beta in ovarian and endometrial clear-cell adenocarcinomas and nonneoplastic endometrium.* Hum Pathol, 2007. **38**(7): p. 1074-80.
25. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
26. Annibaldi, A. and C. Widmann, *Glucose metabolism in cancer cells.* Curr Opin Clin Nutr Metab Care, 2010. **13**(4): p. 466-70.
27. Macheda, M.L., S. Rogers, and J.D. Best, *Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer.* J Cell Physiol, 2005. **202**(3): p. 654-62.
28. Iwasaki, K., et al., *Role of hypoxia-inducible factor-1alpha, carbonic anhydrase-IX, glucose transporter-1 and vascular endothelial growth factor associated with lymph node metastasis and recurrence in patients with locally advanced cervical cancer.* Oncol Lett, 2015. **10**(4): p. 1970-1978.
29. Ma, X., et al., *Clinical significance of COX-2, GLUT-1 and VEGF expressions in endometrial cancer tissues.* Pak J Med Sci, 2015. **31**(2): p. 280-4.
30. Younes, M., et al., *Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers.* Cancer Res, 1996. **56**(5): p. 1164-7.
31. Goldman, N.A., et al., *GLUT1 and GLUT8 in endometrium and endometrial adenocarcinoma.* Mod Pathol, 2006. **19**(11): p. 1429-36.
32. Krzeslak, A., et al., *Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers.* Pathol Oncol Res, 2012. **18**(3): p. 721-8.
33. Kuiper, C., et al., *Low ascorbate levels are associated with increased hypoxia-inducible factor-1 activity and an aggressive tumor phenotype in endometrial cancer.* Cancer Res, 2010. **70**(14): p. 5749-58.
34. Kendall, B.S., et al., *Reproducibility of the diagnosis of endometrial hyperplasia, atypical hyperplasia, and well-differentiated carcinoma.* Am J Surg Pathol, 1998. **22**(8): p. 1012-9.
35. Wang, B.Y., et al., *Immunohistochemical staining of GLUT1 in benign, hyperplastic, and malignant endometrial epithelia.* Cancer, 2000. **88**(12): p. 2774-81.
36. Xiong, Y., Y.Y. Xiong, and Y.F. Zhou, *Expression and significance of beta-catenin, Glut-1 and PTEN in proliferative endometrium, endometrial intraepithelial neoplasia and endometrioid adenocarcinoma.* Eur J Gynaecol Oncol, 2010. **31**(2): p. 160-4.
37. Altgassen, C., et al., *Multicenter validation study of the sentinel lymph node concept in cervical cancer: AGO Study Group.* J Clin Oncol, 2008. **26**(18): p. 2943-51.
38. Cibula, D., et al., *Bilateral ultrastaging of sentinel lymph node in cervical cancer: Lowering the false-negative rate and improving the detection of micrometastasis.* Gynecol Oncol, 2012. **127**(3): p. 462-6.

39. Lecuru, F., et al., *Bilateral negative sentinel nodes accurately predict absence of lymph node metastasis in early cervical cancer: results of the SENTICOL study.* J Clin Oncol, 2011. **29**(13): p. 1686-91.
40. Montgomery, E. and J.I. Epstein, *Anastomosing hemangioma of the genitourinary tract: a lesion mimicking angiosarcoma.* Am J Surg Pathol, 2009. **33**(9): p. 1364-9.
41. Anand, M.S., et al., *Ovarian hemangioma with stromal luteinization and HCG-producing mononucleate and multinucleate cells of uncertain histogenesis: a rare co-existence with therapeutic dilemma.* Indian J Pathol Microbiol, 2012. **55**(4): p. 509-12.
42. Woodruff, J.D., T.J. Williams, and B. Goldberg, *Hormone Activity of the Common Ovarian Neoplasm.* Am J Obstet Gynecol, 1963. **87**: p. 679-98.
43. Savargaonkar, P.R., et al., *Ovarian haemangiomas and stromal luteinization.* Histopathology, 1994. **25**(2): p. 185-8.
44. Longacre, T.A., et al., *Atypical polypoid adenomyofibromas (atypical polypoid adenomyomas) of the uterus. A clinicopathologic study of 55 cases.* Am J Surg Pathol, 1996. **20**(1): p. 1-20.
45. Matsumoto, T., et al., *Clinical management of atypical polypoid adenomyoma of the uterus. A clinicopathological review of 29 cases.* Gynecol Oncol, 2013. **129**(1): p. 54-7.
46. Mazur, M.T., *Atypical polypoid adenomyomas of the endometrium.* Am J Surg Pathol, 1981. **5**(5): p. 473-82.
47. Heatley, M.K., *Atypical polypoid adenomyoma: a systematic review of the English literature.* Histopathology, 2006. **48**(5): p. 609-10.
48. Zhang, H.K. and W.D. Chen, *Atypical polypoid adenomyomas progressed to endometrial endometrioid adenocarcinomas.* Arch Gynecol Obstet, 2012. **286**(3): p. 707-10.
49. Ota, S., et al., *Molecular pathology of atypical polypoid adenomyoma of the uterus.* Human Pathology, 2003. **34**(8): p. 784-788.
50. Takahashi, H., et al., *Frequent beta-catenin gene mutations in atypical polypoid adenomyoma of the uterus.* Human Pathology, 2014. **45**(1): p. 33-40.
51. Cereghini, S., *Liver-enriched transcription factors and hepatocyte differentiation.* FASEB J, 1996. **10**(2): p. 267-82.
52. Mandato, V.D., et al., *HNF1B polymorphism influences the prognosis of endometrial cancer patients: a cohort study.* BMC Cancer, 2015. **15**: p. 229.
53. Painter, J.N., et al., *Fine-mapping of the HNF1B multicancer locus identifies candidate variants that mediate endometrial cancer risk.* Hum Mol Genet, 2015. **24**(5): p. 1478-92.
54. Shen, H., et al., *Epigenetic analysis leads to identification of HNF1B as a subtype-specific susceptibility gene for ovarian cancer.* Nat Commun, 2013. **4**: p. 1628.
55. Noto, H., et al., *Substantially increased risk of cancer in patients with diabetes mellitus: a systematic review and meta-analysis of epidemiologic evidence in Japan.* J Diabetes Complications, 2010. **24**(5): p. 345-53.
56. Terasawa, K., et al., *Epigenetic inactivation of TCF2 in ovarian cancer and various cancer cell lines.* Br J Cancer, 2006. **94**(6): p. 914-21.
57. Enzinger, F.M., *Clear-Cell Sarcoma of Tendons and Aponeuroses. An Analysis of 21 Cases.* Cancer, 1965. **18**: p. 1163-74.
58. Bridge, J.A., et al., *Cytogenetic findings in clear cell sarcoma of tendons and aponeuroses. Malignant melanoma of soft parts.* Cancer Genet Cytogenet, 1991. **52**(1): p. 101-6.
59. Hisaoka, M., et al., *Clear cell sarcoma of soft tissue: a clinicopathologic, immunohistochemical, and molecular analysis of 33 cases.* Am J Surg Pathol, 2008. **32**(3): p. 452-60.

60. Sandberg, A.A. and J.A. Bridge, *Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: clear cell sarcoma (malignant melanoma of soft parts).* Cancer Genet Cytogenet, 2001. **130**(1): p. 1-7.
61. Ewing, J., *Lymphoepithelioma.* Am J Pathol, 1929. **5**(2): p. 99-108 7.
62. Haga, K., et al., *Lymphoepithelioma-like carcinoma of the renal pelvis.* Int J Urol, 2007. **14**(9): p. 851-3.
63. Ramos, M., et al., *Lymphoepithelioma-like gastric carcinoma: clinicopathological characteristics and infection status.* J Surg Res, 2017. **210**: p. 159-168.
64. Chao, A., et al., *Does Epstein-Barr virus play a role in lymphoepithelioma-like carcinoma of the uterine cervix?* Int J Gynecol Pathol, 2009. **28**(3): p. 279-85.
65. Rahimi, S., A. Lena, and G. Vittori, *Endometrial lymphoepitheliomalike carcinoma: absence of Epstein-Barr virus genomes.* Int J Gynecol Cancer, 2007. **17**(2): p. 532-5.
66. Tseng, C.J., et al., *Lymphoepithelioma-like carcinoma of the uterine cervix: association with Epstein-Barr virus and human papillomavirus.* Cancer, 1997. **80**(1): p. 91-7.
67. Ambrosio, M.R., et al., *Lymphoepithelioma-like carcinoma of the endometrium.* Pathologica, 2010. **102**(2): p. 57-61.
68. Jones, H., et al., *Lymphoepithelioma-like carcinoma of the endometrium: immunophenotypic characterization of a rare tumor with microsatellite instability testing.* Int J Gynecol Pathol, 2014. **33**(1): p. 64-73.
69. Makannavar, J.H., H.L. KishanPrasad, and J.K. Shetty, *Lymphoepithelioma-like Carcinoma of Endometrium; A Rare Case Report.* Indian J Surg Oncol, 2015. **6**(2): p. 130-4.
70. Vargas, M.P. and M.J. Merino, *Lymphoepitheliomalike carcinoma: an unusual variant of endometrial cancer. A report of two cases.* Int J Gynecol Pathol, 1998. **17**(3): p. 272-6.
71. Jafarian, A.H., et al., *Association of Epstein Barr virus deoxyribonucleic acid with lung carcinoma.* Indian J Pathol Microbiol, 2013. **56**(4): p. 359-64.
72. Chen, P.C., et al., *Detection of Epstein-Barr virus genome within thymic epithelial tumours in Taiwanese patients by nested PCR, PCR in situ hybridization, and RNA in situ hybridization.* J Pathol, 2002. **197**(5): p. 684-8.
73. Zhao, W., et al., *Primary lymphoepithelioma-like carcinoma of salivary glands: a clinicopathological study of 21 cases.* Int J Clin Exp Pathol, 2014. **7**(11): p. 7951-6.
74. Yang, A.W., et al., *Lymphoepithelioma-like, a variant of urothelial carcinoma of the urinary bladder: a case report and systematic review for optimal treatment modality for disease-free survival.* BMC Urol, 2017. **17**(1): p. 34.
75. Ahn, H., et al., *Lymphoepithelioma-like Carcinoma of the Renal Pelvis: A Case Report and Review of the Literature.* Korean J Pathol, 2014. **48**(6): p. 458-61.
76. Abouelfad, D.M., et al., *Lymphoepithelioma-Like Carcinoma of the Breast Mimicking Granulomatous Mastitis- Case Report and Review of the Literature.* Asian Pac J Cancer Prev, 2017. **18**(7): p. 1737-1741.
77. McCluggage, W.G., *Lymphoepithelioma-like carcinoma of the vagina.* J Clin Pathol, 2001. **54**(12): p. 964-5.
78. Bosmuller, H., et al., *Lymphoepithelioma-like carcinoma of the vulva, an underrecognized entity? Case report with a single inguinal micrometastasis detected by sentinel node technique.* Diagn Pathol, 2011. **6**: p. 4.
79. Lee, S., et al., *Lymphoepithelioma-like carcinoma of the ovary: a case report and review of the literature.* Arch Pathol Lab Med, 2007. **131**(11): p. 1715-8.
80. Forbes, S.A., et al., *COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer.* Nucleic Acids Res, 2011. **39**(Database issue): p. D945-50.
81. Hoang, L.N., et al., *Histotype-genotype correlation in 36 high-grade endometrial carcinomas.* Am J Surg Pathol, 2013. **37**(9): p. 1421-32.

82. Hussein, Y.R., et al., *The Genomic Heterogeneity of FIGO Grade 3 Endometrioid Carcinoma Impacts Diagnostic Accuracy and Reproducibility.* Int J Gynecol Pathol, 2016. **35**(1): p. 16-24.
83. Hussein, Y.R., et al., *Clinicopathological analysis of endometrial carcinomas harboring somatic POLE exonuclease domain mutations.* Mod Pathol, 2015. **28**(4): p. 505-14.
84. Herath, C.H. and R. Chetty, *Epstein-Barr virus-associated lymphoepithelioma-like gastric carcinoma.* Arch Pathol Lab Med, 2008. **132**(4): p. 706-9.
85. Dundr, P., et al., *Large cell neuroendocrine carcinoma of the urinary bladder with lymphoepithelioma-like features.* Pathol Res Pract, 2003. **199**(8): p. 559-63.
86. Tafe, L.J., et al., *Endometrial and ovarian carcinomas with undifferentiated components: clinically aggressive and frequently underrecognized neoplasms.* Mod Pathol, 2010. **23**(6): p. 781-9.