UNIVERZITA KARLOVA V PRAZE
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VZTAH NÁDOROVÉHO GENOTYPU A FENOTYPU K DIAGNOSTICE, PROGNÓZE A PREDIKCI KOLOREKTÁLNÍHO KARCINOMU

RELATION OF TUMOR GENOTYPE AND PHENOTYPE TO DIAGNOSIS, PROGNOSIS AND PREDICTION OF COLORECTAL CANCER

Dizertační práce

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Abstrakt

Kolorektální karcinom je jedním z nejvíce rozšířených typů maligních onemocnění. V jeho biologii a léčbě je stále velké množství otazníků a v této práci jsme se pokusili identifikovat nové prognostické znaky, které by mohly podat nové informace o vývoji tohoto nádoru a pomoci rozpoznat pacienty s těžším průběhem nemoci.

V první části práce jsme se zaměřili na imunohistochemické značení dvou proteinů spojovaných s nádorovými kmenovými buňkami na vzorcích nádoru tlustého střeva a jeho jaterních metastáz. Cílem bylo vyhodnotit vztah mezi hladinou sledovaných proteinů CD44 a CD133 a celkovým a bezpříznakovým přežitím u studovaného souboru pacientů. Pozorovali jsme korelaci mezi zvýšeným množstvím CD133 pozitivních nádorových žlázek a prodloužením bezpříznakového přežití. Tento výsledek je v rozporu s obecným názorem ohledně vlivu CD133 na vývoj nádoru. Možným vysvětlením je použití vysoce specifikovaného souboru pacientů a odlišný metodický přístup, kdy jsme sledovali poměr CD133 pozitivních žlázek ke všem nádorovým žlázkám v zorném poli, nikoli absolutní intenzitu značení.

Následně jsme se zaměřili na studium intenzity transkripce vybraného setu genů v zamražených vzorcích od pacientů s kolorektálním karcinomem. Hlavním cílem bylo detekovat rozdílnou hladinu odpovídajících mRNA mezi zdravou a nádorovou tkání a vliv změny exprese na celkové i bezpříznakové přežití pacientů. Ve studii jsme u 53 pacientů detekovali rozdílné hladiny mRNA u deseti genů. Pro část analýz byl soubor rozdělen na dvě části dle přítomnosti či nepřítomnosti vzdálené metastázy v čase primární operace. Statisticky významně byla s přežíváním pacientů spojena vyšší hladina *VSNL1* v nádorové tkáni, která indikovala prodloužení celkového přežití. U části souboru bez vzdálené metastázy byl popsán vztah mezi vyšší hladinou *SLC26A2* a prodloužením celkového přežití. Prodloužení bezpříznakového přežití bylo spojeno s nižší hladinou *VSNL1* ve zdravé tkáni a vyšší hladinou *SLC26A2* v tkáni nádorové. Porovnání změny úrovně exprese mezi zdravou a nádorovou tkání po statistické analýze ukázalo spojení mezi snížením hladiny *CLDN23* a zkrácením celkového přežití u kompletního souboru pacientů. U části pacientů

bez vzdálené metastázy bylo prokázána vazba mezi výrazným snížením hladiny *SLC26A2* a *ACSL5* a výrazným zvýšením hladiny *LGR5* a prodloužením bezpříznakového přežití.

Jak ukazují výsledky spojené s CD133 proteinem v první a *LGR5* mRNA ve druhé studii, je nutné zkoumat vlastnosti a chování i u znaků, které se již zdají jednoznačné z pohledu jejich efektu na vývoj nádorové tkáně. Pro správnou interpretaci výsledků bude v tomto případě zapotřebí získat více informací o vlastnostech obou proteinů, protože jejich přesná funkce ve fyziologii i patologii buňky není známa.

Studie transkripce vybraných genů u kolorektálního karcinomu ukázala nové znaky s potenciálem upřesnit prognózu pacientů. Tyto znaky by mohly pomoci s výběrem pacientů pro onkologickou léčbu a rovněž přinášejí zprostředkované informace o biologii kolorektálního karcinomu.

Abstract

Colorectal cancer is one of the most common type of malignity. Despite of the existence of numerous studies focused on this carcinoma, there are still many unknown features regarding its diagnosis, treatment or prognosis. In the thesis we focused on the identification of novel prognostics markers that could be useful for the stratification of patients based on the disease outcomes.

In the first study we immunohistochemically assessed expression of two proteins associated with cancer stem cells in the samples of primary colorectal cancer and matched liver metastasis. Goal of the study was to evaluate relation among expression of CD44 and CD133 and overall survival and disease free interval in our set of patients. We observed that increased ratio of CD133 positive compared to CD133 negative tumor glands resulted in longer disease free interval, finding which is opposite to the general view on the CD133 role in the cancer development. Our hypothesis is that we analyzed confined group of patients and followed a bit different goal, where we measured ratio between positive and negative glands in the view-field and not the intensity of staining as the previous studies did.

Our second study was focused on the transcriptional analysis of the selected set of twelve genes using frozen samples from colorectal cancer patients. Main goal was to detect differentially expressed genes between healthy and tumor tissue and relate experimental data to overall survival and disease free interval. In this study we analyzed samples from 53 patients and identified ten genes with altered expression. For portion of analysis we divided patients into two groups based on presence or absence of distant metastasis at the time of primary surgery. Statistically significant associations between gene expression level and clinical data were found. Higher level of *VSNL1* in tumor tissue correlated with longer overall survival. In the group without metastasis we found relation between higher level of *SLC26A2* and longer overall survival, longer disease free survival was related to lower level of *VSNL1* expression in healthy tissue and higher level of *SLC26A2* in tumor tissue. Expression change between healthy and tumor tissue showed that downregulation of *CLDN23* in tumor tissue correlated with shorter overall survival in the complete group

of patients, in the group without distant metastasis we found that downregulation of *SLC26A2* and *ACSL5* and upregulation of *LGR5* related to longer disease free interval.

Our data points out the need to study even markers, whose role in the tumor development seems clear at this moment. To understand our results regarding CD133 protein and *LGR5* mRNA it is necessary to learn more about function of these proteins in biology and pathology of the cell. In the transcription study we showed new markers with the potential to specify prognosis in colorectal cancer patients. These markers could help with the selection of patients for the oncology treatment and could give us indirect insights regarding the tumor biology.

Předmluva a prohlášení

Tato práce byla vytvořena na pracovištích Ústavu histologie a embryologie Lékařské fakulty v Plzni, Univerzity Karlovy v Praze, a Laboratoře experimentální chirurgie Fakultní nemocnice v Plzni, pod vedením Doc. MUDr. Mileny Králíčkové, PhD. a za pomoci cenných rad MUDr. Václava Lišky, PhD.

Prohlašuji, že dizertační práci jsem vypracoval samostatně a všechny literární zdroje jsou řádně citovány. Předkládaná práce nebyla použita k získání jiného nebo stejného titulu. V textové části byly použity části předchozích článků autora, jejichž kompletní znění je k dispozici v přílohové části práce.

Teoretický úvod slouží k přehledu současné klasifikace, diagnózy a typů léčby kolorektálního karcinomu s důrazem na využití změn v genotypu a fenotypu nádorové tkáně pro výše zmíněné cíle. V experimentální části jsou navrženy možnosti nových fenotypových znaků s ohledem na prognózu kolorektálního karcinomu.

V Plzni dne ....

1 Seznam zkratek

5-FU	5-fluorouracil
a.l.	délka amplikonu
ACSL5	acyl-CoA synthetase long-chain family member 5
AKT	v-akt murine thymoma viral oncogene homolog 3
ALDH1	aldehyde dehydrogenase 1
APC	adenomatous polyposis coli
BAX	BCL2-associated X protein
BRAF	v-raf murine sarcoma viral oncogene homolog B
C18	zhoubný novotvar tlustého střeva
C19	zhoubný novotvar rektosigmoideálního spojení
C20	zhoubný novotvar konečníku - rekta
C21	zhoubný novotvar řiti a řitního kanálu
CA19-9	nádorový antigen 19-9
CAPN10	calpain 10
CD133	cluster of differentiation 113
cDNA	komplementární DNA
CEA	karcinoembryonální antigen
CIMP	CpG islang methylator phenotype
CIN	chromozomální nestabilita
CLDN23	claudin 23
CNB	cirkulující nádorová buňka
CpG	cytosin-fosfát-guanin
CRYSTAL	Cetuximab Combined with Irinotecan in First-line Therapy for Metastatic Colorectal Cancer
CSC	nádorové kmenové buňky
CT	počítačová tomografie
CTHRC1	collagen triple helix repeat containing 1
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
DCC	deleted in colorectal carcinoma
DFI	bezpříznakové přežití
DNA	deoxyribonukleová kyselina

DPYD	dihydropyrimidin dehydrogenáza
DSTN	destrin (actin depolymerizing factor)
EGFR	receptor epidermálního růstového faktoru
EpCAM	epithelial cell adhesion molecule
ERCC1	excision repair cross-complementation group 1
ERCC2	excision repair cross-complementation group 2
FANCG	Fanconi anemia, complementation group G
FAP	familiární adenomatózní polypóza
FGFR	fibroblast growth factor receptor
FOBT	Fecal Occult Blood Test
FOS	FBJ murine osteosarcoma viral oncogene homolog
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosin-5‘-trifosfát
HD-CTC	High-Definition Circulating Tumor Cell
HNPCC	hereditární nepolypózní kolorektální karcinom
IBD	Intestinal Bowel Disease
Jak2	Janus kinase 2
KIF11	kinesin family member 11
KRAS	Kirsten rat sarcoma viral oncogene homolog
KRCa	kolorektální karcinom
LGR5	leucine-rich repeat-containing G protein-coupled receptor 5
MAPK1	mitogen-activated protein kinase 1/ERK
MET	MET proto-oncogene, receptor tyrosine kinase
MIER3	mesoderm induction early response 1, family member 3
mKRCa	metastazující kolorektální karcinom
MLH1	mutL homolog 1
moAb	monoklonální protilátka
mRNA	messenger RNA
MSH2	mutS homolog 2
MSH6	mutS homolog 6
MSI	mikrosatelitní nestabilita
MTHFR	metylentetrahydrofolát reduktáza
MYH	mutY homolog

n.t.	nádorová tkáň
NCBI	National Center for Biotechnology Information
NOTCH	notch 1
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
OPUS	Oxaliplatin and Cetuximab in First-line Treatment of Metastatic Colorectal cancer
OS	celkové přežití
PCR	polymerázová řetězová reakce
PDGFR-β	platelet-derived growth factor receptor, beta polypeptide
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PIGF	placentální růstový faktor
PMS2	PMS2 postmeiotic segregation increased 2
POLR2A	polymerase (RNA) II (DNA directed) polypeptide A
PRIME	the Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine the Efficacy
PTEN	phosphatase and tensin homolog
PTPRC	protein tyrosine phosphatase, receptor type, C
qPCR	kvantitativní polymerázová řetězová reakce
RNA	ribonukleová kyselina
S100	S100 calcium binding protein
SAMD3	sterile alpha motif domain containing 3
SLC26A2	solute carrier family 26 (sulfate transporter), member 2
SMAD2	SMAD family member 2
SMAD4	SMAD family member 4
Stat3	signal transducer and activator of transcription 3 (acute-phase response factor)
TGFβRII	transforming growth factor, beta receptor II
TIE-2	TEK tyrosine kinase, endothelial
TKI	tyrosin kinázový inhibitor
tm	teplota nasedání oligonukleotidů

TNM	Tumor-lymph Node-Metastasis
TOKS	test okultního krvácení do stolice
TP53	tumor protein p53
UGT1A1	uridin difosfát glukuronosyl transferáza 1A1
UICC	Union for International Cancer Control
VCAN	versican
VEGF-A	vaskulární-endoteliální růstový faktor A
VEGF-B	vaskulární-endoteliální růstový faktor B
VEGFR	kinase insert domain receptor (a type III receptor tyrosine kinase)
VSNL1	visinin-like 1
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
z.t.	zdravá tkáň
ZN	zhoubný novotvar

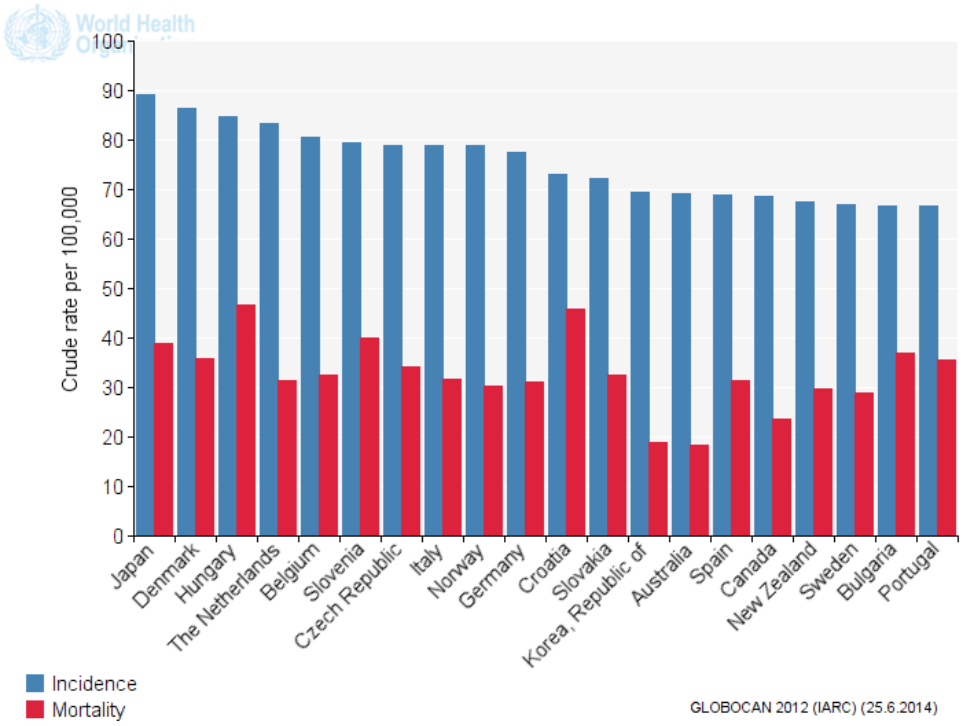
2 Teoretický úvod

2.1 Epidemiologie kolorektálního karcinomu

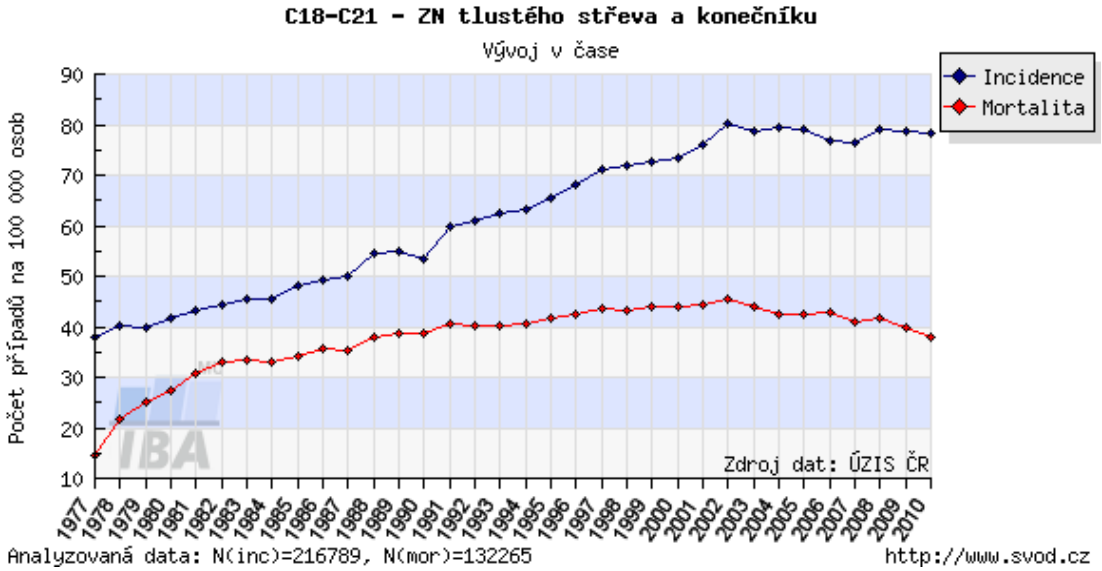
Kolorektální karcinom (KRCa) je celosvětově jedním z nejčastějších maligních onemocnění. V incidenci je u celkové populace na třetím místě za nádory plic a prsu s více než 1,2 milionem případů ročně (téměř 10% všech nádorových onemocnění), z pohledu mortality je KRCa na čtvrté pozici po nádorech plic, žaludku a jater s celkovým počtem 600 tisíc úmrtí (8,1% s nádory spojených úmrtí). Mezi muži jde o třetí nejčastější nádorové onemocnění po nádorech plic a prostaty, u žen jde po nádoru prsu o druhou nejčastější malignitu. Mortalita spojená s KRCa je u mužů obdobná jako v celkové populaci, zatímco u žen je na třetí pozici po nádorech prsu a plic. Celkově je incidence zvýšená v mužské části populace v poměru 1,4:1 oproti ženám (Ferlay et al., 2010). Celoživotní riziko vzniku kolorektálního karcinomu se odhaduje na 5-6% (Grady, 2003a). Incidence i mortalita KRCa je obecně vyšší ve vyspělých zemích než v zemích třetího světa.

V České republice je incidence i mortalita spojená s KRCa jednou z celosvětově nejvyšších (Zavoral, 2009) (obrázek 1). V absolutních hodnotách se v roce 2010 jednalo o 8265 nově diagnostikovaných případů KRCa a 3991 úmrtí s KRCa spojených.

V posledních letech se v České republice incidence všech diagnóz spadajících pod souhrnné označení kolorektální karcinom (C18 – zhoubný novotvar (ZN) tlustého střeva; C19 – ZN rektosigmoideálního spojení; C20 – ZN konečníku – rekta; C21 – ZN řiti a řitního kanálu) i přes vysoká absolutní čísla ustálila a mortalita mírně poklesla (obrázek 2). Tato statistika zahrnuje i diagnózu ZN řiti a řitního kanálu, která je již ektodermálního, nikoliv entodermálního původu. Celkový podíl jednotlivých diagnóz z hlediska nově diagnostikovaných případů KRCa a z hlediska mortality v roce 2010 je shrnut v Tabulce 1. V příštích letech se očekává růst incidence i mortality spojené s KRCa, především v souvislosti s celkovým stárnutím populace. Výše uvedené statistické informace pocházejí z Národního onkologického registru na internetové adrese www.svod.cz.



Obrázek 1 - Graf incidence a mortality KRCa dle dat projektu GLOBOCAN 2012. Česká republika je na sedmém místě z hlediska incidence a šestém z pohledu mortality. Zdroj http://globocan.iarc.fr/. Data normalizovaná na 100 tisíc obyvatel.



Obrázek 2 - Vývoj incidence a mortality KRCa v České republice mezi lety 1977 až 2010. V posledních letech došlo k ustálení incidence a k poklesu mortality díky nově zavedeným postupům v léčbě. Zdroj http://www.svod.cz.

Tabulka 1 - Přehled podílu jednotlivých diagnóz spadajících pod označení kolorektální karcinom na incidenci a mortalitě spojené s KRCa v absolutních počtech případů a v procentech dle dat z roku 2010.

diagnóza	incidence		mortalita	
	absolutní hodnoty	%	absolutní hodnoty	%
C18	4908	59,4%	2245	56,3%
C19	981	11,9%	549	13,8%
C20	2247	27,2%	1140	28,6%
C21	129	1,6%	57	1,4%

2.2 Vývoj a dělení kolorektálního karcinomu

KRCa je dle původu možné rozdělit na dědičné a sporadické. Dědičné formy jsou zodpovědné za přibližně 20-30% případů KRCa. U většiny dědičných případů KRCa je zatím konkrétní mutace neznámá, dobře definované syndromy tvoří 5% všech případů KRCa (Grady, 2003b). Mezi popsané dědičné typy KRCa patří například Lynchův syndrom (též nazývaný hereditární nepolypózní kolorektální karcinom), familiární adenomatózní polypóza, s mutací v genu *MYH* (mutY homolog) spojená *MYH*-asociovanou polypóza a syndrom hyperplastické polypózy (Cunningham et al., 2010; Kalady et al., 2011). Nejvyšší četnosti dosahuje Lynchův syndrom a familiární adenomatózní polypóza, výskyt dalších syndromů je minimální.

Druhou skupinu tvoří sporadické formy KRCa, které jsou zodpovědné za zbývajících 70-80% případů (Lichtenstein et al., 2000; Migliore et al., 2011) a na jejichž vývoji se podílí kombinace environmentálních faktorů a somatických mutací.

2.2.1 Lynchův syndrom

Nejčastější hereditární typ KRCa se vyskytuje přibližně u 1-3% pacientů s KRCa (Hampel et al., 2008). Lynchův syndrom se často označuje jako hereditární nepolypózní kolorektální karcinom (HNPCC). Celoživotní riziko vzniku KRCa u pacientů s Lynchovým syndromem dosahuje 80%. Navíc je toto onemocnění spojeno s dalšími typy nádorů, nejčastěji s nádorem endometria, dále pak například s nádory vaječníků, žaludku, tenkého střeva, slinivky a v podobě Turcotova syndromu s nádory mozku nebo Muir-Torre syndromu

jako kombinace kožního a viscerálního nádoru (Lynch et al., 2009; Shellenberger et al., 2008). Genetickou příčinou Lynchova syndromu jsou zárodečné mutace v genech zodpovědných za opravu DNA, nejčastěji mutace *MLH1* (mutL homolog 1) a *MSH2* (mutS homolog 2). Výsledkem těchto mutací pak nejčastěji bývá nádor s typickou mikrosatelitní nestabilitou (viz část 2.4).

Diagnostika Lynchova syndromu probíhá podle Amsterodamských kritérií I a II (Tabulka 2) či podle revidovaných kritérií z Bethesdy (Tabulka 3). Správná identifikace tohoto syndromu je podstatná pro pacienta samotného i jeho rodinu, jelikož je možné u rizikových osob včasně zahájit preventivní prohlídky a umožnit tak detekci nádoru v raném stádiu.

Tabulka 2 - Diagnostika Lynchova syndromu podle amsterodamských kritérií (Plevová et al., 2009).

Amsterodamská kritéria I	
1.	V rodině jsou alespoň tři pacienti s karcinomem tlustého střeva, jeden z nich je příbuzný prvního stupně ostatních dvou.
2.	Jsou postiženy alespoň dvě generace.
3.	Alespoň jeden nemocný byl mladší 50 let v době diagnózy.
4.	Nádor byl ověřen patologem.
5.	Je vyloučena familiární adenomatózní polypóza.
Amsterodamská kritéria II	
1.	V rodině jsou alespoň tři příbuzní s karcinomem sdruženým s HNPCC (kolorektální karcinom, karcinom endometria, tenkého střeva, ureteru a ledvinové pánvičky), jeden z nich je příbuzný prvního stupně ostatních dvou.
2.	Jsou postiženy alespoň dvě generace.
3.	Alespoň jeden nemocný byl mladší 50 let v době diagnózy.
4.	Nádor byl ověřen patologem.
5.	Je vyloučena familiární adenomatózní polypóza.

Tabulka 3 - Diagnostika Lynchova syndromu podle revidovaných kritérií z Bethesdy (Plevová et al., 2009).

Revidovaná kritéria z Bethesdy	
1.	Kolorektální karcinom diagnostikovaný u pacienta mladšího 50 let.
2.	Přítomnost synchronních nebo metachronních karcinomů střeva nebo jiných nádorů sdružených s HNPCC (karcinom kolorekta, endometria, žaludku, tenkého střeva, ovaria, pankreatu, ureteru a pánvičky, biliárního traktu, mozku – glioblastom, kůže – adenomy sebaceozních žláz a keratoakanthomy), bez ohledu na věk.
3.	Kolorektální karcinom s histologií odpovídající vysokému stupni mikrosatelitní nestability, diagnostikovaný u pacienta mladšího 60 let (přítomnost tumor-infiltrujících lymfocytů, lymfocytární reakce podobná Crohnově chorobě, mucinózní charakter, medulární růst, prstencové buňky v nádoru).
4.	Kolorektální karcinom diagnostikovaný u jednoho nebo více příbuzných prvního stupně s nádorem charakteristickým pro HNPCC, jeden z nádorů je diagnostikován před 50. rokem věku.
5.	Kolorektální karcinom diagnostikovaný u dvou nebo více příbuzných prvního nebo druhého stupně s nádory sdruženými s HNPCC, bez ohledu na věk.

2.2.2 Familiární adenomatózní polypóza

Zárodečná mutace v genu *APC* (adenomatous polyposis coli) je příčinou méně než jednoho procenta kolorektálních karcinomů (Patel and Ahnen, 2012). Klinickým projevem je přítomnost vysokého množství polypů v tlustém střevě při nízkém věku pacienta, obvykle do 35 let. Benigní polypy je možné považovat za prekancerózní stádia a riziko vzniku KRCa u neléčených pacientů s familiární adenomatózní polypózou (FAP) ve věku 50 let dosahuje 95% (Jasperson et al., 2010). Stejně jako v případě Lynchova syndromu, i u FAP se vyskytují nádory mimo oblast tlustého střeva – nejčastěji karcinomy tenkého střeva a papilární karcinom štítné žlázy (Anaya et al., 2008).

Kromě plně rozvinuté formy FAP rozeznáváme i atenuovanou formu FAP, která se projevuje menším množstvím adenomů v tlustém střevě a nástupem v pozdějším věku (Jasperson et al., 2010).

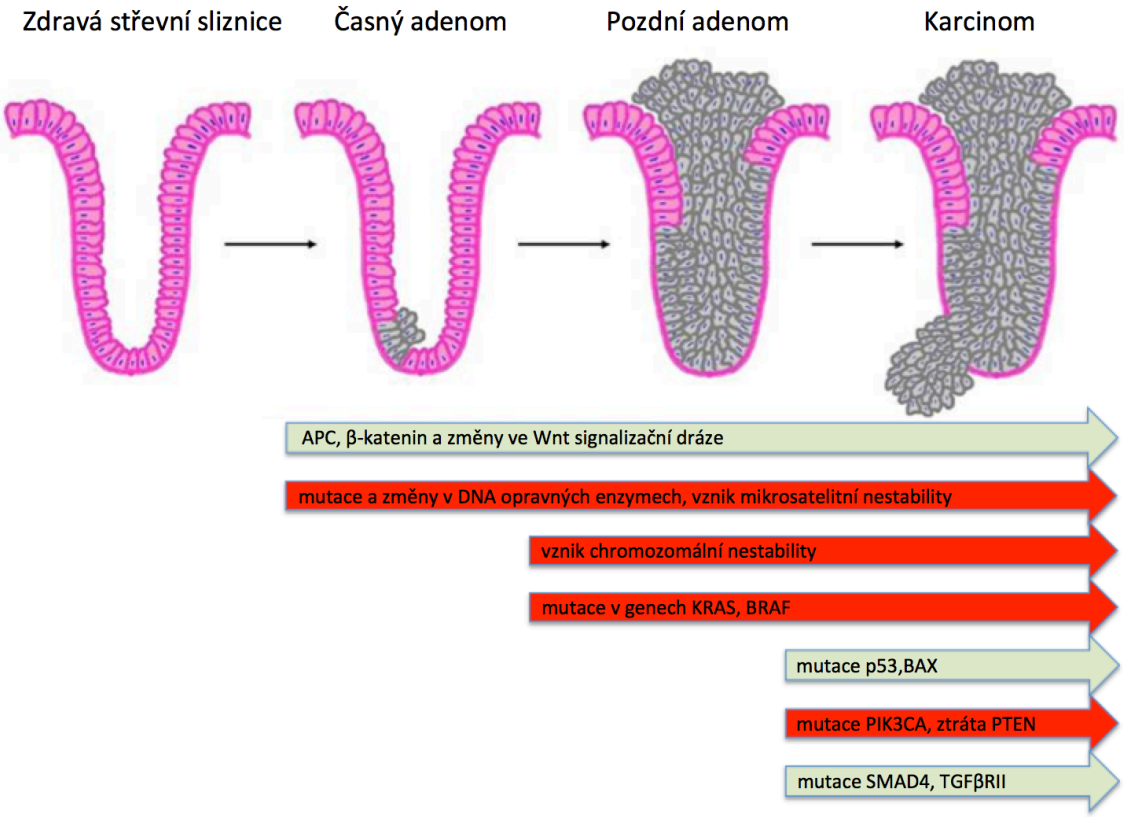
2.2.3 Sporadický kolorektální karcinom

Sporadické případy tvoří většinu KRCa a jejich vznik není vysvětlitelný přítomností některé ze v současnosti popsaných zárodečných mutací. V etiologii sporadického KRCa hraje roli velké množství faktorů, od genetického pozadí přes somatické mutace a epigenetické regulace až po vliv vnějšího prostředí, přičemž na vzniku KRCa se většinou podílí kombinace několika faktorů.

V současnosti uznávanou teorií vzniku a vývoje kolorektálního karcinomu je model vícestupňové kancerogeneze, také nazývaný Vogelsteinův model kancerogeneze. Podle něj dochází postupným hromaděním mutací k přeměně zdravého střevního epitelu na dysplastické léze, které se dále vyvíjejí přes časný a pozdní adenom až do stádia karcinomu (Fearon and Vogelstein, 1990).

Vývoj směrem ke kolorektálnímu karcinomu je potencován vznikem konkrétních somatických mutací. První a zásadní je mutace v genu *APC* (podobně jako u familiární adenomatózní polypózy), která je detekovatelná již v dysplastických lézích (Powell et al., 1992). Dalším krokem při vývoji adenomu je mutace v genu *KRAS* (Kirsten rat sarcoma viral oncogene homolog), který patří do rodiny malých GTP (guanosin-5'-trifosfát) vazebných proteinů a hraje roli v přenosu signálů od receptorů pro růstové faktory. Tato mutace je dle některých studií přítomna ve více než 50% KRCa (Haigis et al., 2008) a její přítomnost pravděpodobně dává maligním buňkám růstovou výhodu, především v kombinaci s mutací v genu *APC* (Haigis et al., 2008). Během vývoje adenomu z časného k pokročilému se objevuje delece na dlouhém raménku chromozomu 18, která zasahuje kondicionální tumor supresor/proto-onkogen *DCC* (deleted in colorectal carcinoma) (Lea et al., 2009). Posledním krokem při přechodu od adenomu ke karcinomu je mutace v tumor supresorovém genu *TP53* (tumor protein p53), která se vyskytuje u 75% KRCa, s nižším výskytem u nádorů proximální části tlustého střeva a u Lynchova syndromu. Mutace v *TP53* se jen velmi zřídka vyskytují v adenomech, zdá se tedy, že jsou klíčové pro vznik karcinomu. Pokud mutace vedou k vyřazení funkce proteinu p53, způsobují akumulaci dalších mutací a zablokování p53-mediované apoptózy (Houlston, 2001). Kromě mutace v *TP53* může být přechod z adenomu na

karcinom provázen i mutacemi DNA (deoxyribonukleová kyselina) opravných genů *MLH1* a *MSH2*, podobně jako u Lynchova syndromu (Houlston, 2001). Sekvence typických mutací při vývoji KRCa je shrnuta v obrázku 3.



Obrázek 3 - Vývoj sporadického kolorektálního karcinomu. Zelené šipky znázorňují inaktivaci nádorových supresorů, červené naopak změny v proto-onkogenech a celkové genetické změny v nádorových buňkách. Převzato z (Pitule et al., 2013).

Odlišným genetickým pozadím a především rozdílnou morfologií se postupnému vývoji nádoru sekvencí adenom-karcinom vymyká část sporadických KRCa vzniklých takzvanou pilovitou cestou kancerogeneze, které tvoří celkově 10% KRCa. Rozlišujeme čtyři typy pilovitých lézí – sesilní (přisedlé) pilovité polypy, sesilní pilovité adenomy, tradiční pilovité adenomy a hyperplastické polypy, které jsou předchozím typům podobné morfologicky, ale mají velmi nízký maligní potenciál (Yamane et al., 2014). Významnou roli v této dráze hraje zvýšená metylace CpG (cytosin-fosfát-guanin) oblastí

v genomu a mikrosatelitní nestabilita doprovázená mutacemi v BRAF (v-raf murine sarcoma viral oncogene homolog B) proto-onkogenu (Guarinos et al., 2012).

Kromě geneticky podmíněných rizikových faktorů hrají významnou roli v etiologii sporadického karcinomu i environmentální a další negenetické faktory. Za nejvýznamnější lze považovat vyšší věk, protože většina případů KRCa se vyskytuje u osob starších 50 let (92% případů), respektive 60 let (72% případů). Dále byly jako rizikové faktory popsány nízká fyzická aktivita, nízká konzumace ovoce a zeleniny, nízký příjem vlákniny a dieta bohatá na tuky, vysoká hmotnost až obezita, kouření a nadměrná konzumace alkoholu (Benito et al., 1991; Wu et al., 1987).

2.2.4 Kolorektální karcinom a zánětlivá střevní onemocnění

Zvýšené riziko výskytu kolorektálního karcinomu bylo dle některých studií pozorováno u osob se zánětlivými střevními onemocněními (IBD, Intestinal Bowel Disease) jako jsou Crohnova choroba či ulcerózní kolitida, které se vyskytují u zhruba 2% pacientů s KRCa (Triantafillidis et al., 2009). Kolorektální karcinomy vzniklé na pozadí zánětu mají podobné procento chromozomálních a mikrosatelitních nestabilit jako čistě sporadické KRCa (Cunningham et al., 2010), ale vyskytují se v mladším věku (Rhodes and Campbell, 2002). Mezi rizikové faktory vzniku KRCa na pozadí IBD patří chronický zánět, při kterém dochází ke dráždění střevní sliznice, což zvyšuje pravděpodobnost vzniku nádorových buněk, dále délka trvání onemocnění či věk pacientů při propuknutí zánětlivých střevních onemocnění (Andersen and Jess, 2013).

I přes rizikové faktory není vliv střevních zánětlivých onemocnění na riziko výskytu KRCa zcela jednoznačný – pacientům s IBD je totiž věnována vyšší péče spojená s pravidelnými kolonoskopiemi od časného věku, jedním z důležitých léčebných zákroků jsou částečné kolektomie a léčba podávaná pacientům s IBD může mít protektivní efekt při vzniku pokročilých neoplázií či KRCa. Protektivní role je přiřazována například thiopurinům, i když nedávná meta-analýza jejich vliv na riziko vzniku KRCa neprokázala (Jess et al., 2014; van Schaik et al., 2012). Přestože některé práce odhalily zvýšené riziko vzniku

KRCa u pacientů s IBD (Herrinton et al., 2012), další studie z posledních let provedené na odlišné populaci nárůst incidence KRCa u této skupiny pacientů neprokazují (Jess et al., 2012, 2013).

2.2.5 Nádorové kmenové buňky u kolorektálního karcinomu

V současnosti byla u většiny solidních i hematologických nádorů identifikována minoritní buněčná populace nazvaná nádorové kmenové buňky (CSC, cancer stem cells). Tyto buňky jsou na začátku hierarchického uspořádání nádorové tkáně, která byla poprvé pozorována u akutní myeloidní leukemie v roce 1997 (Bonnet and Dick, 1997). Později byly podobné populace nalezeny například i u karcinomu prsu (Al-Hajj et al., 2003), prostaty (Collins et al., 2005), tlustého střeva (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) či slinivky (Li et al., 2007).

Nádorové kmenové buňky sdílejí velké množství svých znaků s fyziologickými kmenovými buňkami. Oba typy se dělí asymetricky, jsou schopny sebeobnovy, diferenciace v další buňky dané tkáně či nádoru, dlouhého přežívání i za nepříznivých podmínek a mají relativně dlouhý buněčný cyklus (Aguilar-Gallardo and Simón, 2013). Další podobností je zvýšená odolnost proti chemo- a radioterapii, což z nich činí velice obtížné cíle pro běžné léčebné režimy. Tímto jsou CSC potenciálními zdroji pozdějších recidiv onemocnění či půdou pro selekci rezistentních klonů pomocí suboptimálních dávek onkologických terapeutik (Ishii et al., 2008). S rozvojem poznání o CSC se vytvářejí i speciální terapie založené na prvotní indukci diferenciace CSC a následnému použití chemoterapie či cílené biologické léčby k usmrcení kompletní masy nádorových buněk (Friedman et al., 2013).

Pro identifikaci CSC byly využity různé, většinou povrchové znaky. U kolorektálního karcinomu byla jako první použita pozitivita značení protilátkou proti proteinu CD133 (cluster of differentiation 133), kdy CD133 pozitivní buňky vykazovaly vyšší schopnost tvořit nádory než buňky CD133 negativní (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). V dalších studiích byly mezi znaky přidány například CD44 a CD166 (Dalerba et al., 2007), CD29, CD24 a LGR5 (leucine-rich repeat-containing G protein-coupled receptor 5) (Vermeulen et al., 2008) či ALDH1 (aldehyde dehydrogenase 1) (Huang et al., 2009). Řada těchto

znaků se ale nachází i na normálních kmenových buňkách tlustého střeva, což komplikuje oddělení těchto populací, navíc ani jeden z těchto znaků neurčuje CSC s absolutní jistotou – například i mezi CD133 negativními buňkami existují kmenové buňky schopné tvořit nádory se stejnou frekvencí jako buňky CD133 pozitivní (Shmelkov et al., 2008).

Spojení mezi fyziologickými a nádorovými kmenovými buňkami je pro vývoj nádoru podstatné. Jelikož většina střevních epiteliálních buněk má rychlý životní cyklus a k nahromadění dostatečného počtu mutací je zapotřebí déle žijících buněk, jsou cílem mutací pravděpodobně fyziologické kmenové buňky střevního epitelu, ze kterých se nádorové kmenové buňky vyvíjejí a dále zajišťují zachování a růst nádoru (Vermeulen and Snippert, 2014). Na základě studia nádorových kmenových buněk by bylo možné připravit efektivnější léčebná schémata s cílem eliminovat vývoj KRCa.

2.3 Diagnostika kolorektálního karcinomu

Úspěšnost léčby pacientů s KRCa je vysoká, pokud je nemoc diagnostikována v časném stádiu. Rozdíl v pětiletém přežití mezi stádii I a II a stádii III a IV dle starší klasifikace UICC 2002 (Union for International Cancer Control) je enormní - 93,2% a 82,5% pro stádia I a II, 59,5% a 8,1% pro stádia III a IV (O'Connell et al., 2004). Přesnost a spolehlivost diagnostických metod je zásadní pro zachycení KRCa v raném stádiu a tedy pro umožnění léčby s maximální efektivitou.

2.3.1 Klasifikace kolorektálního karcinomu

Pro správné nasazení léčby a odhad vývoje KRCa je potřebné provést přesnou klasifikaci nádoru a staging na základě některého z používaných klasifikačních systémů – v současnosti se využívá TNM systém (tumor - lymph node - metastasis), Dukův systém a číselný staging. Dalším kategorizačním způsobem je rozdělení KRCa na základě určení dediferenciace nádoru dle grading systému.

TNM klasifikaci je možné rozdělit na klinickou (předoperační) a patologickou (na základě patologického vyšetření operačního nálezu). V současnosti se používá nejčastěji 6. verze (UICC 2002) a 7. verze (UICC 2009) klasifikace. Část T charakterizuje primární nádor a jeho vlastnosti, část N popisuje přítomnost nádorových buněk v místních lymfatických uzlinách a část M poté přítomnost vzdálených metastáz (podrobný popis 7. verze viz Tabulka 4). Dukův a číselný staging systém rozdělují nádory do několika kategorií dle jejich pokročilosti (Tabulka 5 a 6). Převodní vztahy mezi jednotlivými systémy jsou shrnuty v Tabulce 7. Jednotlivé kategorie grading systému dle patologického vyšetření shrnuje Tabulka 8.

Tabulka 4 - Jednotlivé hodnoty parametrů TNM klasifikace a jejich význam.

TNM staging	
hodnota	popis stavu
Tx	primární nádor nemůže být ohodnocen
T0	zdravá tkáň bez přítomnosti nádoru
Tis	karcinom *in situ* - nádor neopustil epitel či invaze jen do lamina propria
T1	nádor invaduje do podslizničního vaziva
T2	nádor invaduje do slizniční svaloviny
T3	nádor invaduje skrze slizniční svalovinu do okolních tkání
T4a	nádor penetruje viscerální peritoneum
T4b	nádor penetruje či přisedá k dalším orgánům
Nx	lymfatické uzliny nemohou být hodnoceny
N0	bez metastáz v místních lymfatických uzlinách
N1	1-3 metastázy v místních lymfatických uzlinách (podkategorie N1a-c)
N2	4 a více metastáz v místních lymfatických uzlinách (podkategorie N2a-b)
M0	bez vzdálených metastáz
M1	přítomnost vzdálených metastáz
M1a	vzdálené metastázy jen v jednom orgánu
M1b	metastázy ve více orgánech či peritoneu

Tabulka 5 - Hodnoty Dukova systému a jejich význam.

Dukův systém	
hodnota	popis stavu
A	nádor prorostl ke svalové vrstvě stěny střeva
B	nádor invaduje skrze celou stěnu střeva, bez metastáz v lymfatických uzlinách
C	detekována metastáza v místních lymfatických uzlinách
D	detekována vzdálená metastáza

Tabulka 6 - Hodnoty číselného stagingu a jejich význam na základě 7. vydání UICC klasifikace.

Číselný staging	
hodnota	popis stavu
0	karcinom *in situ*
I	nádor prorostl ke svalové vrstvě stěny střeva
IIa	nádor invaduje skrze celou stěnu střeva, bez metastáz v lymfatických uzlinách
IIb	nádor invaduje okolní tkáně, ale nedetekovány metastázy v lymfatických uzlinách
IIIa	nádor prorostl maximálně ke svalové vrstvě stěny střeva, ale byly detekovány 1-3 metastázy v lymfatických uzlinách
IIIb	nádor invaduje skrze celou stěnu střeva či do okolních tkání, a byly detekovány 1-3 metastázy v lymfatických uzlinách
IIIc	nádor má jakoukoliv velikost, ale byly detekovány 4 a více metastázy v lymfatických uzlinách a žádná vzdálená metastáza
IVa	nádor jakékoliv velikosti, ale s detekovanou vzdálenou metastázou
IVb	nádor jakékoliv velikosti, ale s detekovanou vzdálenou metastázou ve více orgánech

Tabulka 7 - Převodní tabulka mezi jednotlivými typy staging systémů na základě 7. vydání UICC klasifikace.

číselný staging	Dukův systém	T	N	M
0	-	Tis	N0	M0
I	A	T1	N0	M0
	A	T2	N0	M0
IIa	B	T3	N0	M0
IIb	B	T4	N0	M0
IIIa	C	T1-2	N1	M0
	C	T1	N2a	M0
IIIb	C	T3-4a	N1	M0
	C	T2-3	N2a	M0
	C	T1-2	N2b	M0
IIIc	C	T4a	N2a	M0
	C	T3-T4a	N2b	M0
	C	T4b	N1-2	M0
IVa	D	jakékoli T	jakékoli N	M1a
IVb	D	jakékoli T	jakékoli N	M1b

Tabulka 8 - Kategorie grading systému a jejich význam.

Grading	
hodnota	popis stavu
1	nádorové buňky podobné fyziologickým buňkám střeva (dobře diferencované)
2	nádorové buňky vizuálně abnormální, rychlejší buněčné dělení
3	nádorové buňky s vysoce změněnou morfologií, rychlý buněčný cyklus, potenciál k šíření nádoru (nediferencované buňky)

2.3.2 Zavedené screeningové a diagnostické metody

Vývoj KRCa dle klasického modelu od zdravé sliznice přes adenom po karcinom (Fearon and Vogelstein, 1990) probíhá obvykle velmi pomalu, v průměru se uvádí 7-10 let (Miller and Steele, 2012). Takto dlouhý časový horizont umožňuje použití preventivních screeningových testů, které mohou vést k časnému zachycení onemocnění. Přesto bylo v letech 2006 až 2010 v České republice diagnostikováno s časným stádiem KRCa (stádium I a II) jen 45% pacientů, s pokročilým KRCa (stádium III a IV) 47% pacientů a u zbylých 8% pacientů bylo stádium neznámé (www.svod.cz).

Plošný screening kolorektálního karcinomu v běžné populaci se v České republice provádí od roku 2000. V roce 2009 byla zavedena nová pravidla, která zvýšila pokrytí populace. Od roku 2013 se zavádí systém adresného zvaní na preventivní prohlídku s cílem přiblížit pokrytí populace k hodnotám typickým u západoevropských států, tedy 50% cílové populace (Kral and Seifert, 2013; Zavoral et al., 2014).

Primární screeningovou metodou je test okultního krvácení ve stolici (TOKS, v anglické literatuře Fecal Occult Blood Test, FOBT), kde jsou původní testy využívající guajakovou pryskyřici postupně nahrazovány testy imunohistochemickými, které mají vyšší senzitivitu i specificitu (Zhu et al., 2010). Tento test mohou zdarma absolvovat v ročním intervalu osoby ve věku 50-54 let. Při dovršení 55 let je možné pokračovat v TOKS s dvouletým intervalem, či podstoupit primární screeningovou kolonoskopii, kde v případě negativního výsledku dojde na dobu deseti let k přerušení screeningu - zde se využívá pomalého vývoje KRCa (Kral and Seifert, 2013).

Výhodou neinvazivních testů je vyšší účast pacientů a tedy kvalitnější pokrytí populace. Kromě již zavedených testů je ve vývoji široké spektrum nových diagnostických metod, které by mohly senzitivitou, specificitou či určením konkrétních informací o daném nádoru překonat současný standard v podobě testů okultního krvácení do stolice. Nevýhodou těchto testů je vyšší cena a často i nároky na personál a vybavení laboratoří.

Mezi další metodiky, které je možné použít v diagnostice KRCa patří flexibilní sigmoideoskopie a radiologické testy, především irigografie a CT kolonografie (CT, počítačová tomografie) (Levin et al., 2008). Ani jeden z těchto postupů ale nedosahuje dostatečné spolehlivosti, a proto je v současnosti nejvýhodnější použít TOKS kombinovaný v případě pozitivního nálezu s kompletní kolonoskopií. Použití kolonoskopie umožňuje i odběr bioptického vzorku z případné léze a získání podstatných informací o nádoru.

2.3.3 Nové metodiky využitelné pro diagnostiku a screening KRCa

Volná DNA ve stolici

Zajímavou alternativou ke guajakovému i imunohistochemickému TOKS je testování DNA (deoxyribonukleová kyselina) extrahované ze stolice. Detekce je založená na genetických a epigenetických změnách v nádorové tkáni (Ahlquist, 2010). DNA uvolněná z epiteliálních buněk trávicího traktu představuje jen minimální množství celkových nukleových kyselin ve stolici, které z více než 99,9% tvoří DNA bakterií střevní mikroflóry (Klaassen et al., 2003). Díky existenci vysoce senzitivních metod pro detekci mutací je přesto možné zachytit změny v onkogenech, které mohou být spojeny s přítomností nádoru v trávicí soustavě (Deng et al. 2012; Li et al. 2012; Zou et al. 2009). Kromě mutací je možné analyzovat i metylační profil DNA, který je v případě nádorů rovněž odlišný od běžného střevního epitelu. Poslední generace testů DNA ve stolici pracují s upravenými panely sledovaných znaků, které zvyšují specificitu a senzitivitu především pro detekci adenomů a časných stádií karcinomu kolorekta (Ahlquist et al., 2012). Při správném nastavení panelu sledovaných znaků je možné zachytit nádory nejen v oblasti tlustého střeva a konečníku, ale i nádory v dalších částech trávicí soustavy (slinivka, tenké střevo či žaludek), u nichž jsou testy na detekci krve ve stolici nepoužitelné (Ahlquist, 2009; Kisiel et al., 2012). Zásadní nevýhodou testu DNA ve stolici je jeho vyšší cena a přísnější nároky na kvalitu vzorku.

Cirkulující nádorové buňky

Buňky uvolněné z nádorové masy karcinomů je možné detekovat v periferní krvi pacientů vzhledem k jejich odlišným vlastnostem od běžných krevních buněk. První popis cirkulující nádorové buňky pochází již z roku 1869 od australského lékaře Thomase Ashwortha (Sleijfer et al., 2007). V posledních letech se začíná využívat cirkulujících nádorových buněk (CNB) pro diagnostiku a především prognózu onemocnění (nejprve byl tento test do klinické praxe zaveden u metastatického nádoru prsu, později i u metastatického karcinomu prostaty

a kolorektálního karcinomu), s výhledem pro možné využití CNB i pro další typy malignit (Liberko et al., 2013).

Největší komplikací při stanovení CNB je jejich malé množství v poměru k fyziologickým buňkám krve. Ve většině detekčních postupů je proto prvním krokem obohacení studovaného vzorku o populaci buněk, ve které se budou s nejvyšší pravděpodobností CNB vyskytovat. V této části postupu se využívá odlišného fenotypu buněk uvolněných z epiteliálního nádoru oproti buňkám krve. V současnosti jediný systém schválený pro klinické použití – CellSearch – používá tři kroky pro identifikaci CNB: obohacení vzorku pomocí protilátky proti EpCAM (epithelial cell adhesion molecule; povrchový znak epiteliálních buněk), následné označení protilátkami proti cytokeratinům pro identifikaci buněk epiteliálního původu a protilátkou proti CD45 neboli PTPRC (protein tyrosine phosphatase, receptor type, C) pro identifikaci buněk hematopoetického původu (Riethdorf et al., 2007). Posledním krokem je kvantifikace buněk splňujících parametry CNB.

Kromě systému CellSearch existuje řada dalších metod izolace, identifikace a kvantifikace CNB. Například systém AdnaTest funguje na základě detekce několika epiteliálních znaků pomocí kvantitativní polymerázové řetězové reakce (qPCR) u buněk předem izolovaných protilátkou proti EpCAM (Andreopoulou et al., 2012).

Značná část výzkumu je věnována mikrofluidním zařízením, které jsou na základě různých afinitních metod schopné zachytit velké množství potenciálních nádorových buněk, které je následně možné analyzovat. Existují metodiky založené na průtokové cytometrii, ty se ale potýkají s nízkým množstvím CNB v krvi. Na počátku výzkumu CNB byla pro jejich izolaci využita i odlišná velikost oproti leukocytům, kdy leukocyty jsou obvykle mnohem menší než uvolněné epiteliální buňky. Nicméně CNB mohou nabývat poměrně široké škály rozměrů, což komplikuje zisk definované populace buněk (Park et al., 2012).

Zajímavý postup používá pro detekci CNB mikroskopická metoda zvaná HD-CTC (High-Definition Circulating Tumor Cell), která je schopna analyzovat všechny jaderné krevní buňky, čímž není limitována jako další metody prací s určitou subpopulací buněk (Marrinucci et al., 2012). Díky tomu je možné

zachytit i odlišné populace cirkulujících buněk v krvi, například cirkulující endotelové buňky (Bethel et al., 2014), a teoreticky i CNB, které ztratily svoje epiteliální charakteristiky například cestou epitelo-mesenchymální tranzice. Tato buněčná populace, pravděpodobně velmi podstatná pro vznik sekundárních nádorů, není pomocí klasických testů zachycena kvůli ztrátě povrchových epiteliálních molekul, pomocí kterých dochází k počáteční selekci buněk pro následnou charakterizaci.

Volné cirkulující nukleové kyseliny

Do tělních tekutin se kromě intaktních nádorových buněk uvolňují i nukleové kyseliny pocházející z odumřelých buněk tumoru, které je tak možné využít k diagnostice i sledování průběhu onemocnění. Nejčastěji se v této souvislosti studují volné cirkulující krátké RNA (ribonukleová kyselina) a volná cirkulující DNA v plasmě či séru. Obě tyto nukleové kyseliny je možné použít pro neinvazivní diagnostiku KRCa. U DNA je navíc možnost identifikovat mutační či metylační stav sledovaných genů.

Krátké RNA, označované jako microRNA, jsou obvykle 20 až 24 nukleotidů dlouhé, protein ne-kódující RNA s regulační funkcí (Jansson and Lund, 2012). V posledních letech se testuje využití detekce jednotlivých microRNA i komplexních panelů v séru pro diagnostiku KRCa a prognózu onemocnění. Z diagnostického pohledu dokázala rozdělit pacienty s KRCa od zdravých kontrol hladina miR-378 v plasmě (Zanutto et al., 2014) či dva komplexní panely, první s devíti microRNA (Luo et al., 2013) a druhý s použitím změření sérové hladiny šesti microRNA (Wang et al., 2014). Roli v prognóze KRCa ukázala například i miR-29c, jejíž zvýšená hladina v séru korelovala s časným relapsem onemocnění (Yang et al., 2013), nebo miR-21, u níž byla snížená hladina v séru spojena se zvýšeným rizikem lokální recidivy a zvýšenou mortalitou pacientů (Menéndez et al., 2013).

V případě DNA může být k diagnostice použito množství DNA cirkulující v krvi, kde pacienti s KRCa mají vyšší koncentraci volné DNA oproti zdravým kontrolám (Schwarzenbach et al., 2008). Cirkulující DNA lze testovat i na

přítomnost mutací typických pro KRCa, například změny v genech *KRAS* a *BRAF* (Spindler et al., 2013; Thierry et al., 2014).

Metabolomický přístup

Nádorová tkáň je typická změněným metabolismem, který vede k produkci metabolitů odlišných od tkáně zdravé. Tento rozdíl lze využít v diagnostice nádorových chorob. Metodicky se používá například plynová chromatografie kombinovaná s hmotnostní spektrometrií (Ikeda et al., 2012), kdy vstupním vzorkem může být krevní sérum, moč, exkrementy či dech. Díky tomu je metabolomický přístup velice vhodný, protože není invazivní a pro pacienta nepřináší žádná rizika (Di Lena et al., 2013).

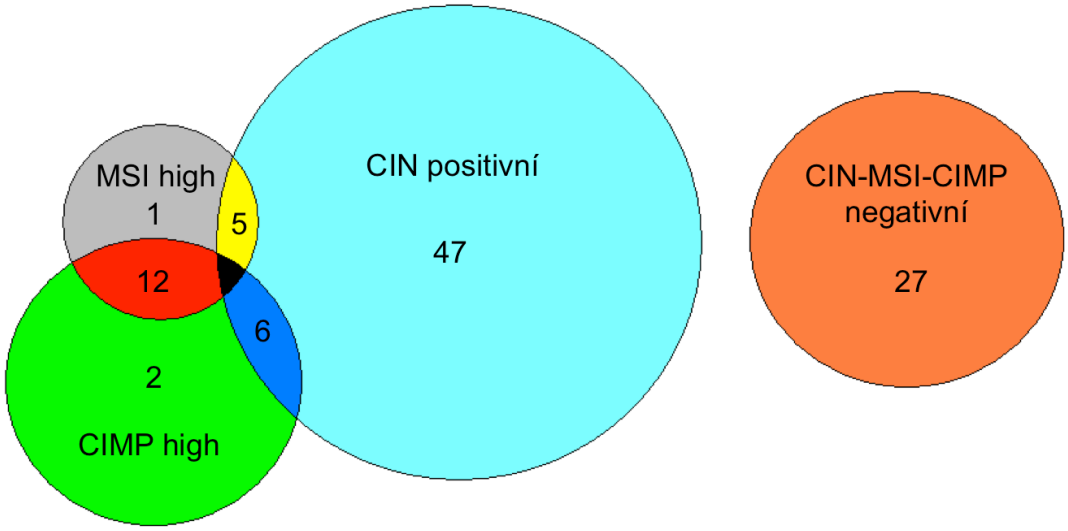
U kolorektálního karcinomu proběhly v posledních letech metabolomické studie využívající dech a sérum pacientů (Altomare et al., 2013; Nishiumi et al., 2012). Při diagnostice onemocnění ze séra bylo pomocí modelu založeného na čtyřech metabolitech dosaženo senzitivity 83,1%, specificity 81% a přesnosti 82%, navíc s výraznou schopností odlišit pacienty s nízkým stádiem KRCa od pacientů zdravých (Nishiumi et al., 2012). Studie založená na odlišných metabolitech v dechu identifikovala 15 metabolitů, jejichž použitím bylo dosaženo senzitivity 86%, specificity 83% a přesnosti 85%. U validační skupiny tento systém dosáhl přesnosti 76% (Altomare et al., 2013).

Metabolomika skýtá potenciál pro detekci nádorových onemocnění, navíc s možností diagnózy přesného typu nádoru, protože ty se mezi sebou pravděpodobně liší rozdílným profilem metabolitů. V současnosti již tento přístup proniká do i diagnostiky a sledování odpovědi na léčbu u nenádorových chorob jako laktózové intolerance či cystické fibrózy (Colombo et al., 2011; Marton et al., 2012) a větší rozšíření do nádorové biologie je jen otázkou času.

2.4 Molekulární podtypy kolorektálního karcinomu

Z molekulárního hlediska je kolorektální karcinom vysoce heterogenní onemocnění. V současnosti rozeznáváme tři hlavní podtypy kolorektálního karcinomu, které se kromě mechanizmů vývoje liší i klinickým průběhem

a odpovědí na určité typy léčby. Z genetického pohledu odlišujeme KRCa s chromozomální nestabilitou (CIN), s mikrosatelitní nestabilitou (MSI) a posledním typem jsou epigeneticky definované KRCa s vysoce metylovanými CpG ostrůvky (tzv. CpG island methylator phenotype, CIMP) (Bogaert and Prenen, 2014; Markowitz and Bertagnolli, 2009). Procentuální poměry mezi jednotlivými podtypy jsou znázorněny na obrázku 4.



Obrázek 4 - Procentuální zastoupení jednotlivých genetických a epigenetických subtypů KRCa. Chromosomální nestabilitu (CIN positivní) vykazuje 58% KRCa, mikrosatelitní nestabilitu (MSI high) 18% případů KRCa, metylaci CpG (CIMP high) ostrůvků 20% KRCa a negativitu ve všech třech znacích 27% KRCa. Převzato z (Pitule et al., 2011).

KRCa s chromosomální nestabilitou

Chromosomální nestabilita stojí za vývojem největší části sporadických KRCa, u 80-85% případů je možné detekovat přestavby chromozomů na různé úrovni od malých změn na úrovni jednobázových mutací přes změny v počtu chromosomů (aneuploidie) a přestavby chromosomů až ke genové amplifikaci (Grady and Carethers, 2008). S chromosomální nestabilitou jsou spojeny i ztráty heterozygosity v určitých oblastech genomu, což je velice efektivní

mechanizmus fyzického odstranění oblastí často spojených s tumor supresorovými geny.

Byly popsány různé molekulární mechanizmy, které mohou vést k CIN, například špatná funkce mitotického kontrolního bodu vedoucí k poruchám segregace chromozomů (Pino and Chung, 2010; Wang, 2004), centrosomální abnormality (Ganem et al., 2009; Lassmann et al., 2009) či nestability telomer a rozdílná aktivita telomerázy (Bertorelle et al., 2014).

Společně s významnými karyotypovými změnami nalézáme u CIN typů KRCa typické mutace, ale v současnosti není zcela jasné, zda hromadění těchto mutací je výsledkem CIN či naopak (Pino and Chung, 2010). Jednotlivé mutace velmi často zasahují dráhy podstatné pro vývoj kolorektálního karcinomu. Nejčastěji mutovanými nádorovými supresory jsou *APC* a *TP53* a geny na dlouhém raménku chromozomu 18 - *SMAD2* (SMAD family member 2), *SMAD4* (SMAD family member 4) a *DCC*, které jsou často postiženy i zmíněnou ztrátou heterozygosity, která se v této oblasti vyskytuje u více než 70% případů KRCa (Fearon and Vogelstein, 1990). Mezi mutované proto-onkogeny patří gen *CTNNB1* (catenin (cadherin-associated protein), beta 1, 88kDa) kódující β-katenin, který hraje podstatnou roli při vývoji KRCa a buněčné proliferaci (White et al., 2012), a geny *KRAS* a *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha), které hrají roli v přežívání a rovněž potencují proliferaci buněk (Samuels and Waldman, 2010). Komplex typických mutací na pozadí chromozomální nestability se nazývá chromosomálně nestabilní dráha vývoje KRCa (Tejpar and Van Cutsem, 2002).

Z hlediska klinického pohledu jsou CIN pozitivní KRCa spojeny s horší prognózou než v případě KRCa s mikrosatelitní nestabilitou (Popat et al., 2005; Walther et al., 2008). Popis drah vedoucích k vývoji CIN je podstatný z hlediska možnosti jejich zablokování, což by mohlo hrát roli při léčbě pacientů s tímto typem KRCa. V současnosti se testuje interference s některými proteiny s rolí v jednotlivých drahách vývoje CIN pomocí nízkomolekulárních inhibitorů, například zablokování Aurora kinázy či proteinu KIF11 (kinesin family member 11), mitotického kinesinu spojeného se segregací chromozomů (Nakai et al.,

2009; Palani et al., 2013) (data o klinických studiích jsou k dispozici na serveru www.clinicaltrials.gov).

KRCa s mikrosatelitní nestabilitou

Mikrosatelitní nestabilita stojí za vznikem a vývojem 15% případů sporadického kolorektálního karcinomu. Mikrosatelity jsou opakující se 1 až 6 bázové sekvence, také označované jako krátké tandemové repetice. Jednotlivé alely genů s mikrosatelitními oblastmi se od sebe mohou lišit počtem opakování dané krátké oligonukleotidové sekvence. U sporadického kolorektálního karcinomu je vznik MSI spojen s inaktivací DNA opravných mechanismů mutací nebo snížení exprese klíčových genů pomocí hypermetylace jejich promotorů (Söreide et al., 2006). Nejčastěji změněnými geny jsou *MLH1*, *PMS2* (PMS2 postmeiotic segregation increased 2), *MSH2* a *MSH6* (mutS homolog 6) (Yim, 2012). Zárodečné mutace v těchto genech jsou spojeny s dědičným Lynchovým syndromem. Nejběžnějším způsobem vzniku MSI pozitivních sporadických KRCa je mutace *MLH1* nebo *MSH2* a epigenetická inaktivace *MLH1* promotoru (Wheeler et al., 1999), které jsou dohromady zodpovědné za více než 90% MSI pozitivních sporadických KRCa (Gryfe et al., 2000). Výsledkem těchto změn je hromadění somatických mutací v mikrosatelitních oblastech ovlivňujících především jejich délku. Pokud se repetice vyskytnou v kódujících oblastech genů, dochází k posunu čtecího rámce a tvorbě zkrácených či nefunkčních proteinů. Tímto mechanismem jsou u kolorektálního karcinomu inaktivovány například geny *PTEN* (phosphatase and tensin homolog), *BAX* (BCL2-associated X protein) či *TGFβRII* (transforming growth factor, beta receptor II) (Iacopetta et al., 2010).

Zatímco určení hlavních typů rozvinuté chromozomální nestability je metodicky spolehlivé, u MSI záleží na stanovovaném panelu znaků. Původní panel obsahoval 5 sledovaných oblastí pokrývající dvě jednonukleotidové a tři dvounukleotidové repetice (Boland et al., 1998), nicméně specificita panelu nebyla dostatečná a v řadě případů docházelo ke špatné klasifikaci jednotlivých nádorů (Murphy et al., 2006). V posledních letech se prosazují nové kombinace

znaků v panelu, především analýza pěti oblastí s jednonukleotidovými repeticemi (Suraweera et al., 2002).

Pro klinickou praxi je stanovení MSI podstatné z prognostického pohledu, jelikož řada prací ukázala na lepší přežívání pacientů s MSI pozitivními KRCa ve stadiu II a III oproti pacientům s CIN pozitivními či mikrosatelitně stabilními nádory (Popat et al., 2005). Prognostický efekt mikrosatelitní stability může být dále potencován kombinací s určitými mutacemi, například MSI negativita spojená s mutací v *BRAF* se ukazuje jako vysoce negativní prognostický znak pro délku přežívání pacientů (Pai et al., 2012). KRCa s mikrosatelitní nestabilitou vznikají převážně v proximální oblasti tlustého střeva a vyznačují se nižší diferenciací a vysokým počtem tumor infiltrujících lymfocytů (Boland and Goel, 2010). Mikrosatelitní status je možné využít i jako prediktivní znak pro léčbu založenou na 5-FU (5-fluorouracil), jelikož část působení 5-FU vyžaduje funkční mechanizmus opravy DNA, který je u pacientů s MSI pozitivními nádory narušen. Některé studie ukazují nepřítomnost benefitu při adjuvantní léčbě 5-FU u MSI pozitivních pacientů či dokonce zhoršení stavu pacientů (Sargent et al., 2010), zatímco jiné tento závěr nepotvrdily (Hemminki et al., 2000; Hutchins et al., 2011).

Kolorektální karcinom s aberantní metylací CpG ostrůvků

Třetí hlavní podtyp KRCa je spojen s aberantní metylací CpG ostrůvků v DNA (Issa, 2004; Toyota et al., 1999), což jsou úseky bohaté na cytosin a guanin lokalizované v oblastech promotorů a prvního exonu u zhruba 70% lidských genů (Saxonov et al., 2006). V základním stavu jsou tyto oblasti většinou bez metylových skupin, aby mohla probíhat transkripce jimi kódovaných genů. Opačná situace je u CpG ostrůvků mimo promotorové oblasti, které jsou většinou opatřeny metylovou skupinou. Ve fyziologické situaci jsou metylovány například promotorové CpG oblasti u imprintovaných genů či u genů na inaktivovaném X chromosomu (Cotton et al., 2011; Reik and Lewis, 2005). Během vývoje kolorektálního karcinomu dojde ke změně promotorové metylace u více než 5% genů (Schuebel et al., 2007), což je větší množství, než kolik je jich v tomto onemocnění ovlivněno mutacemi (Wood et al., 2007).

Určení CIMP statutu u kolorektálního karcinomu komplikuje neexistence standardizovaných panelů pro určení hladiny metylace. V současnosti se používají dva panely založené na pěti studovaných oblastech (Chan et al., 2002; Weisenberger et al., 2006) a jeden zkoumající osm oblastí (Ogino and Goel, 2008), přičemž data získaná jednotlivými panely nelze mezi sebou jednoznačně srovnávat z důvodu odlišné senzitivity i specificity. Jednotlivé testy i odlišně interpretují získaná data. KRCa je tak možné dělit na dvě skupiny – CIMP pozitivní a CIMP negativní (Weisenberger et al., 2006); na tři skupiny – CIMP-high, CIMP-low a CIMP negativní (Shen et al., 2007); respektive skupiny čtyři – CIMP high, CIMP-low a dvě skupiny CIMP negativní dle mutačního stavu genu *TP53* (Hinoue et al., 2012).

CIMP je ve vývoji KRCa často spojen s mikrosatelitní nestabilitou z důvodu metylace promotorové oblasti u *MLH1*. Z tohoto pohledu je pak možné dle CIMP a MSI stavu rozdělit KRCa na čtyři až šest skupin s odlišným klinickým chováním. Například CIMP-high/MSI-pozitivní nádory jsou typické lokalizací v proximální oblasti střeva, častějším výskytem u žen, vyšším věkem pacientů a lepší prognózou. Naopak velice negativní prognózu mají pacienti s KRCa se znaky CIMP-high/MSI-negativní v kombinaci s mutací genu *BRAF*.

Nové způsoby typizace kolorektálního karcinomu

S rozvojem metodik schopných analyzovat tisíce znaků v jednom běhu, jako jsou například různé typy mikročipů pro analýzu genové exprese, se otevírá nový způsob klasifikace KRCa. Tři podstatné práce z minulých let rozdělily KRCa do tří, čtyř a šesti podtypů (Perez-Villamil et al., 2012; Sadanandam et al., 2013; De Sousa E Melo et al., 2013).

V první studii byly identifikovány tři typy KRCa dle expresních profilů, z nichž se dva výrazně překrývaly s klasickými CIN a MSI subtypy, poslední typ pak vykazoval vysokou stabilitu mikrosatelitů, vyšší metylaci CpG ostrůvků, a dle svého profilu byl příbuzný s přisedlým pilovitým adenomem. Tento podtyp byl spojen s negativní prognózou a častou rezistencí k léčbě protilátkami proti EGFR (receptor epidermálního růstového faktoru) a jeho identifikace je tedy z klinického pohledu velmi podstatná (De Sousa E Melo et al., 2013).

Přidanou hodnotou studie rozdělující KRCa do šesti skupin bylo spojení těchto podtypů s typem léčby, který by mohl být pro dané nádory nejvhodnější. Tři podtypy byly spojeny s výrazně prodlouženým bezpříznakovým přežitím (DFI) po chirurgickém zákroku, a tito pacienti by tedy v případě lokalizovaného nádoru mohli být ušetřeni následné zatěžující chemoterapie či biologické léčby. Dva podtypy s negativní prognózou byly spojeny s dobrou odpovědí na adjuvantní léčbu režimem FOLFIRI, a u posledního podtypu, rezistentního na léčbu anti-EGFR protilátkami, byla odhalena potenciální možnost a účinnost léčby inhibitory kinázy MET (MET proto-oncogene, receptor tyrosine kinase) (Sadanandam et al., 2013).

Na základě charakteristických expresních profilů se podařilo KRCa rozdělit i do čtyř odlišných podtypů, které se nacházely napříč nádory od stádia I do stádia IV. Autoři použili dělení na nádory s nízkým a vysokým procentem stromální komponenty, s imunoglobulinem spojené nádory a mucinózní nádory. Rozdílné expresní profily byly vztaženy k odlišným drahám aktivujícím karcinogenezi a mohou pomoci k indikaci odlišné léčby u jednotlivých nádorových podtypů (Perez-Villamil et al., 2012).

2.5 Léčba kolorektálního karcinomu

Terapie kolorektálního karcinomu sestává z kombinace chirurgické léčby, radioterapie, chemoterapie a cílené biologické léčby. Jedinou metodou zaručující kompletní eliminaci nádoru je radikální chirurgický zákrok, který je možný především v případě lokalizovaného KRCa. Právě častý přechod lokalizovaného KRCa do metastatické nemoci představuje pro pacienty výrazné riziko – synchronní či metachronní metastáza se vyskytuje až u 50% z nich (Kindler and Shulman, 2001). Výskyt metastatické nemoci znamená výrazné zkrácení pětiletého přežití. I přes značné pokroky související se zavedením nových typů léčby je dlouhodobé přežívání těchto pacientů stále velmi nízké (Manfredi et al., 2006).

Základním postupem při léčbě lokalizovaného KRCa je chirurgické odstranění nádoru, ať už v podobě radikální operace, při které dojde k odstranění kompletní nádorové tkáně, či v podobě paliativního zákroku, který může obnovit

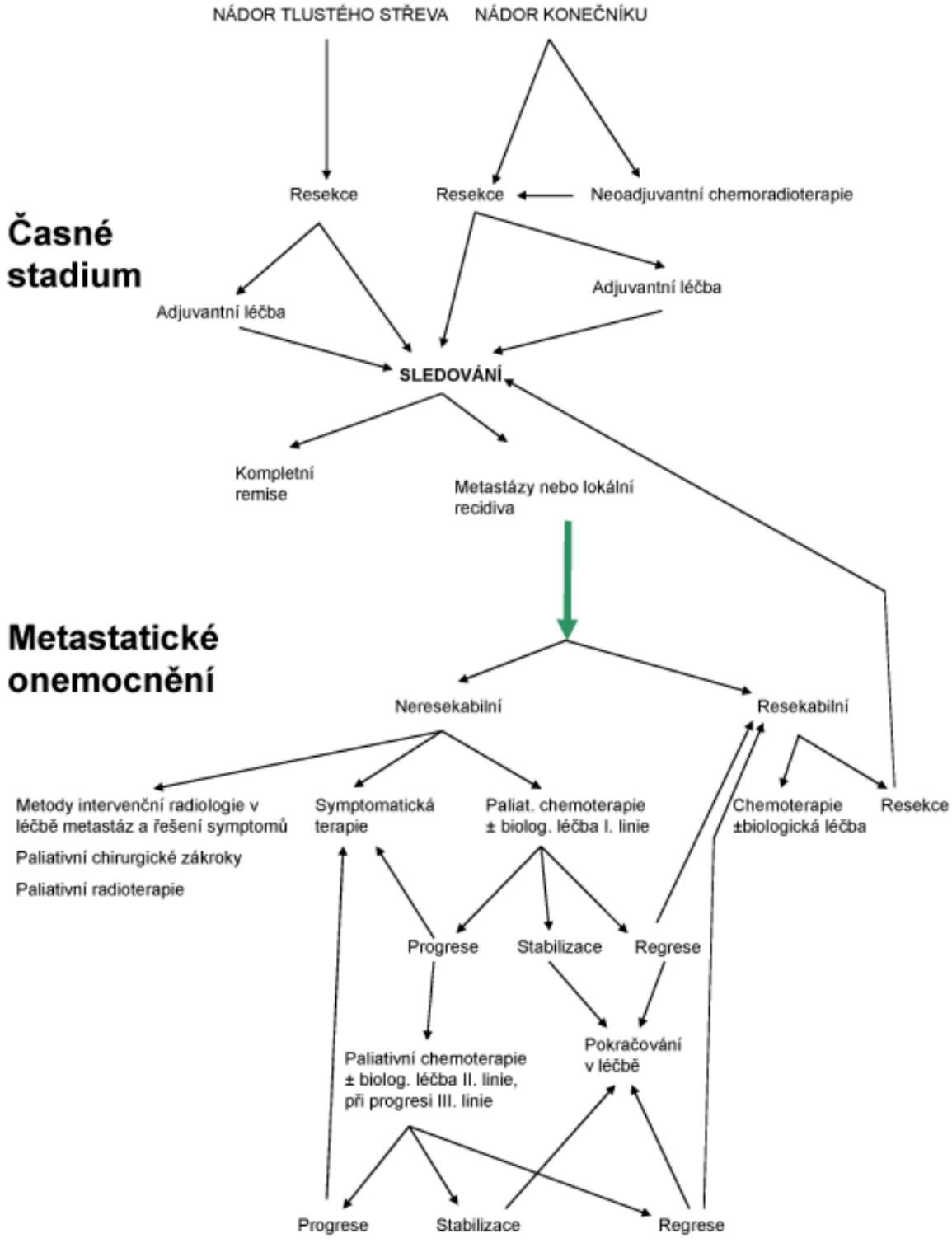
průchodnost střeva či sloužit jako příprava pro další typy léčby (Halámková et al., 2013). Kromě nejběžnější otevřené operace jsou v posledních letech častěji zaváděny i laparoskopické operační postupy, které kladou menší nároky na pacienty a jejichž úspěšnost a procento komplikací či relapsů onemocnění je v současnosti srovnatelné s klasickým přístupem (Biondi et al., 2013). Operační zákrok je možné doplnit celou řadou léčebných schémat založených na radioterapii, chemoterapii i cílené biologické léčbě. Základní léčebné schéma lokalizovaného i metastazujícího KRCa (mKRCa) je shrnuto na obrázku 5.

2.5.1 Radioterapeutická léčba

Radioterapie se indikuje u karcinomu rekta, zatímco u karcinomu tlustého střeva se v současnosti nevyužívá. U nádoru rekta je používána nejčastěji jako předoperační radioterapie či v kombinaci s chemoterapeutickými režimy jako neoadjuvantní chemoradioterapie. Hlavním cílem je zmenšení nádoru rekta a následné zvýšení operability, spojené často se snahou nepoškodit anální svěrače a poskytnout tak pacientovi vyšší kvalitu života (Damin and Lazzaron, 2014).

V současné době je aktivní celá řada klinických studií, které stanovují účinnost jednotlivých chemoradioterapeutických postupů, které mohou nejen usnadnit následnou operaci, ale v některých případech mohou vést až k makroskopické remisi nádoru (Glynne-Jones et al., 2013).

V řadě případů má předoperační radioterapie minimální vliv na prodloužení celkového přežití (OS), ale výrazně zvyšuje kvalitu života pacientů zmenšením rizika lokální recidivy a nutnosti opakovat operační zákrok (Glimelius, 2013; Rahbari et al., 2013). Recentní studie ukázala, že pacienti, kteří podstoupili neo-adjuvantní nebo adjuvantní radioterapii, dosahují vyššího procenta desetiletého přežití oproti pacientům bez radioterapie (Peng et al., 2014).



Obrázek 5 - Základní schéma rozhodování o léčbě u pacientů s KRCa. Převzato z http://www.kolorektum.cz/index.php?pg=pro-odborniky--diagnostika-a-lecba-kolorektalniho-karcinomu.

2.5.2 Onkologická léčba

Chemoterapie je nejčastějším způsobem léčby pacientů s KRCa hned po chirurgické intervenci. Mezi základní chemoterapeutika patří 5-fluorouracil, oxaliplatina a irinotecan. Chemoterapii lze použít jako adjuvantní (zajišťovací) chemoterapii po kompletním chirurgickém odstranění nádoru (obrázek 6) a paliativní chemoterapii, která je použita u pacientů s neresekabilními primárními či sekundárními nádory, či u pacientů, u nichž nebylo možné provést radikální operační zákrok (obrázek 7 a 8). Při použití jako neoadjuvantní chemoterapie je jejím cílem zmenšení nádorové hmoty do stavu, ve kterém by bylo možné tumor odstranit chirurgickým zákrokem (nejčastěji používána u primárně inoperabilních jaterních metastáz nebo s cílem zmenšení velikosti či snížení stagingu nádoru) (Ćwiertka, 2008; Leonard et al., 2005).

Nejstarším používaným chemoterapeutikem je pyrimidinový analog 5-fluorouracil. 5-FU patří mezi antimetabolity a jeho hlavním mechanismem působení je inhibice thymidylát syntázy, enzymu nezbytného pro syntézu thymidinu (Wilson et al., 2014). Efekt mají i metabolity 5-FU - fluorodeoxyuridin trifosfát se může přímo inkorporovat do DNA, kde blokuje její reparaci, a fluorouridin trifosfát do syntetizované RNA, čímž blokuje její další zpracování (Longley et al., 2003). 5-FU je často používán v kombinaci s leukovorinem, derivátem kyseliny listové, který potencuje účinnost léčby 5-FU (Advanced Colorectal Cancer Meta-Analysis Project, 1992). Na 5-FU je založen i lék kapecitabin. Tento prekurzor 5-FU má výhodu v možnosti orálního podání, vyšší odpovědi na léčbu a nižšímu procentu komplikací oproti infuznímu podání u 5-FU (Twelves, 2002).

5-FU i kapecitabin se úspěšně používají s chemoterapeutiky nové generace v různých kombinačních režimech (základní režimy jsou shrnuty v Tabulce 9).

Oxaliplatina je derivát platiny, který se při léčbě KRCa používá pouze v kombinaci s dalšími chemoterapeutiky, nejčastěji 5-FU. Spolu s karboplatinou a cisplatinou patří do rodiny léků založených na platině, i když mechanismus působení jednotlivých medikamentů se liší (Rixe et al., 1996). U kolorektálního karcinomu dosahuje oxaliplatina vyšší efektivity, nižší toxicity a menší úrovně

rezistence než cisplatina (Virag et al., 2012). Přesto je dlouhodobější použití oxaliplatiny spojeno s chronickou neuropatií i se vznikem rezistence (Misset, 1998; Seetharam et al., 2009). Mechanizmem fungování oxaliplatiny je tvorba adduktů platiny s DNA, které interferují s replikací a transkripcí a vedou k aktivaci DNA opravných mechanizmů či spuštění apoptózy. Oxaliplatina je kromě toho schopna aktivovat expresi řady buněčných genů, včetně těch s rolí v drahách podstatných pro vývoj nádoru jako *FOS* (FBJ murine osteosarcoma viral oncogene homolog) a *NOTCH1* (notch 1), ale její celkové působení na nádorové buňky stále není dostatečně podrobně popsáno (Alian et al., 2012).

Irinotecan je dalším z běžně používaných chemoterapeutik pro léčbu kolorektálního karcinomu. Používá se většinou v kombinaci s dalšími látkami ve všech liniích léčby KRCa. Mechanizmus jeho působení je založen na inhibici topoizomerázy I aktivním metabolitem irinotecanu SN-38 (Pommier et al., 2010). Topoizomeráza I je enzym zodpovědný za rozbalení DNA během její replikace či transkripce a jeho zablokování efektivně zastaví oba tyto procesy (Pommier, 2013). Nevýhodou irinotecanu je jeho poměrně vysoká toxicita, která se projevuje především průjmem a neutropenií (Paulík et al., 2012).

Tabulka 9 - Kombinační léčebné režimy u KRCa. Vypsána je pouze kombinace léčiv, nikoliv dávkování. Zdroj - Modrá kniha České onkologické společnosti, 18. vydání.

režim	kombinace léčiv
FU/FA (DeGramont)	5-fluorouracil, leukovorin
FU/FA (Mayo)	
Machover	
AIO	
FOLFOX	oxaliplatina, leukovorin, 5-fluorouracil
FLOX	
FOLFIRI	irinotekan, leukovorin, 5-fluorouracil
XELIRI	kapecitabin, irinotecan
XELOX	kapecitabin, oxaliplatina
FOLFOXIRI	oxaliplatina, irinotecan, leukovorin, 5-fluorouracil

2.5.3 Biologická léčba

Nejnovější látky v terapii především metastatického KRCa jsou monoklonální protilátky (moAb) používané pro cílenou biologickou léčbu. Jako první byl pro použití schválen bevacizumab, protilátka proti vaskulárnímu-endoteliálnímu růstovému faktoru A (VEGF-A), následovaly protilátky proti EGFR cetuximab a panitumumab. Zařazení cílených léčiv do léčebných režimů může stát za prodloužením OS a DFI pacientů s metastatickým KRCa během posledních let (Messori et al., 2014).

Bevacizumab je humanizovaná monoklonální protilátka, která je schopná vazbou na VEGF-A blokovat angiogenezi, která je nezbytná pro růst nádoru. Díky tvorbě nových cév v nádoru může nádor dále růst bez rizika přílišné nekrózy v centru tumoru. Blokování pro-angiogenních signálů je tedy cestou ke zmenšení objemu nádorové tkáně (Kubota, 2012). Přínos bevacizumabu byl nejprve pozorován při přidání k první linii léčby chemoterapeutickými kombinacemi 5-FU s leukovorinem a ironotecanu s 5-FU a leukovorinem, kde přinesl prodloužení přežití i času do progrese a zvýšení odpovědi na léčbu (Marshall, 2005). Následně byl jeho pozitivní efekt prokázán i pro další režimy včetně použití v dalších liniích léčby (Pavlidis and Pavlidis, 2013). Přes pozitivní efekt u paliativní léčby nebyl pozorován žádný přínos přidání bevacizumabu do adjuvantní léčby pacientů po operaci pokročilého kolorektálního karcinomu bez metastatické nemoci (Van Cutsem et al., 2011).

Cetuximab je chimerická protilátka (kombinace myší a lidské části) proti EGFR. Mechanizmus působení je blokace signalizace přes EGFR, která při normálním průběhu aktivuje dělení nádorových buněk a přispívá k jejich přežívání (Ciardiello and Tortora, 2001). Cetuximab byl nejprve testován v monoterapii i jako součást kombinační léčby s irinotecanem jako další linie léčby u irinotecan – rezistentních pacientů a prokázal určitý benefit především v podobě zvýšené odpovědi na léčbu (Cunningham et al., 2004). V současnosti je při správné selekci pacientů možné použít cetuximab v kombinaci s řadou chemoterapeutik, kde pomáhá zvýšit jejich účinnost či obejít mechanizmy resistence (Gerber and Choy, 2010), či za určitých podmínek i jako monoterapie prodlužující přežití pacientů (Mekata et al., 2013). Cetuximab je rovněž možné

použít v neo-adjuvantním režimu kvůli jeho efektu na zmenšení nádorů a zvýšení jejich resekability u nádoru rekta či u metastatického KRCa (Folprecht et al., 2010; Gomez et al., 2013).

Panitumumab je plně humanizovaná protilátka proti EGFR. Výhodou oproti cetuximabu je menší množství komplikací při jeho administraci a možnost aplikovat panitumumab bez nutnosti premedikace (Saif and Cohenuram, 2006). Stejně jako v případě cetuximabu je důležitá správná selekce pacientů, kteří mohou profitovat z přidání anti-EGFR protilátek do léčebného schématu. Panitumumab se při dodržení těchto pravidel při použití v první linii léčby ukázal jako látka prodlužující čas do progrese onemocnění i odpověď na léčbu ve studii PRIME (the Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine the Efficacy) (Douillard et al., 2010). Panitumumab přinesl benefit i při použití ve druhé a třetí linii léčby (Hocking and Price, 2014). Přidání panitumumabu do kombinace s chemoterapií a bevacizumabem naopak znamenala zhoršení stavu pacientů a pro léčbu se nedoporučuje (Hecht et al., 2008).

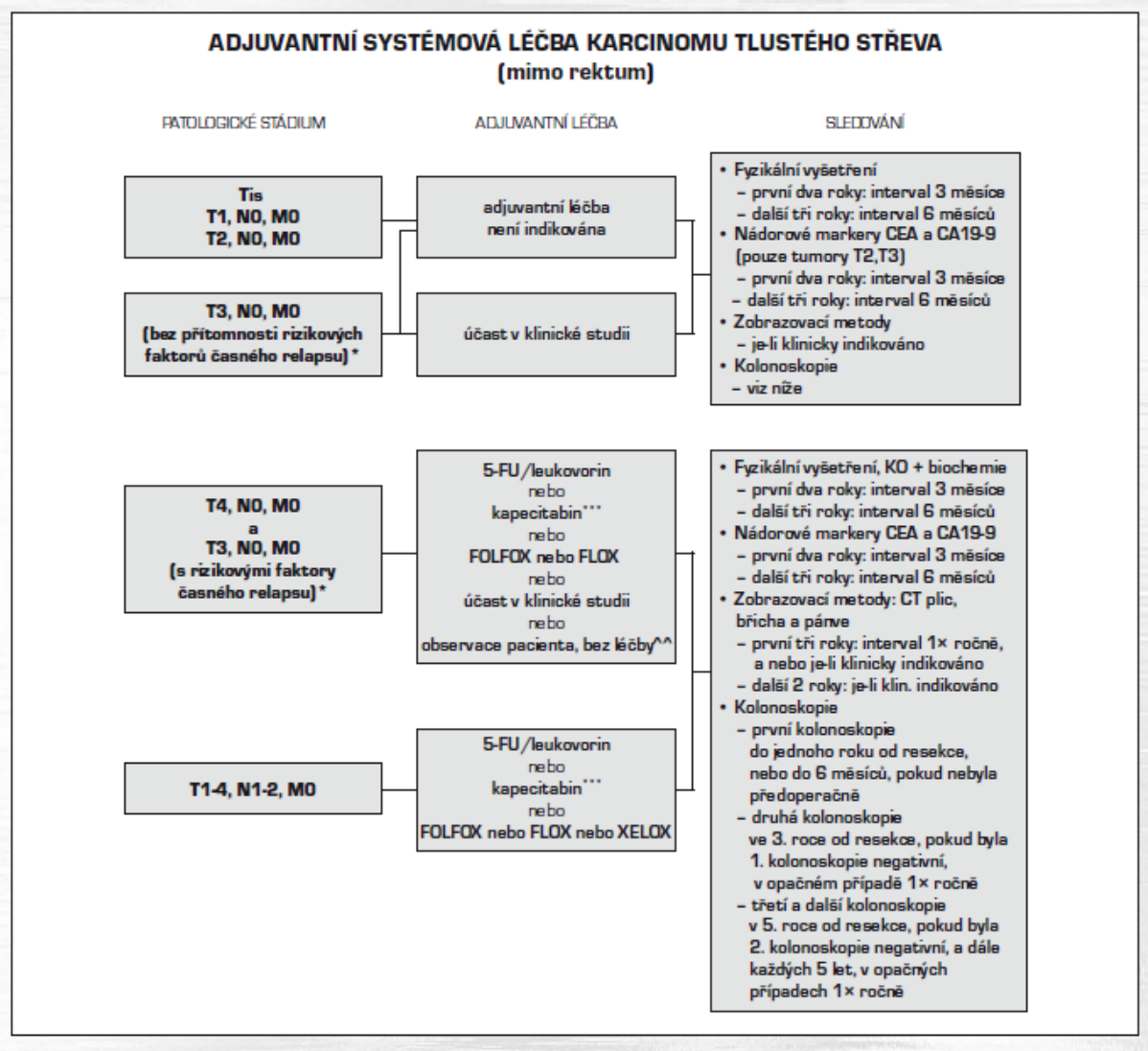
2.5.4 Další používané a perspektivní látky pro léčbu KRCa

Kromě zmíněných látek jsou v současnosti v různých fázích použití i klinického výzkumu další látky využitelné pro léčbu KRCa. Výzkum jde především směrem k cílené léčbě. Mezi anti-angiogenní látky schválené pro léčbu patří například zif-aflibercept a regorafenib. Zif-aflibercept je rekombinantní protein s vysokou afinitou k VEGF-A, VEGF-B (vaskulární-endoteliální růstový faktor B) a placentálnímu růstovému faktoru (PlGF). Vazbou na tyto proteiny zabrání jejich navázání na buněčné receptory a tím efektivně zablokuje signalizaci (Wang and Lockhart, 2012). Zif-aflibercept je možné použít v kombinaci s režimem FOLFIRI v druhé linii léčby KRCa, kde zlepšuje celkové přežití i čas do progrese onemocnění (Van Cutsem et al., 2012).

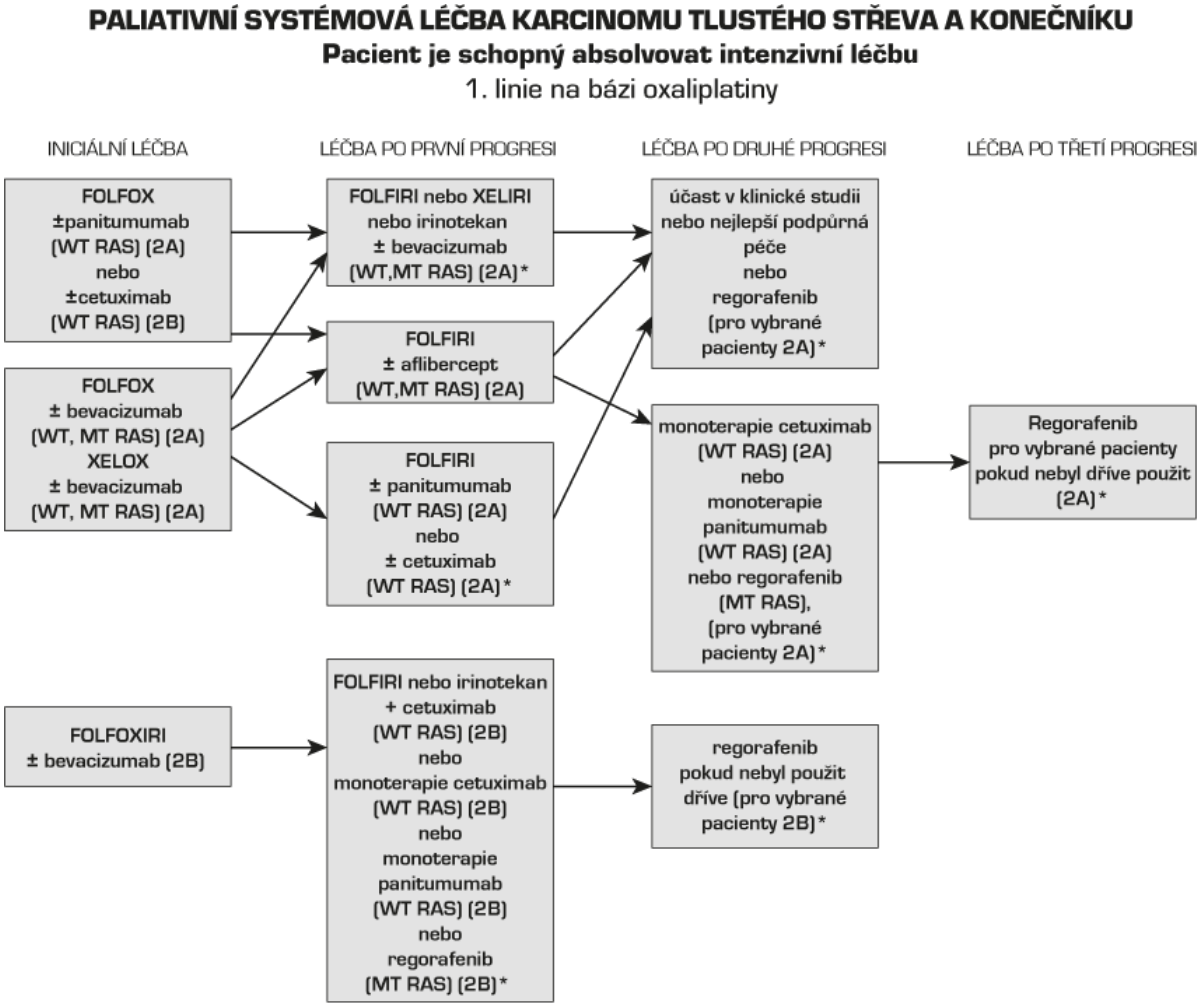
Regorafenib je multimodální kinázový inhibitor, který blokuje angiogenezi skrze inhibici VEGFR (kinase insert domain receptor (a type III receptor tyrosine kinase)) a TIE-2 (TEK tyrosine kinase, endothelial), ovlivňuje nádorové stroma blokováním PDGFR-β (platelet-derived growth factor receptor, beta

polypeptide) a FGFR (fibroblast growth factor receptor) a intracelulární pro-onkogenní dráhy blokováním funkce BRAF (Khan et al., 2014). Použití regorafenibu přináší benefit v podobě delšího celkového přežití u pacientů s metastastickým KRCa, u nichž selhaly všechny dostupné režimy léčby (Grothey et al., 2013).

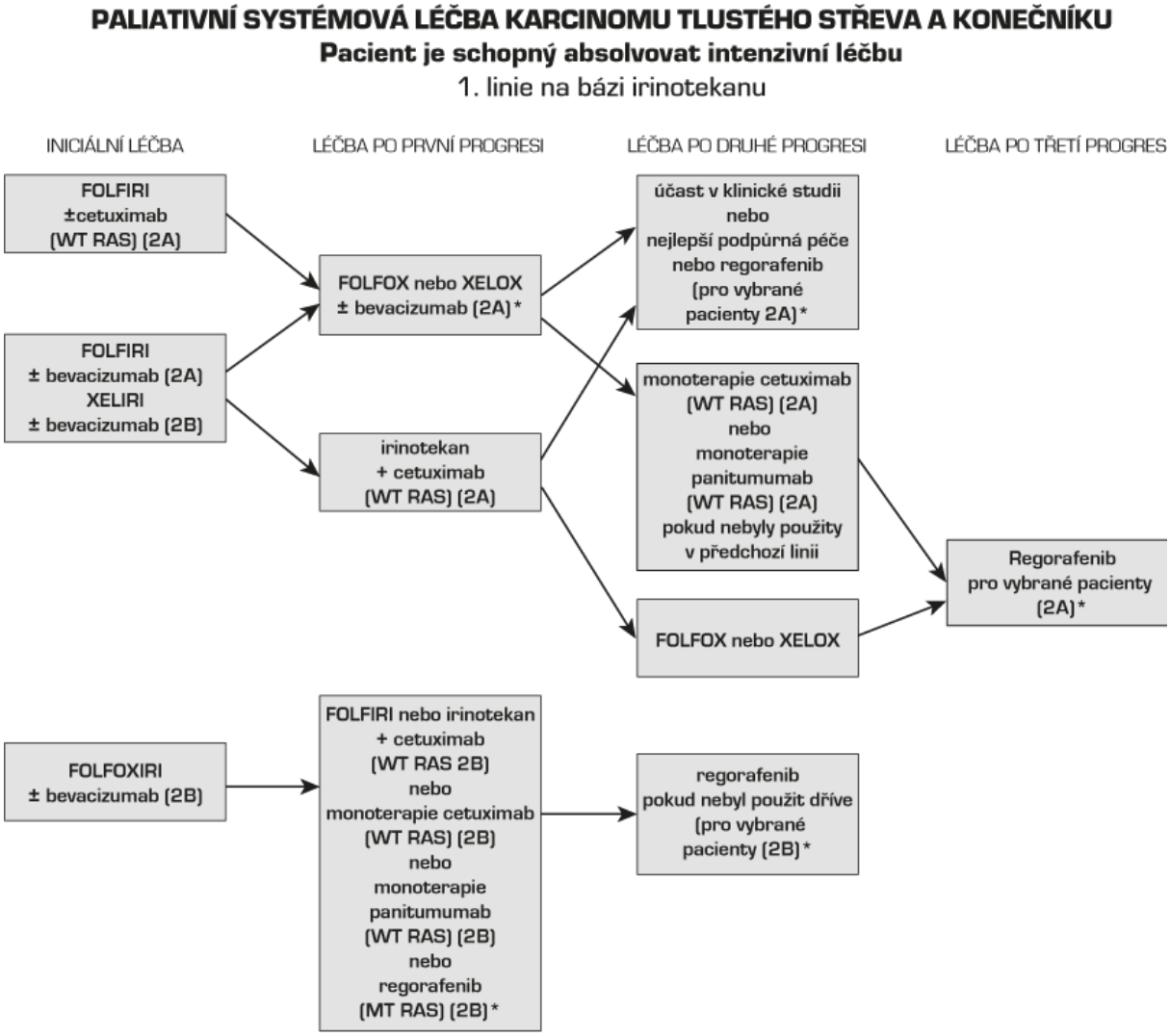
Přestože blokace EGFR protilátkami přináší prospěch pro část pacientů, podobný efekt nebyl pozorován u malých tyrosin kinázových inhibitorů (TKI), jako jsou erlotinib a gefitinib. Obě tyto látky byly zkoumány u metastatického KRCa v mono i kombinační terapii bez pozorovatelného efektu (Mahipal et al., 2014). Poslední studie ale ukazují možnost využití těchto inhibitorů v nových režimech, například jako kombinace erlotinibu s bevacizumabem v podobě udržovací léčby (Muñoz et al., 2014).



Obrázek 6 - Možnosti adjuvantní léčby nádoru tlustého střeva v závislosti na diagnóze. Zdroj Modrá kniha pro Zhoubný novotvar kolorekta - http://www.linkos.cz/informace-pro-praxi/modra-kniha/5-zhoubny-novotvar-kolorekta-c18-20/.



Obrázek 7 - Možnosti paliativní léčby s první linií založenou na oxaliplatině. Zdroj Modrá kniha pro Zhoubný novotvar kolorekta - http://www.linkos.cz/informace-pro-praxi/modra-kniha/5-zhoubny-novotvar-kolorekta-c18-20/.



Obrázek 8 - Možnosti paliativní léčby s první linií založenou na irinotecanu. Zdroj Modrá kniha pro Zhoubný novotvar kolorekta - http://www.linkos.cz/informace-pro-praxi/modra-kniha/5-zhoubny-novotvar-kolorekta-c18-20/.

2.6 Prognostické a prediktivní znaky KRCa

U kolorektálního karcinomu podobně jako u dalších malignit a nemocí obecně nalézáme znaky, které nás mohou informovat o průběhu nemoci či efektivitě použité léčby. První kategorii nazýváme prognostické znaky – ty napovídají o dalším průběhu choroby, její agresivitě a o rizicích pro pacienta. Prediktivní znaky pak dokáží předpovědět efektivitu léčby daným přípravkem. Obě kategorie jsou důležité pro klinickou praxi, jelikož prognostické znaky mohou napovědět o nutnosti léčby a prediktivní o jejím typu, což v kombinaci přináší benefit pro pacienta, který může být ušetřen organismus zatěžující a potenciálně nefunkční léčby. Některé znaky mohou nést prognostickou i prediktivní funkci zároveň. Informace k prognostickému a prediktivnímu efektu jednotlivých klasických genetických a epigenetických subtypů KRCa byly shrnuty v jednotlivých odpovídajících kapitolách, v následujících odstavcích se budeme věnovat změnám na úrovni jednotlivých genů.

2.6.1 Prediktivní a prognostické znaky pro anti-angiogenní léčbu

V současnosti neexistují žádné prognostické nebo prediktivní znaky pro anti-angiogenní léčbu, například pro použití bevacizumabu. Výsledky získané na preklinických modelech se ve studiích s pacienty nepodařilo ověřit (Luo and Xu, 2014).

2.6.2 Prediktivní znaky pro léčbu protilátkami proti receptoru epidermálního růstového faktoru

V současné době jsou v cílené léčbě KRCa zaměřené proti receptoru epidermálního růstového faktoru používané protilátky erbitux a panitumumab. Pro obě z nich lze do značné míry využít stejné prediktivní znaky.

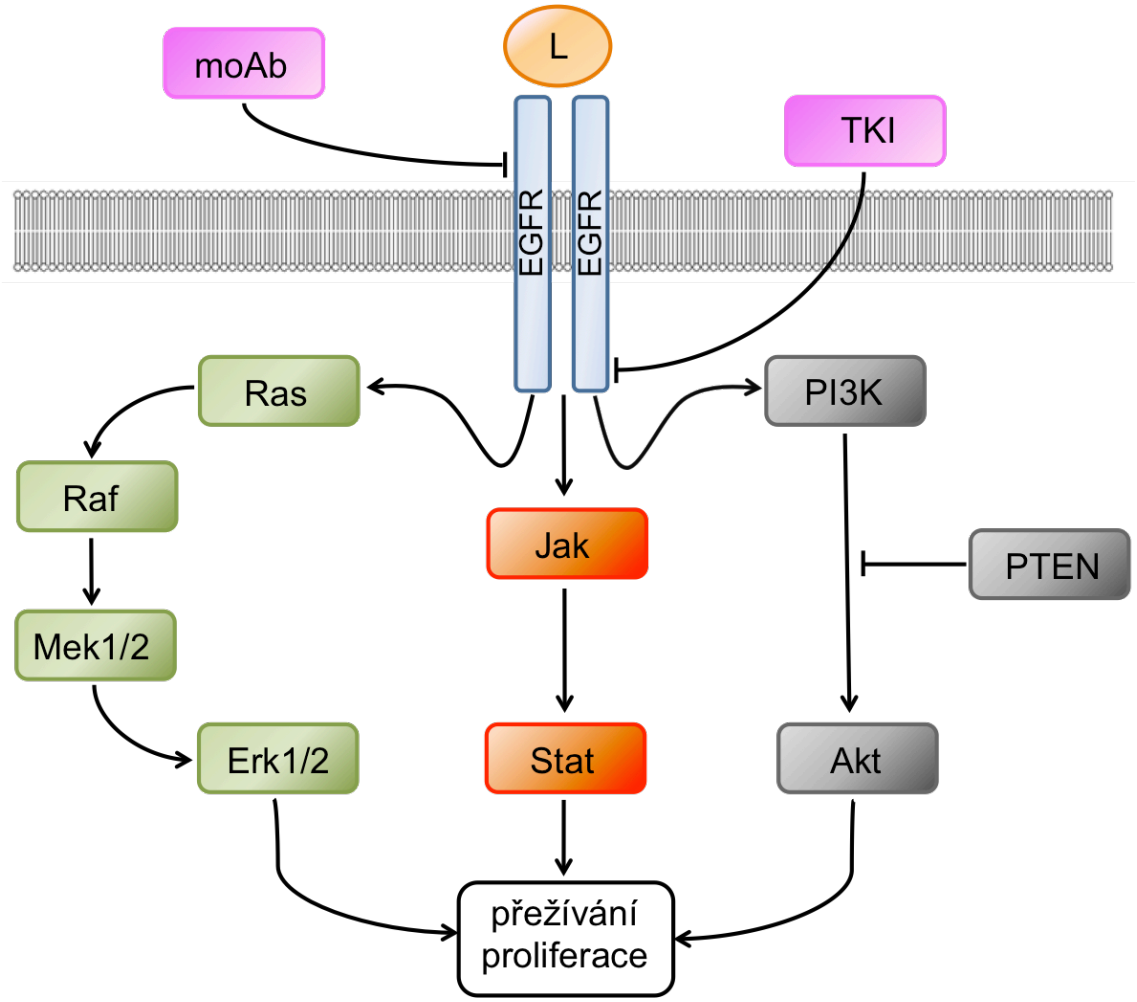
EGFR

EGFR je receptor tyrosinová kináza patřící do rodiny receptorů epidermálních růstových faktorů (Warren and Landgraf, 2006). Jedná se o transmembránový glykoprotein, který po aktivaci specifickými ligandy vytváří dimer (homodimer či

heterodimer s dalšími členy své proteinové rodiny). Dimerizace vede k aktivaci intracelulární kinázové domény, která fosforyluje další vnitrobuněčné signální proteiny, z nichž majoritní je protein KRAS. Mezi signální kaskády aktivované EGFR patří mitogen-aktivovaná protein kinázová dráha, PI3K (phosphatidyl-inositol-3-kináza) a AKT (v-akt murine thymoma viral oncogene homolog 3) dráha nebo Jak2/Stat3 (Janus kinase 2 / signal transducer and activator of transcription 3 (acute-phase response factor)) dráha, které všechny hrají významnou roli v udržení homeostázy uvnitř buňky (Lurje and Lenz, 2009; Yarden and Sliwkowski, 2001) (obrázek 9). Deregulací těchto signalizačních drah může dojít k aberantní proliferaci buněk se všemi jejími důsledky (Spano et al., 2005). Role EGFR ve vývoji KRCa se zdá být zásadní, přestože fyziologická funkce EGFR v intestinálním epitelu není příliš jasná. Podle současných informací by mohl hrát významnou úlohu při udržování populace intestinálních kmenových buněk a v obnově a ochraně střevního epitelu (Nautiyal et al., 2012; Xu et al., 2011).

Exprese EGFR na nádorových buňkách byla při zavedení protilátek proti EGFR do klinické praxe nezbytným znakem pro jejich využití v léčbě. Exprese byla stanovována imunohistochemicky na vzorcích primárních a částečně i sekundárních KRCa (Siena et al., 2009). Další výzkumy ukázaly, že exprese EGFR nekoreluje s odpovědí na léčbu anti-EGFR protilátkami (Rodríguez et al., 2010; Saltz et al., 2004) a že i pacienti s nádory negativními na EGFR mohou mít benefit z léčby těmito protilátkami (Chung et al., 2005). S nutností lépe stratifikovat pacienty, kteří by měli benefit z anti-EGFR léčby, byly hledány nové prediktivní faktory, a jako podstatné se ukázaly mutace v genu *KRAS* (Lièvre et al., 2006).

Z pohledu dalších možných změn *EGFR* samotného jsou jeho mutace u kolorektálního karcinomu vzácné a jejich přítomnost nenese prediktivní roli pro léčbu anti-EGFR protilátkami (Tsuchihashi et al., 2005). U KRCa naopak dochází s vysokou frekvencí ke zmnožení genu pro *EGFR*, které bylo spojeno s lepší objektivní odpovědí na léčbu anti-EGFR protilátkami a celkově lepší prognózou pro pacienty (Jiang et al., 2013).



Obrázek 9 - Základní dělení dráhy od receptoru pro epidermální růstový faktor. Převzato z (Pitule et al., 2013). Ukázka místa účinku léčby tyrosin kinázovými inhibitory a monoklonálními protilátkami.

KRAS

KRAS mutace se vyskytuje u 40% pacientů (Amado et al., 2008) a nejzásadnější pro KRCa jsou mutace v kodónech 12 a 13 druhého exonu. Změny v tomto exonu narušují vnitřní GTPázovou aktivitu, nedochází tak k degradaci vázaného guanosintrifosfátu na guanosindifosfát a protein zůstává permanentně v aktivovaném stavu. Role *KRAS* mutace byla zpočátku pozorována v menších retrospektivních studiích (Lièvre et al., 2008; De Roock et al., 2008), poté byla potvrzena ve velkých prospektivních studií jako CRYSTAL (Cetuximab Combined with Irinotecan in First-line Therapy for Metastatic Colorectal Cancer) (Van Cutsem et al., 2009), OPUS (Oxaliplatin

and Cetuximab in First-line Treatment of Metastatic Colorectal cancer) (Bokemeyer et al., 2009) a PRIME (Douillard et al., 2010) – dle závěrů těchto prací vyplývá, že benefit z použití monoklonálních protilátek v léčbě mKRCa mají pouze pacienti s nemutovanou verzí proteinu KRAS. Tento závěr je velice podstatný, protože umožňuje stratifikovat pacienty dle přítomnosti či nepřítomnosti mutace a nenasazovat tak u pacientů s mutovanou verzí *KRAS* léčbu, která by nepřinášela užitek. Další práce naznačily, že je podstatné určit i konkrétní typ mutace – pacienti se změnou *KRAS* v 13. kodónu (p.G13D) vykazovali částečnou odpověď na léčbu cetuximabem na rozdíl od pacientů s *KRAS* mutovaným v jiných pozicích (De Roock et al., 2010a; Tejpar et al., 2012).

Ani skupina pacientů s nemutovaným *KRAS* neodpovídá na léčbu moAb homogenně a i po vyřazení pacientů s mutovaným *KRAS* proteinem byla pozitivní reakce na léčbu pozorována jen u 20 – 40 % z nich (Vecchione et al., 2011). Studiem členů signalizačních drah spojených s EGFR byly odhaleny další molekulárně biologické změny, které jsou příčinou rezistence nádorů k anti-EGFR moAb. Nejdůležitější z těchto změn jsou mutace v genech *BRAF* a *PIK3CA*, zastavení exprese proteinu PTEN, hlavního negativního regulátoru PI3K dráhy, a rovněž mutace v genu *NRAS* (neuroblastoma RAS viral (v-ras) oncogene homolog), který patří do stejné rodiny jako gen *KRAS* (Grossmann and Samowitz, 2011). *NRAS* je mutován v méně než 3% KRCa a prvotní výsledky ukazují, že by jeho mutace mohla rovněž být jednou z příčin rezistence KRCa k použití cetuximabu (De Roock et al., 2010b).

BRAF

BRAF je jednou z kináz v takzvané mitogen-aktivované protein kinázové dráze (Chong et al., 2003). Protein kódovaný *BRAF* genem je u 10-15% KRCa postižen bodovou mutací V600E, která zapříčiní konstitutivní aktivitu jeho kinázové domény (Davies et al., 2002; Samowitz et al., 2005). Podobně jako u *KRAS* mutace je změněná varianta proteinu BRAF spojována s rezistencí nádorů na léčbu moAb proti EGFR (Loupakis et al., 2009; Di Nicolantonio et al., 2008). Analýzy velkých randomizovaných studií OPUS a CRYSTAL nepřinesly

jednoznačné závěry o prediktivní hodnotě *BRAF* mutace pro léčbu moAb, konkrétně cetuximabem (Bokemeyer et al., 2012). Ukázaly ale prognostický efekt mutace V600E, jelikož pacienti s nádory nesoucími tuto změnu měli kratší celkové přežívání než pacienti s nádory s nemutovaným *BRAF* (Bokemeyer et al., 2012; Roth et al., 2010; Yokota et al., 2011). Vzhledem k tomu, že *KRAS* a *BRAF* proteiny jsou členy jedné signalizační dráhy, stačí k aktivaci nádorového programu v buňce pouze jedna změna v dané signalizační dráze. Nádory s mutací v *KRAS* i *BRAF* zároveň se nacházejí velice vzácně (Sahin et al., 2013).

PIK3CA a PTEN

Druhou zásadní drahou vedoucí od EGFR je signální kaskáda, na jejímž začátku stojí PI3K. Signalizace touto drahou vedoucí přes její základní uzel, kinázu AKT, zajišťuje v buňce široké spektrum funkcí – regulaci glukózového metabolismu, antiapoptotické děje nebo regulaci genové exprese (Vivanco and Sawyers, 2002). Z celkového pohledu jde o dráhu regulující přežívání a metabolismus buněk. S KRCa jsou spojeny dvě změny v této signální kaskádě, a to mutace v katalytické jednotce PI3K a změna exprese negativního regulátoru dráhy, proteinu PTEN. Mutace *PIK3CA* genu, který kóduje p100α katalytickou podjednotku PI3K, se vyskytují u 15 – 25% (De Roock et al., 2010b; Samuels et al., 2005) případů KRCa a jsou rozděleny mezi exony 9 a 20 (dle studií přibližně 70% mutací v exonu 9, 30% v exonu 20). Závěry několika prací zaměřených na prediktivní hodnotu *PIK3CA* mutace ve vztahu k odpovědi na léčbu moAb proti EGFR jsou nejednoznačné (Perrone et al., 2009; Prenen et al., 2009; Sartore-Bianchi et al., 2009) a ukazuje se, že prediktivní funkce závisí na přítomnosti dalších mutací (především v genech *KRAS* a *BRAF*), a že podobně jako u genu *KRAS* i zde budou mít jednotlivé mutace odlišný biologický efekt (Mao et al., 2012; De Roock et al., 2010b).

U proteinu PTEN je problém s absencí standardizovaných metod, a proto různé studie docházejí k mírně odlišným výsledkům, přesto je pozorováno spojení mezi ztrátou exprese PTEN a rezistencí k EGFR cíleným léčivům (Colakoglu et al., 2008; Sawai et al., 2008; Sood et al., 2012). U *PI3KCA* i PTEN je nutné

ověřit skutečný efekt jejich mutací nebo exprese pro predikci odpovědi na léčbu cetuximabem v rozsáhlejší randomizované studii. Z prognostického pohledu je role *PIK3CA* mutací rovněž neznámá. Nedávná práce objevila potenciálně horší chování nádorů s mutací v obou sledovaných kodonech genu *PIK3CA* (Liao et al., 2012).

2.6.3 Prediktivní znaky pro léčbu fluoridovanými pyrimidiny

Základním prediktivním znakem pro léčbu 5-FU či kapecitabinem je thymidylát syntáza, enzym, přes jehož inhibici je zprostředkován hlavní účinek těchto léčiv. Prediktivní hodnotu má zvýšená hladina thymidylát syntázy, které je docíleno například genovou amplifikací či zmnožením zesilovacích oblastí v promotoru tohoto genu (Gibson, 2006; Marsh and McLeod, 2001; Wang et al., 2004a). Vyšší množství thymidylát syntázy má za následek nižší odpověď na léčbu 5-FU, a je tedy považováno za negativní prediktivní faktor (Kornmann et al., 2012).

Dalším ze znaků s vlivem na léčbu 5-FU je dihydropyrimidin dehydrogenáza kódovaná genem *DPYD*. Tento enzym je z 80% zodpovědný za katabolismus 5-FU. Alelické varianty genu *DPYD* mohou způsobovat zvýšené riziko toxicity 5-FU, především alela označovaná jako *DPYD*2A*, která vede k vynechání jednoho exonu a následné syntéze proteinu se sníženou aktivitou (Wei et al., 1996).

Dalšími studovanými prediktivními znaky léčby 5-FU jsou thymidin fosforyláza a metylentetrahydrofolát reduktáza (*MTHFR*), u které jsou ale výsledky studií jednotlivých polymorfismů nejednotné. *MTHFR* tak není v současnosti využitelná jako znak vhodný k predikci účinku léčby (van Huis-Tanja et al., 2013). Thymidin fosforyláza je poslední enzym v kaskádě přeměny kapecitabinu na 5-FU. Její vyšší hladina je spojena s lepší odpovědí na léčbu kapecitabinem například u metastatického nádoru prsu (Andreetta et al., 2009). U kolorektálního karcinomu je pozorovatelné podobné spojení mezi hladinou thymidin fosforylázy a účinnosti kapecitabinu (Petrioli et al., 2010).

Prediktivním znakem pro léčbu 5-FU je i stabilita mikrosatelitních oblastí DNA. V případě pozitivity na mikrosatelitní nestabilitu způsobenou chybějícími DNA

opravnými proteiny jako MLH1, nemají pacienti žádný nebo pouze minimální benefit z léčby. K účinné funkci 5-FU je totiž potřebný fungující DNA opravný systém, který pak dokáže zastavit buněčné dělení v případě detekce vysokého množství DNA poškození (Ribic et al., 2003).

2.6.4 Prediktivní znaky pro léčbu oxaliplatinou

Prediktivní znaky pro použití oxaliplatiny jsou studovány ze dvou pohledů – vzhledem k její toxicitě predikují účinnost a vedlejší účinky léčby změny v genech spojených s buněčnou detoxifikací, vzhledem k jejímu mechanismu fungování jsou pak jako predikční faktory studované především geny spojené s opravou DNA poškození.

Zástupcem první skupiny je glutathion-S-transferáza P1, jeden z hlavních detoxifikačních enzymů, kde je polymorfizmus I105V spojen se zvýšeným rizikem oxaliplatinou-indukované neuropatie (Lecomte et al., 2006).

Vztah k léčbě oxaliplatinou byl nalezen u polymorfizmů *ERCC1* a *ERCC2* (excision repair cross-complementation group 1 a 2), genů s rolí v nukleotidové excizní opravě DNA. Polymorfizmy mají rozdílný efekt v závislosti na populaci, u asijské populace se ukázala prediktivní hodnota *ERCC1* C118T, kde T alela byla spojena se sníženou odpovědí na léčbu, kratším obdobím do progrese a celkovým přežitím. U evropské populace byl stejný efekt popsán pro G alelu u *ERCC2* T751G (Yin et al., 2011).

Prediktivní hodnota byla spojena i s polymorfizmem genu *XRCC1* (X-ray repair complementing defective repair in Chinese hamster cells 1), který kóduje protein podstatný pro bázovou excizní reparaci. Změna R399Q byla popsána jako signifikantní negativní faktor pro odpověď na léčbu režimem FOLFOX (Suh et al., 2006), přestože její skutečný efekt bude pravděpodobně jako v případě *ERCC1* a *2* limitován typem studované populace (Huang et al., 2011).

Prediktivní hodnotu pro léčbu oxaliplatinou může mít i mutace v genu *KRAS*. Pokusy na buněčných liniích ukázaly změnu senzitivity na oxaliplatinu v závislosti na *KRAS* mutaci, která ovlivňuje expresní hladinu *ERCC1* (Lin et al., 2012).

2.6.5 Prediktivní znaky pro léčbu irinotecanem

Podobně jako u fluorouracilu i v případě irinotecanu jsou hlavními prediktivními faktory polymorfizmy v genech spojených s metabolismem tohoto léčiva. Hlavní vliv na predikci mají alely uridin difosfát glukuronosyl transferázy 1A1 (*UGT1A1*). Alela *UGT1A1*28* je spojena se zvýšeným rizikem neutropenie a její stanovení je doporučeno u pacientů s plánovaným nasazením irinotecanu do léčby (Hoskins et al., 2007). U asijské populace má podobný efekt i alela *UGT1A1*6* (Jada et al., 2007).

2.6.6 Prognostické znaky u KRCa

Znaky pro prognózu onemocnění jsou podstatné především pro ty pacienty, u kterých je obtížné zhodnotit benefit terapie (chemoterapie i biologické léčby) oproti zhoršení kvality života a případným komplikacím (například toxicitě léčby). U KRCa se jedná především o indikaci adjuvantní chemoterapie u pacientů s nádorem ve stadiu UICC II (Gray et al., 2007).

Vztah k prognóze byl studován u genů typicky mutovaných u kolorektálního karcinomu, například *TP53*, u kterého je vliv na prognózu prokazatelný jen u pokročilých stádií onemocnění (Iacopetta et al., 2006). Podobným případem jsou i další typické změny u KRCa, mutace genu *APC* a ztráta části chromozomu 18, kde je jejich prognostický efekt nejistý. Z prognostického hlediska byly zkoumány i mutace genů podstatných pro predikci odpovědi na léčbu monoklonálními protilátkami, ale v případě nadměrné exprese *EGFR* ani mutace genu *KRAS* nebyl pozorován výrazný vztah k prognóze (Walther et al., 2009).

S prognózou onemocnění mohou být spojovány i celé podtypy KRCa, kde lepší prognózu pozorujeme u pacientů s vysokým stupněm mikrosatelitní nestability (Popat et al., 2005). Pokud se u pacientů vyskytne kombinace mikrosatelitní nestability a mutace genu *BRAF* vedoucí k záměně aminokyseliny V600E, znamená to pro pacienta vysoce negativní prognózu onemocnění (French et al., 2008). Podobně pacienti s přítomností CIMP jakékoli úrovně vykazují zkrácení OS (Juo et al., 2014).

Průběh onemocnění se v klinické praxi může sledovat pomocí hladin nádorových znaků CEA (karcinoembryonální antigen) a CA19-9 (nádorový antigen 19-9), které jsou stanovovány ze séra či plazmy. Vypovídací hodnota těchto znaků není příliš vysoká, ale v hrubých rysech může informovat o rozvoji onemocnění či ztrátě odpovědi na používanou léčbu (Byström et al., 2012; Duffy et al., 2014).

K prognóze onemocnění lze použít i kombinace znaků, které mohou dohromady přinést vyšší senzitivitu i specificitu testu a lépe stratifikovat pacienty. V současnosti existují testy MammaPrint a OncoTypeDX založené na genové expresi pro lepší stratifikaci pacientek po operaci nádoru prsu. Podobné snahy existují i v případě kolorektálního karcinomu (Jorissen et al., 2009; O'Connell et al., 2010; Wang et al., 2004b; Yothers et al., 2013), ale z navržených panelů se zatím žádný v klinické praxi rutinně nepoužívá. Příkladem komerčně dostupných panelů mohou být OncoPrint a OncoTypeDX Colorectal Cancer (Kelley and Venook, 2011).

Z prognostického pohledu jsou sledovány i imunitní buňky infiltrující nádorovou tkáň. Jejich detekce se obvykle provádí imunohistochemicky a studované leukocyty jsou identifikovány podle přítomnosti typických, nejčastěji povrchových znaků. Například infiltrace nádoru CD57 a CD68 pozitivními buňkami je pro pacienta pozitivní informace (Chaput et al., 2013). U metastatického KRCa byla popsána lepší prognóza u pacientů s vysokou infiltrací CD45RO pozitivními buňkami (Lee et al., 2013). V naší předchozí studii jsme jako pozitivní prognostický faktor popsali i infiltraci nádoru CD57 a S100 (S100 calcium binding protein) pozitivními imunitními buňkami (Liska et al., 2012).

U kolorektálního karcinomu se studuje řada dalších znaků s potenciálem přispět k prognóze onemocnění. Z recentní literatury se jedná například o expresi CD26 proteinu, jehož zvýšená hladina detekovaná imunohistochemicky v nádorové tkáni je spojena se zhoršenou prognózou (Lam et al., 2014), či zvýšené množství inhibitoru buněčné smrti survivinu, které je spojeno se zhoršenou prognózou a metastatickým fenotypem (Krieg et al., 2013). Celou

skupinu prognostických znaků tvoří microRNA, z nichž některé studované byly zmíněny v kapitole 2.3.3.

3 Cíle a hypotézy dizertační práce

Základním cílem předkládané dizertační práce bylo získat nové poznatky o biologickém chování kolorektálního karcinomu, jednoho z nejčastějších typů nádorových onemocnění. Především se jednalo o identifikaci znaků, které mají potenciál přinést nové informace k prognóze pacientů v různých stádiích vývoje KRCa.

V této práci jsou soustředěny výsledky dvou projektů, které přistupovaly k identifikaci prognostických znaků odlišným a částečně komplementárním přístupem. Experimentálně získaná data byla vztažena ke klinickým a patologickým údajům a statisticky analyzována.

Cílem první studie bylo využití rutinně dostupné metodiky, jakou je imunohistochemické vyšetření nádorové tkáně, ke studiu vztahu přítomnosti znaků nádorových kmenových buněk a prognózy pacientů s metastazujícím kolorektálním karcinomem. Imunohistochemie jako hlavní metodika byla vybrána z důvodu její dostupnosti v patologických laboratořích, v případě pozitivních výsledků by tedy jejímu uplatnění v klinické praxi nebránila dostupnost specializovaných přístrojů.

Druhá studie jako hlavní metodický princip využívala kvantitativní polymerázovou řetězovou reakci pro detekci rozdílné expresní hladiny studované skupiny genů mezi vzorkem zdravé a nádorové tkáně u pacientů s KRCa. Díky tomuto přístupu bylo možné sledovat více znaků během jednoho experimentu. Nevýhodou tohoto přístupu je specializovaná příprava a zpracování vzorku, proto tato práce spíše než na rychlé zavedení výstupů ke klinické praxi směřovala k získání nových informací o nádorové biologii, o které bychom se mohli opřít v dalších navazujících studiích.

Hypotézy:

I. Při imunohistochemickém stanovení přítomnosti CD44 a CD133 proteinu v nádorové tkáni budou identifikovány vztahy mezi expresní hladinou v primárním a sekundárním nádoru z téhož pacienta.

II. Intenzita značení proteinů CD44 a CD133 bude souviset s celkovým a bezpříznakovým přežitím pacienta – na základě převažujících literárních údajů předpokládáme snížení obou ukazatelů se vzrůstající intenzitou značení.

III. Analýza relativní expresní hladiny u genů zařazených do druhé studie ukáže rozdíly mezi zdravou a nádorovou tkání.

IV. Expresní hladina studovaných genů bude korelovat se sledovanými klinickými parametry a expresní rozdíl bude použitelný pro stanovení prognózy u pacientů s KRCa.

4 Materiál a metody

4.1 Imunohistochemická detekce CD44 a CD133

4.1.1 Soubor pacientů

Do studie byli zařazeni pacienti operovaní mezi lety 1996 až 2010 pro primární kolorektální karcinom a následně metastázu kolorektálního karcinomu do jater. Parafínové bločky byly získány z archivu Šiklova patologického ústavu Lékařské fakulty UK v Plzni a Fakultní nemocnice v Plzni. Podstatné bylo, aby u pacientů zařazených do studie byly k dispozici kompletní klinické údaje, které byly získány z informačního systému Fakultní nemocnice v Plzni. Z podstatných údajů byly sledovány především parametry TNM klasifikace a grading nádoru, datum obou operačních zákroků, pohlaví pacientů a věk v době diagnózy. Všechna data byla před dalším zpracováním anonymizována. Popis studovaného souboru v čase diagnózy KRCa je k dispozici v Tabulce 10.

4.1.2 Imunohistochemické zpracování vzorků

Parafínové bločky byly připraveny podle standardního protokolu fixací 8% formaldehydem a následným zalitím do parafínu. Tloušťka použitých řezů byla 5 μm a jako přehledné barvení preparátů byla zvolena kombinace hematoxylinu a eozinu.

Pro imunohistochemickou analýzu byly použity protilátky CD44 (DF1485, 1:100, Dako, Glostrup, Dánsko) a CD133/1 (AC133, 1:100, Miltenyi Biotech, Bergisch Gladbach, Německo). Vzorky nebyly vystaveny žádné speciální aktivaci antigenů. Primární protilátky byly vizualizovány pomocí senzitivního komplexu streptavidin – biotin – peroxidáza (Biogenex, San Ramon, Kalifornie, USA). Specificita barvení byla ověřena potřebnými pozitivními a negativními kontrolami.

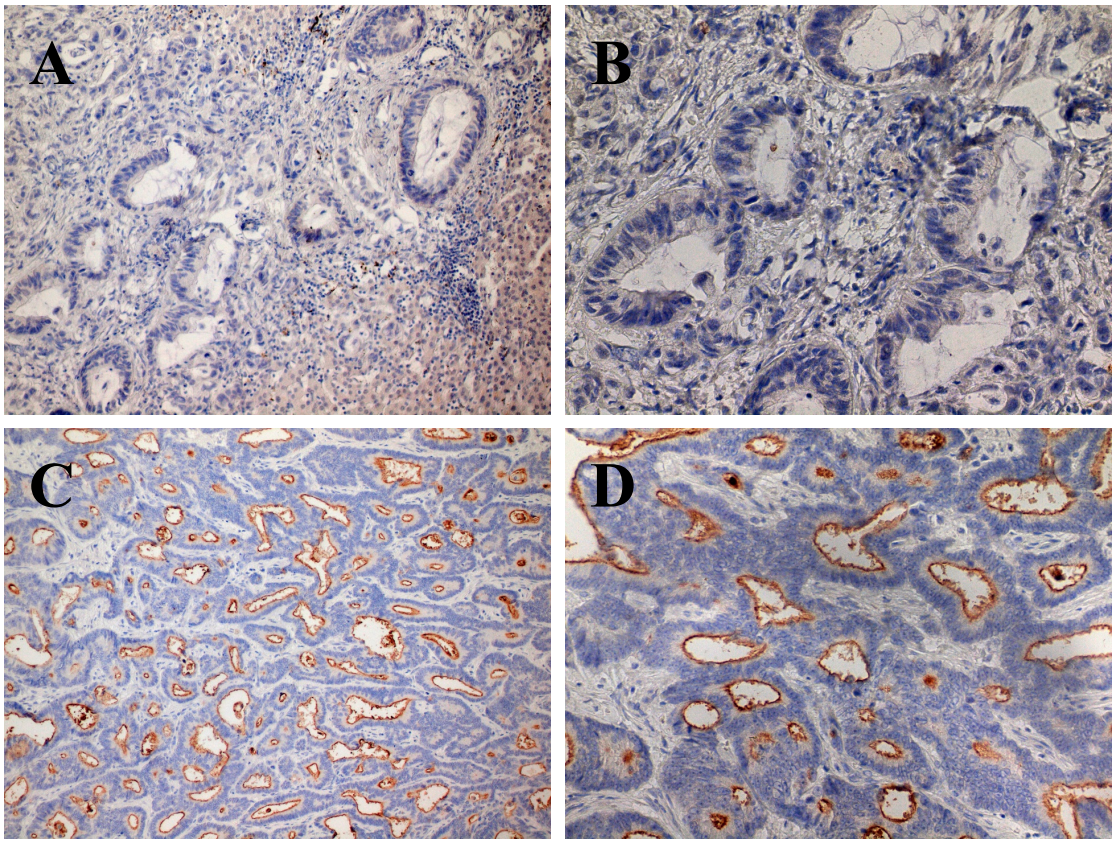
Tabulka 10 - Popis souboru pacientů vstupujících do studie.

parametr	počet
celkový počet	94
pohlaví (muži / ženy)	57/37
věk v čase primární operace (roky)	
medián	61,9
mezikvartilový rozsah	12,4
věk v čase operace jater (roky)	
medián	63,2
mezikvartilový rozsah	11,9
Velikost tumoru (T)	
T1	3
T2	3
T3	73
T4	9
neznámá	6
Lymfatické uzliny (N)	
N0	27
N1	37
N2	16
neznámé	14
Vzdálená metastáza (M)	
M0	45
M1	49
Grading (G)	
G1	19
G2	53
G3	13
neznámý	9

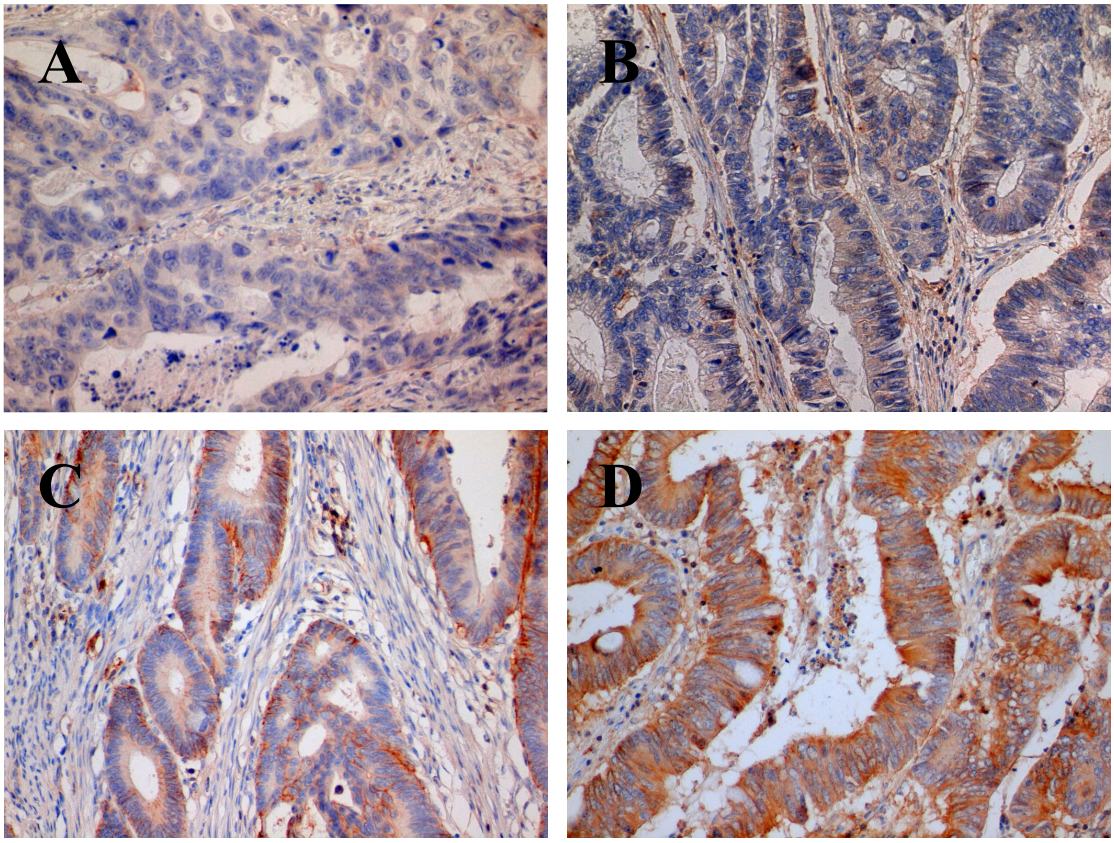
4.1.3 Semikvantitativní analýza preparátů

Pro hodnocení intenzity obarvení byla použita metodika na základě naší předchozí studie (Liska et al., 2012). Všechny obarvené preparáty byly nezávisle hodnoceny třemi výzkumníky. Intenzita značení CD44 protilátkou byla stanovena v oblasti nádorové tkáně pomocí 10x objektivu na škále 0 (negativní) až 3 (silně pozitivní). Semikvantitativní měřítko bylo založeno na intenzitě signálu pozorovatelného v buňkách nádoru, nikoli v nádorovém stromatu. Byla hodnocena pozitivita značení na cytoplazmatické membráně, kde by měl být CD44 protein lokalizován. Pro hodnocení CD133 byl 40x objektivem lokalizován nádor a v pěti mikroskopických polích bylo spočítáno množství pozitivních

nádorových žlázek a množství všech nádorových žlázek v daném poli. CD133 protein se vyskytuje na apikální straně nádorových buněk, signál byl tedy detekován pouze na části buněk směřujících do lumen nádorových žlázek. U vzorků jaterních metastáz bylo použito pozitivní značení žlučovodů jako interní kontrola a preparáty s negativními žlučovody byly z další analýzy vyloučeny. Příklady hodnocení intenzity značení jsou na obrázcích 10 a 11.



Obrázek 10 – Značení tkáně protilátkou proti CD133. Nádor negativní na přítomnost CD133 (A,B) a nádor pozitivní na přítomnost CD133 proteinu (C,D). Zvětšení 200x (A,C), detaily ve zvětšení 400x (B,D).



Obrázek 11 - Značení tkáně protilátkou proti CD44. Na zvolené semikvantitativní škále od nuly (negativní) do tří (nejvíce pozitivní) jsou zde ukázky intensity 0 (A), intensita 1 (B), intensita 2 (C) a intenzita 3 (D). Všechny příklady jsou ve zvětšení 400x.

4.1.4 Statistická analýza

Data pro CD44 a CD133 značení (v případě CD44 celková pozitivita preparátu, u CD133 poměr pozitivních/negativních žlázek) od třech výzkumníků byla zprůměrována pro každý sledovaný preparát a v případě nadměrného rozptylu odečtených hodnot došlo k opětovnému prozkoumání konkrétních preparátů. Výsledné hodnoty se pohybovaly v rozmezí od 0 do 3 v případě CD44 a od 0 do 1 v případě CD133 (průměrná hodnota poměru CD133 pozitivních žláz ku všem žlázám ve sledovaných oblastech vzorku).

Celkové přežití bylo u každého pacienta stanoveno dvakrát, jednou pro dobu od primární operace a podruhé jako doba od operace jaterní metastázy. DFI bylo stanoveno jako doba od operace metastázy do další recidivy onemocnění.

Skupiny pacientů pro porovnání byly definovány nezávisle pro každou hodnocenou proměnou rozdělením souboru dle mediánu této proměnné. Analýzy přežití byly provedeny pomocí Kaplan-Meier křivek s Cox-Mantel testem. Pozitivní výsledky ($p < 0,05$) byly dále validovány Coxovým modelem proporcionálních rizik s následným chí-kvadrát testem. Vztahy mezi kategoriemi TNM či gradingu tumoru a CD44 či CD133 pozitivitou byly hodnoceny Mann-Whitney U testem. Korelace mezi intenzitou značení oběma protilátkami v KRCa a jaterní metastáze byly testovány pomoci Spearmanova koeficientu pořadové korelace. Všechny statistické analýzy byly provedeny v softwaru STATISTICA (StatSoft, Tulsa, OK, USA).

4.2 Expresní profil vybraných genů u kolorektálního karcinomu

4.2.1 Výběr kandidátních genů

Geny pro studii byly vybrány na základě prací věnovaných kolorektálnímu karcinomu a využívajících mikročipové metodiky k určení rozdílné abundance mRNA (messenger RNA) ve sledovaných vzorcích (Kleivi et al., 2007; Kwong et al., 2005). Cílem bylo získat set genů, u kterých byla pozorována expresní změna pomocí těchto postupů, ale tato změna nebyla potvrzena dalšími metodikami a ověřena na definovaném setu pacientů. Získaný set genů byl následně podroben studiu literatury, kdy došlo k vyřazení většiny dobře prozkoumaných genů. Do experimentální části byly zařazeny geny, které byly shledány potenciálně podstatnými nejen dle literárních informací a chování v jiných typech malignit, ale i díky jejich funkci či interakčním partnerům. Seznam genů vstupujících do studie shrnuje Tabulka 11.

Z celkových 12 vybraných genů byly *MAPK1* a *LGR5* (vysvětlení zkratek viz Tabulka 11) do studie zařazeny jako kontrolní s již známou expresní změnou pozorovanou u kolorektálního karcinomu, s cílem pokusit se identifikovat vztah mezi expresní změnou a klinickými údaji.

Referenční geny *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) a *POLR2A* (polymerase (RNA) II (DNA directed) polypeptide A) byly vybrány na základě dat získaných ve společném projektu s našimi spolupracovníky (Hlavata et al., 2012) a na základě vlastních výsledků.

Tabulka 11 - Seznam genů vstupujících do studie. GeneID je unikátní identifikátor genu z databáze NCBI (National Center for Biotechnology Information), stejně jako mRNA kód.

GeneID	zkratka	název genu	mRNA kód
7447	VSNL1	visinin-like 1	NM_003385.4
11132	CAPN10	calpain 10	NM_023083.3
1462	VCAN	versican	NM_004385.4
137075	CLDN23	claudin 23	NM_194284.2
11034	DSTN	destrin (actin depolymerizing factor)	NM_006870.3
154075	SAMD3	sterile alpha motif domain containing 3	NM_001017373.2
5594	MAPK1	mitogen-activated protein kinase 1/ERK	NM_002745.4
115908	CTHRC1	collagen triple helix repeat containing 1	NM_138455.2
166968	MIER3	mesoderm induction early response 1, family member 3	NM_152622.3
51703	ACSL5	acyl-CoA synthetase long-chain family member 5	NM_016234.3
1836	SLC26A2	solute carrier family 26 (sulfate transporter), member 2	NM_000112.3
8549	LGR5	leucine-rich repeat-containing G protein-coupled receptor 5	NM_003667.2

4.2.2 Soubor pacientů

Do studie bylo zařazeno 53 pacientů, kteří byli operováni na Chirurgické klinice Fakultní nemocnice v Plzni mezi lety 2008 až 2010. Pacienti byli operováni pro kolorektální karcinom a jednalo se o pacienty bez předchozí chemoterapeutické léčby. Soubor byl rozdělen do dvou skupin na pacienty paliativně operované, u kterých se v době primární operace nacházela vzdálená metastáza nejčastěji v játrech (N=25), a radikálně operované, u kterých nebylo patrné metastatické ložisko ve vzdálených orgánech (N=28) a u nichž po operačním zákroku následovala adjuvantní onkologická léčba. Obě skupiny se mezi sebou nelišily ve skladbě pohlaví, ve věku, v T a N stádiu TNM klasifikace, ani v gradingu onemocnění. Rozdíly byly pozorovány v M kategorii a i v lokalizaci nádoru, kde většina radikální skupiny měla nádor lokalizovaný v tlustém střevě, zatímco u pacientů paliativní skupiny převažovala lokalizace v rektosigmoideu či rektu. Celková data jsou shrnuta v Tabulce 12.

Odběr vzorků probíhal přímo během operace. Od každého pacienta byly odebrány vzorky tkáně z nádoru a ze zdravé střevní sliznice. Od každého typu tkáně byly odebírány tři vzorky o maximální velikosti 1 cm^3 pro rychlé zamražení a minimalizaci destrukce tkáně. Za zdravou tkáň byla považována makroskopicky zdravá střevní sliznice na okraji resekátu (obvykle ve vzdálenosti 15 cm od nádorového ložiska). Vzorky byly uchovávány v kryozkumavkách v hlubokomrazícím boxu při teplotě -80°C.

Klinická data byla získána ze záznamů se zaměřením především na patologické informace o nádoru (TNM, grading a histologická examinace nádoru) a na informace o pacientech – pohlaví, věk, datum diagnózy, datum operačního zákroku, datum poslední kontroly pacienta, případná recidiva a úmrtí. Všechna data byla získána zdravotnickým personálem a před předáním výzkumníkům a statistikům byla anonymizována.

Studie byla schválena Etickou komisí Lékařské fakulty a Fakultní nemocnice v Plzni a pacienti s odběrem materiálu předem vyjádřili svůj písemný souhlas.

4.2.3 Izolace RNA

RNA byla získána ze zamražených tkání pomocí TRI REAGENT®RT (Molecular Research Center, Cincinnati, OH, USA) s použitím standardního protokolu výrobce. Ve stručnosti, tkáň byla přenesena ze zkumavky do třecí misky vychlazené tekutým dusíkem, kde byla rozemleta na jemný prášek. Rozemletá tkáň byla přenesena do zkumavky s 1 ml vychlazeného TRI REAGENT®RT a lyzována. Po přidání 50 μl bromoanisolu a důkladném promixování se vzniklá směs nechala centrifugovat, čímž se oddělily jednotlivé fáze. Vrchní čirá část o objemu 500 μl se smíchala se stejným objemem isopropyl alkoholu a po pěti minutové inkubaci následovala centrifugace a dvojí omytí získané pelety 70% ethanolem. Po celkovém vysušení byla peleta rozpuštěna v čisté vodě bez RNáz a DNáz (Ambion, Carlsbad, CA, USA) v objemu odpovídajícím velikosti pelety (obvykle v rozmezí 20-120 μl).

Množství RNA bylo stanoveno měřením absorbance na přístroji Infinite M200 (Tecan, Männendorf, Švýcarsko). Kvalita RNA byla určena pomocí elektroforézy v agarózovém gelu. Pouze RNA s poměrem absorbance

230nm/260nm > 1,7 a bez degradace viditelné na elektroforetickém gelu byla použita pro další analýzu. U části vzorků bylo navíc stanoveno „RNA integrity number“ pomocí přístroje 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Tabulka 12 - Klinické parametry studovaného souboru, uvedeny jsou vždy počty pacientů zařazených do dané kategorie.

	Skupina A (paliativní)	Skupina B (radikální)	p	test
Pohlaví (muži/ženy)	17/8	19/9	1	Fisherův exaktní
Věk v době diagnózy (roky)				
Medián	65	63,5	0,485	Mann-Whitney U
Mezikvartilový rozsah	7	12		
Velikost tumoru (T)				
T2	1	1	0,989	Pearsonův Chi-kvadrát
T3	19	21		
T4	5	6		
Lymfatické uzliny (N)				
N0	7	11	0,681	Pearsonův Chi-kvadrát
N1	11	10		
N2	7	7		
Vzdálená metastáza (M)				
M0	0	28	<0.001	Fisherův exaktní
M1	25	0		
Grading (G)				
G1	4	3	0,101	Pearsonův Chi-kvadrát
G2	20	18		
G3	1	7		
Lokalizace primárního nádoru				
Tlusté střevo	9	19	0,025	Fisherův exaktní
Rektosigmoideum nebo konečník	16	8		

4.2.4 Reverzní transkripce

K syntéze komplementární DNA (cDNA) byl využit RevertAid First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA). Do každé reakce o objemu 20 μl vstupovalo 500 ng celkové RNA. Před samotnou reverzní transkripcí byla RNA inkubována s DNázou I (Top-Bio, Vestec, Česká republika) po dobu

5 minut při pokojové teplotě kvůli odstranění potenciální kontaminace genomickou DNA. Pro priming reverzní transkripce byla využita směs oligo(dT)18 a náhodných hexamerů s finální koncentrací každého z nich 2,5 μM. V dalších krocích se pokračovalo dle protokolu výrobce.

Kvalita získané cDNA byla otestována pomocí PCR (polymerázová řetězová reakce) amplifikace genu GAPDH a výsledný produkt byl detekován v agarozovém gelu pomocí barvy SYBR Safe (Life Technologies, Carlsbad, CA, USA). Díky využití příslušných kontrol bylo možné odhalit případnou kontaminaci izolované RNA genomickou DNA či kontaminaci chemikálií externím zdrojem nukleových kyselin.

4.2.5 Kvantitativní real-time PCR

K designu oligonukleotidů pro detekci množství mRNA jednotlivých genů byl využit software Primer-3 s cílem navrhnout oligonukleotidy s podobnými nasedacími teplotami, délkou produktu a umístěním produktu na exon-exonové rozhraní. Navržené oligonukleotidy byly syntetizovány společností Sigma Aldrich (St. Louis, MO, USA). Seznam sekvencí je shrnut v Tabulce 13.

Pro detekci produktů byl zvolen Power SYBR Green PCR master mix (Life Technologies, Carlsbad, CA, USA) kompatibilní s použitým přístrojem 7500 Fast (Life Technologies, Carlsbad, CA, USA). Vzhledem k použití sekvenčně nespecifického barviva bylo nutné před analýzou určit optimální protokol a nasedací teploty pro jednotlivé dvojice oligonukleotidů, které byly stanoveny pomocí měření sensitivity, specificity a efektivity jednotlivých reakcí. Na základě této analýzy byly geny rozděleny do dvou skupin podle nasedací teploty (58°C pro *CLDN23, SLC26A2, VSNL1, CAPN10, VCAN, MAPK1* a 60°C pro *LGR5, DSTN, MIER3, ACSL5, CTHRC1, SAMD3*). Parametry reakcí byly následovné: počáteční zahřátí 50°C po 20 s, počáteční denaturace 95°C po 10 min a následně 42 cyklů složených z denaturace 95°C po 15 s, přisednutí a polymerizace při 58°C (60°C) po 1 min. Po skončení amplifikace následovalo stanovení křivky tání v jednotlivých jamkách s cílem určit případné kontaminace či nespecifické amplifikace. Výsledky byly zpracovány v softwaru dodávanému

k přístroji 7500 Fast a základní statistická analýza provedena softwarem REST2009 (Qiagen, Hildesheim, Německo).

Tabulka 13 - Seznam sekvencí oligonukleotidů použitých pro qPCR. tm – teplota nasedání oligonukleotidů ve stupních Celsia vypočtená při jejich návrhu, a.l. – délka amplikonu syntetizovaného při použití dané dvojice oligonukleotidů.

název	sekvence 5´- 3´	tm	a.l.
F_hVSNL1	agaactgttgagttttatcattttcg	59	90
R_hVSNL1	caggggccagtttgctatt	60	
F_hDSTN	cctggcatcttggaaatcat	59	99
R_hDSTN	aaagcagattacaatgtagccctaa	59	
F_hSAMD3	catgcaaacagaagcagctc	59	96
R_hSAMD3	ttttcagctggatagaaagatgg	59	
F_hLGR5	aatcccctgcccagtctc	60	74
R_hLGR5	cccttgggaatgtatgtcaga	59	
F_hVCAN	gcacctgtgtgccaggata	60	70
R_hVCAN	cagggattagagtgacattcatca	60	
F_hMIER3	ttgaggaaggaaataatgattggt	60	113
R_hMIER3	caccaaagtaactggtcttcgtt	59	
F_hMAPK1	ccgtgacctcaagccttc	59	72
R_hMAPK1	gccaggccaaagtcacag	60	
F_hSLC26A2	ggttggcagcactgtaacct	60	64
R_hSLC26A2	cacttgaaagaagcccatcg	60	
F_hCLDN23	ttgcatcaatataattattgggtttt	59	66
R_hCLDN23	agtttgcatggcaaggagtt	59	
F_hCAPN10	tgccagagggaggatgtg	60	73
R_hCAPN10	gctcgtaggacccatggac	60	
F_hACSL5	ttcctgtctcttgcataaaggtt	59	95
R_hACSL5	ccaattcggagatgatccac	60	
F_hCTHRC1	ccaaggggaagcaaaagg	60	74
R_hCTHRC1	cccttgtaagcacattccatta	59	

4.2.6 Statistická analýza

Ke všem statistickým testům byl použit software STATISTICA (StatSoft, Tulsa, OK, USA). Statistická významnost rozdílu mezi expresí sledovaných genů v nádorové a zdravé tkáni byla testována pomocí Wilcoxonova párového testu. Dalším krokem byla analýza přežití s cílem identifikovat vztahy mezi expresními daty a celkovým a bezpříznakovým přežitím. Vztah mezi expresní hladinou každého genu a OS byl hodnocen pro celý soubor pacientu i zvlášť pro

radikální a paliativní skupinu. U radikální skupiny pacientů byl navíc hodnocen vztah expresních dat a DFI po operaci primárního nádoru. Pro každý sledovaný gen byly skupiny pacientů rozděleny na základě mediánu expresních hodnot daného genu a následně došlo k porovnání jednotlivých vzniklých podskupin. Pro každou analýzu byly vytvořeny Kaplan-Meier křivky a rozdíl byl testován Gehan-Wilcoxon, Cox-Mantel a log-rank testem.

Vztah lokalizace tumoru a genové exprese byl stanoven pomocí Mann-Whitney U testu poté, co byli pacienti rozděleni do dvou skupin dle lokalizace nádoru v tlustém střevě či v rektosigmoideu a rektu.

Korelace mezi jednotlivými expresními změnami byly testovány pomocí Spearmanova korelačního koeficientu.

5 Výsledky a diskuze

5.1 Imunohistochemická detekce proteinů CD44 a CD133

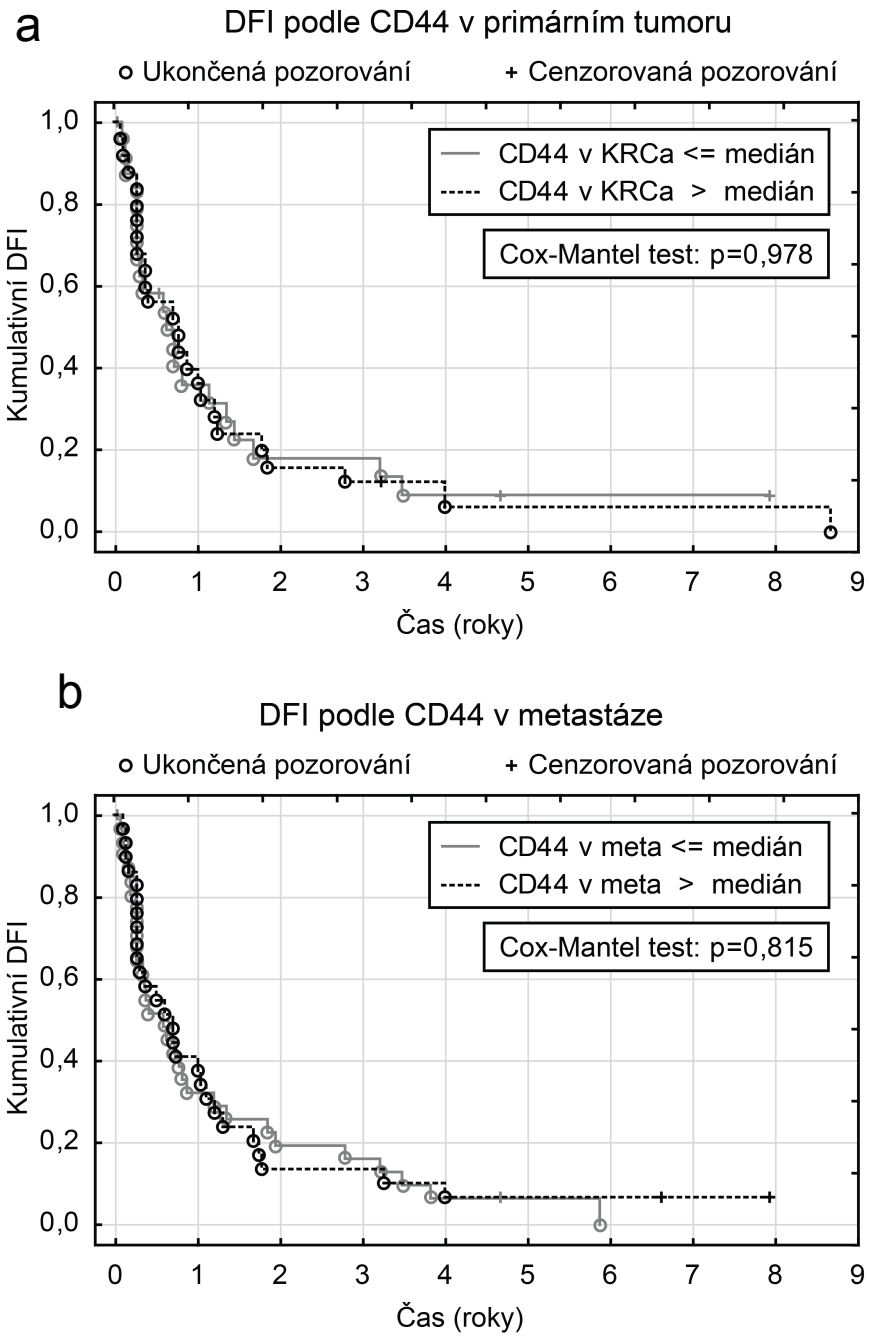
5.1.1 Výsledky

Celkově bylo do studie zahrnuto 94 pacientů s primárním KRCa a sekundárním ložiskem v jaterní tkáni. Vzorky s nízkou kvalitou obarvení byly z dalšího výzkumu vyřazeny. Ve sledovaném souboru dosahovalo celkové přežití v 1, 3 a 5 roce po operaci jaterní metastázy 88%, 65% a 35%, u bezpříznakového přežití byl podíl pacientů ve stejných časových okamžicích 38%, 16% a 8%.

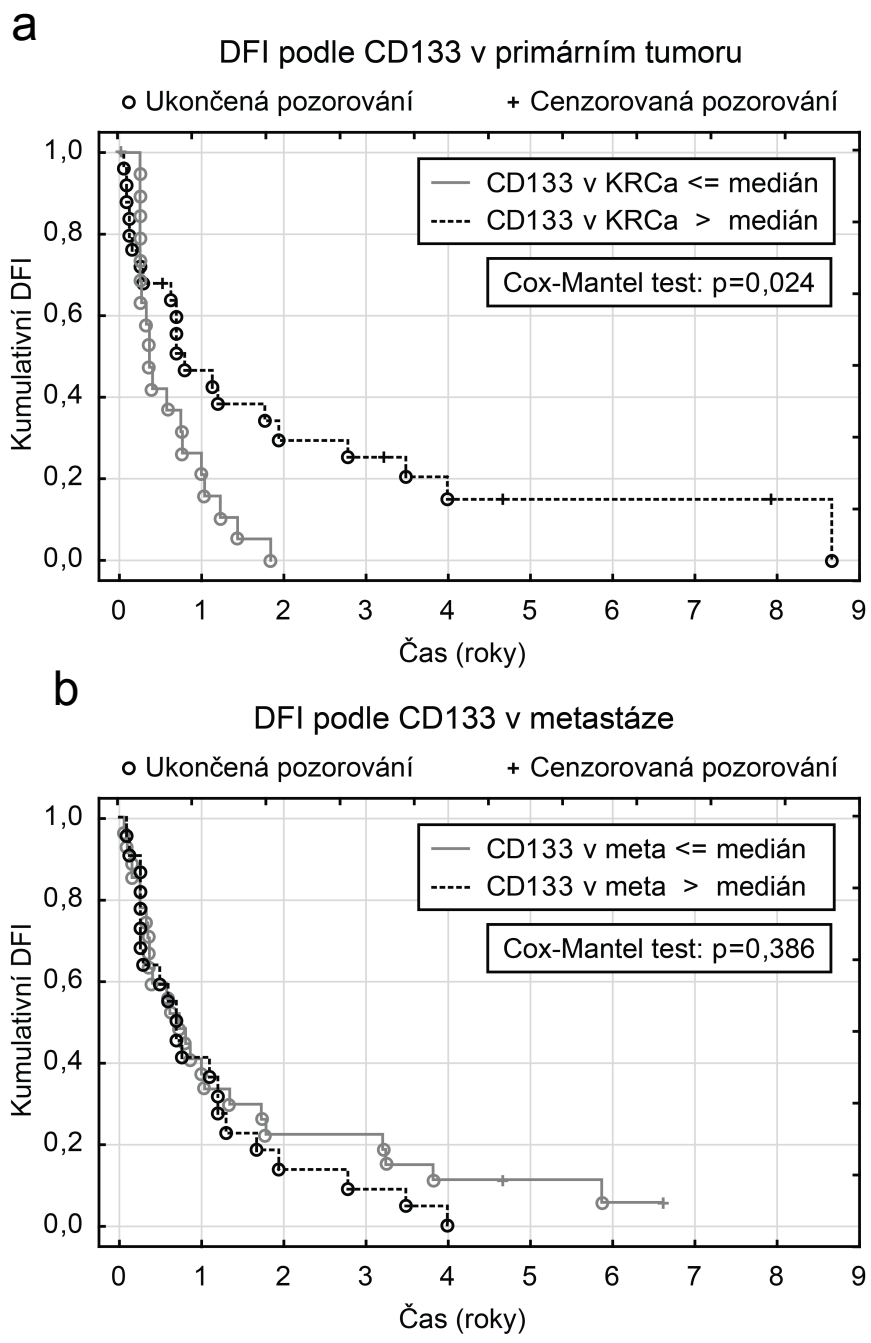
U CD44 intenzity nebyl pozorován žádný vztah mezi OS nebo DFI ani u jednoho typu vzorku (Kaplan-Meier křivky pro DFI jsou znázorněny v obrázku 12a a 12b). U CD133 značení v KRCa bylo pozorováno delší DFI u pacientů s pozitivitou nad medián studovaného souboru (Cox-Mantel p = 0,0244) (obrázek 13a), u jaterní metastázy žádný vztah značení k OS ani DFI pozorován nebyl (Kaplan-Meier graf pro DFI je na obrázku 13b). CD133 intenzita v KRCa se tak v našem souboru pacientů chovala jako pozitivní prognostický faktor. Toto pozorování bylo potvrzeno i Coxovým modelem proporcionálních rizik s použitím hodnoty CD133 pozitivity jako jediné nezávislé proměnné (chí-kvadrát p = 0,0137).

Poměr CD133 pozitivních a negativních žlázek se lišil v závislosti na gradingu nádoru, kdy v KRCa byla nižší pozitivita u nádoru stádia G1 oproti stádiu G2 (Mann-Whitney U test p = 0,0248) (obrázek 14a) a u CD133 v jaterní metastáze byla nižší pozitivita u pacientů stádia G1 v porovnání s pacienty stádia G2 nebo G3 (Mann-Whitney U test p = 0,0470) (obrázek 14b). Porovnání s TNM klasifikací ukázalo rozdíly v CD44 intenzitě, kdy vyšší intenzita byla u stádia N0 v porovnání se stádii N1 a N2 (Mann-Whitney U test p = 0,0287) (obrázek 15a) a rovněž při porovnání N0 pouze se skupinou N2 (Mann-Whitney U test p = 0,0212) (obrázek 15b).

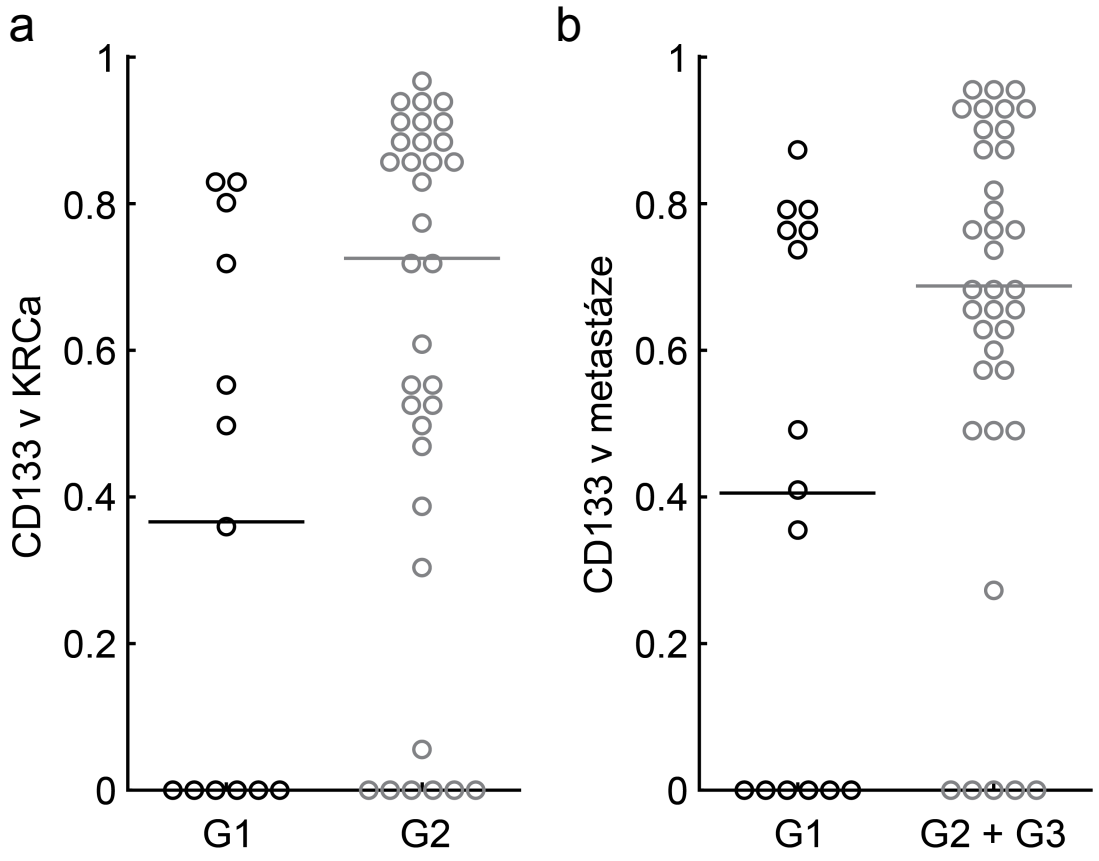
Spearmanova korelace odhalila vztah mezi CD133 pozitivitou u KRCa a jaterní metastázy (Spearman R = 0,5466, p = 0,00068).



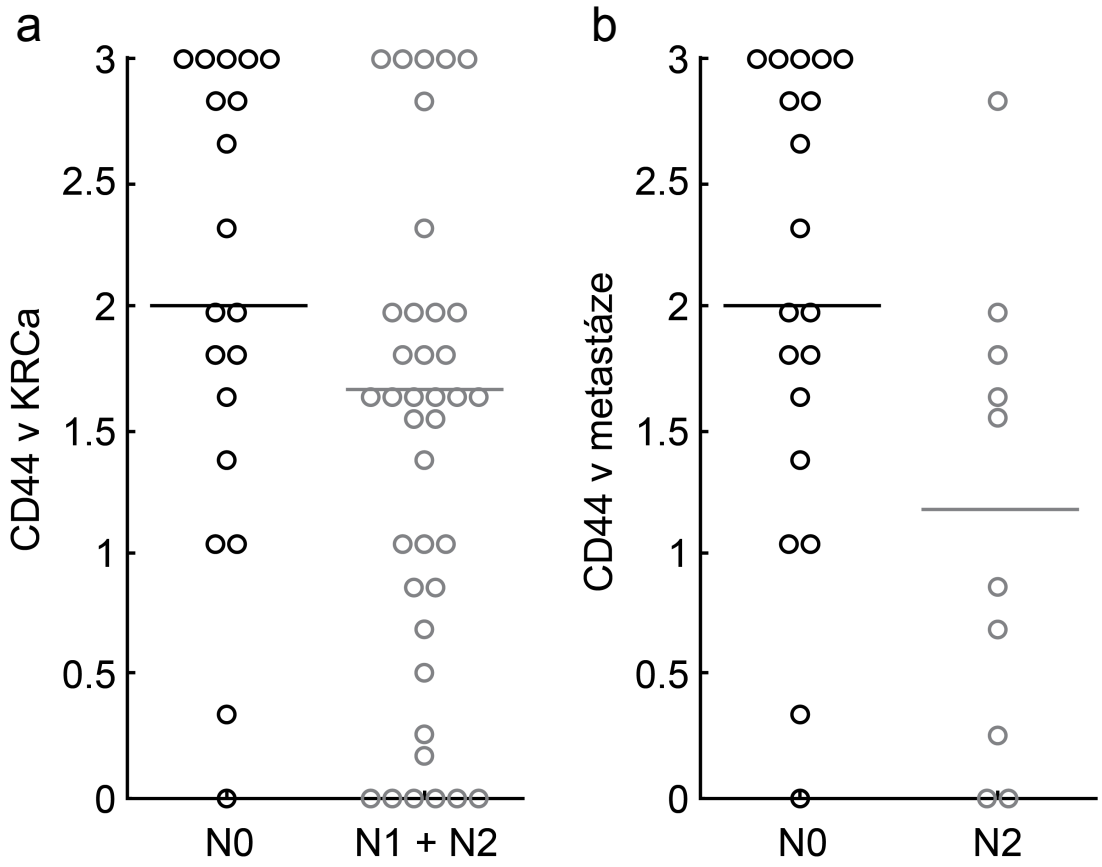
Obrázek 12 - Kaplan-Meier křivky porovnávající DFI u skupin pacientů ze studovaného souboru rozdělených dle mediánu intenzity značení proti CD44 v KRCa (12a) a v jaterní metastáze (12b). V obou případech se porovnávané skupiny mezi sebou signifikantně nelišily.



Obrázek 13 - Kaplan-Meier křivky porovnávající DFI u skupin pacientů ze studovaného souboru rozdělených dle mediánu intenzity značení proti CD133 v KRCa (13a) a v jaterní metastáze (13b). Vyšší procento CD133 pozitivních žlázek v KRCa bylo spojeno s prodloužením DFI.



Obrázek 14 - Korelace klinických dat s procentem CD133 pozitivních žlázek. (a) rozdílné hodnoty CD133 pozitivity v KRCa dle odlišného gradingu primárního tumoru, (b) rozdílné hodnoty CD133 pozitivity v jaterní metastáze na základě gradingu odpovídajícího primárního nádoru.



Obrázek 15 - Korelace klinických dat s intenzitou CD44 značení. (a) rozdílné hodnoty CD44 intenzity u KRCa dle odlišné invaze nádoru do lymfatických uzlin, (b) rozdílné hodnoty CD44 intenzity u jaterní metastázy na základě odlišné infiltrace uzlin odpovídajícího primárního nádoru.

5.1.2 Diskuze

Koncept nádorových kmenových buněk a jejich významu pro vývoj nádoru je široce přijímaný, nicméně spojení jednotlivých znaků CSC s klinickými údaji je stále velmi obtížné (Ren et al., 2013). U CD44 je problémem velké množství sestřihových variant s odlišnou funkcí a studie vlivu CD44 na klinické chování nádoru většinou neuspěly v nalezení vztahu k OS či DFI (Galizia et al., 2012; Langan et al., 2012; Li et al., 2013). Ke stejnému výsledku došla i předložená studie. CD44 tedy není možné použít jako jediného znaku ke zpřesnění prognózy pacientů. Nicméně byl v naší studii pozorován pokles intenzity barvení v primárním nádoru v sekvenci N0 – N1 – N2, tedy pacienti s nejvyšším postižením lymfatických uzlin (N2) vykazovali nejnižší pozitivitu na CD44. Tento

jev by mohl být vysvětlován snadnější invazí nádorových buněk díky snížení CD44 – dependentní vazby na extracelulární hmotu (Galizia et al., 2012).

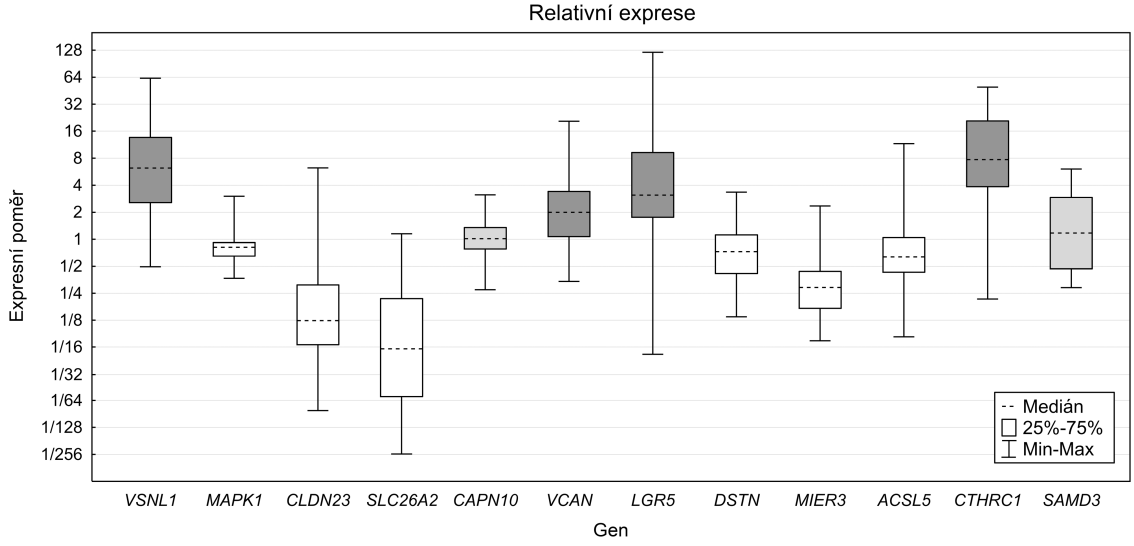
CD133 byl první znak používaný k identifikaci CSC u kolorektálního karcinomu (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). Problémem při imunohistochemické detekci CD133 může být maskování epitopu pro protilátku AC133/1 posttranslační modifikací během diferenciace CSC, což může vést ke snížení vazby protilátky k cílové molekule (Kemper et al., 2010). Některé studie ukazují, že nikoliv pouhá pozitivita či negativita, ale že množství CD133 proteinu může odlišit buňky s odlišnou růstovou kapacitou (Liao et al., 2010). Rozsáhlá meta-analýza CD133 exprese v KRCa ukázala, že zvýšené množství CD133 proteinu koreluje s některými klinicko-patologickými faktory a že je možné zvýšené množství CD133 považovat za negativní prognostický znak (Chen et al., 2013). V našem úzce vymezeném souboru pacientů byl CD133 expresi přiřazen opačný vliv, kdy pacienti s větším množstvím CD133 pozitivních žlázek měli delší bezpříznakové přežití. Již dříve byla exprese CD133 spojena spíše s dobře a středně diferencovanými nádory v porovnání s nediferencovanými KRCa, které jsou spíše CD133 negativní (Horst et al., 2008). U metastatického KRCa bylo zjištěno, že CD133 jsou častěji v G1/G0 fázi buněčného cyklu než v S či G2/M fázi (Gharagozloo et al., 2012). Na základě těchto informací můžeme soudit, že CD133 je produkováno především v pomaleji se dělících buňkách a také že je spojováno spíše s nádory s lepší prognózou. Tato data mohou osvětlit pozitivní prognostický efekt CD133 pozorovaný v naší studii.

Na základě pozorované asociace mezi prodloužením DFI a CD133 v KRCa a nikoliv v jaterní metastáze je možné spekulovat, že vlastnosti primárního nádoru mohou být pro recidivu onemocnění podstatnější než charakter jaterních metastáz.

5.2 Expresní profil vybraných genů u kolorektálního karcinomu

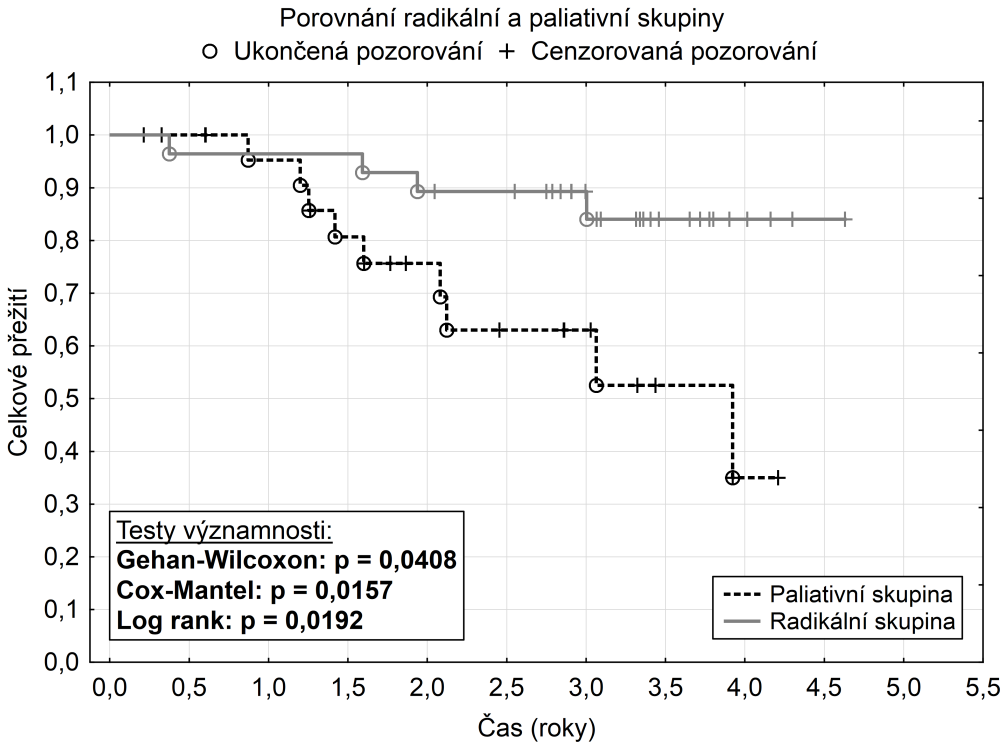
5.2.1 Výsledky

Z dvanácti studovaných genů vykazovalo deset rozdílnou expresní hladinu mezi zdravou a nádorovou tkání v celkovém souboru 53 pacientů. U čtyř genů byla detekována vyšší exprese v nádorové tkáni (*LGR5, CTHRC1, VSNL1* a *VCAN*), u šesti genů byla exprese v nádoru snížena (*DSTN, MIER3, ACSL5, MAPK1, CLDN23* a *SLC26A2*) (obrázek 16). Obě podskupiny pacientů (radikální a paliativní) kopírovaly expresní změny zjištěné u celého souboru s výjimkou genu DSTN, jehož relativní exprese byla snížena u radikální skupiny a v případě paliativní skupiny se mezi zdravou a nádorovou tkání nelišila.



Obrázek 16 - Expresní změna studovaných genů při porovnání zdravé a nádorové tkáně. Bílé obdélníky znázorňují geny se sníženou expresí ve zdravé tkáni, tmavě šedé obdélníky geny se zvýšenou expresní hladinou v nádorové tkáni a světle šedé obdélníky geny, u nichž nebyla detekována statisticky významná změna expresní hladiny.

Ve studovaném souboru pacientů byl pozorovatelný rozdíl v celkovém přežití u pacientů zařazených do paliativní skupiny v porovnání s pacienty v radikální skupině (Cox-Mantel p = 0,016, Wilcoxon p = 0,041 a log-rank p = 0,019) (obrázek 17).



Obrázek 17 – Porovnání celkového přežití mezi radikální a paliativní skupinou pacientů.

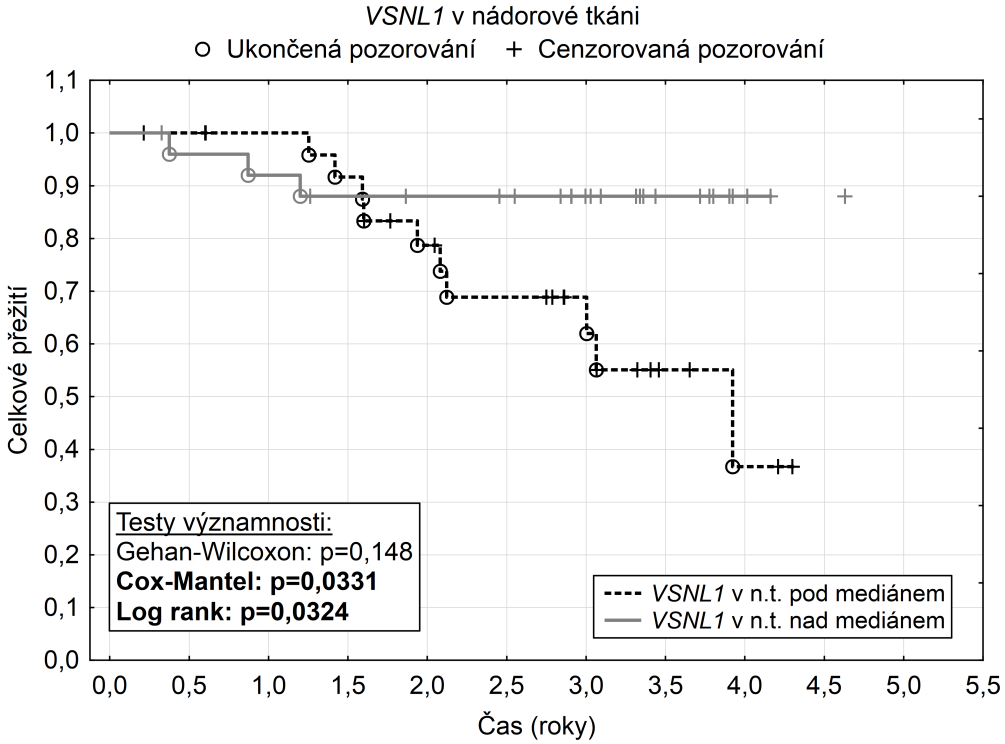
Při korelaci relativních expresních hladin s klinickými daty nebyl u žádného genu prokázán vztah s věkem, parametry TNM klasifikace ani s gradingem tumoru.

Celkové a bezpříznakové přežití bylo hodnoceno z pohledu exprese sledovaných genů v jednotlivých tkáních v porovnání s referenčními geny a rovněž z pohledu expresního rozdílu mezi zdravou a nádorovou tkání. V prvním případě byl na hladině významnosti p = 0,05 objeven vliv exprese *VSNL1* v nádorové tkáni na OS v kompletním souboru pacientů, kdy exprese *VNSL1* nad medián znamenala prodloužení OS (Cox-Mantel p = 0,033 a log-rank p = 0,032) (obrázek 18). Při rozdělení pacientů do jednotlivých

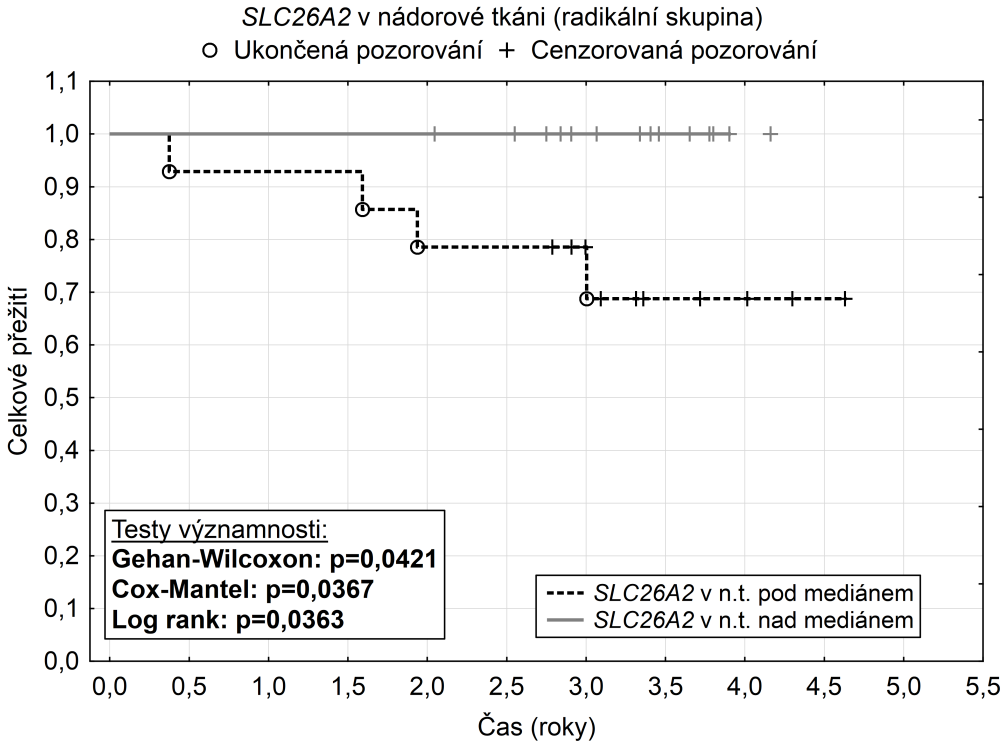
sledovaných podskupin byl u radikální skupiny pozorován vztah mezi expresí *SLC26A2* v nádorové tkáni nad medián souboru a delším OS (Cox-Mantel p = 0,036) (obrázek 19). V případě DFI bylo v radikální skupině pacientů pozorováno prodloužení DFI při expresi *VSNL1* ve zdravé tkáni pod medián sledované populace (Cox-Mantel p = 0,022 a log-rank p = 0,020) (obrázek 20) a při expresi *SLC26A2* ve zdravé tkáni nad medián sledované populace (Cox-Mantel p = 0,012, Wilcoxon p = 0,014 a log-rank p = 0,011) (obrázek 21).

Hodnocení vztahu relativní exprese mezi zdravou a nádorovou tkání a OS a DFI přineslo celkem čtyři statisticky významné výsledky. V kompletním souboru pacientů znamenalo výrazné snížení expresní hladiny *CLDN23* zkrácení OS (Wilcoxon p = 0,045) (obrázek 22). Další tři výsledky se týkaly pouze pacientů v radikální skupině, kdy méně výrazné snížení expresní hladiny *SLC26A2* a *ACSL5* značilo delší DFI (Wilcoxon p = 0,046, Cox-Mantel p = 0,045 a log-rank p = 0,041 pro *SLC26A2*, Wilcoxon p = 0,040 pro *ACSL5*) (obrázek 23 a 24). U *LGR5* bylo prokázáno prodloužení DFI u pacientů s vyšším nárůstem expresní hladiny (Wilcoxon p = 0,046, Cox-Mantel p = 0,028 a log-rank p = 0,026) (obrázek 25).

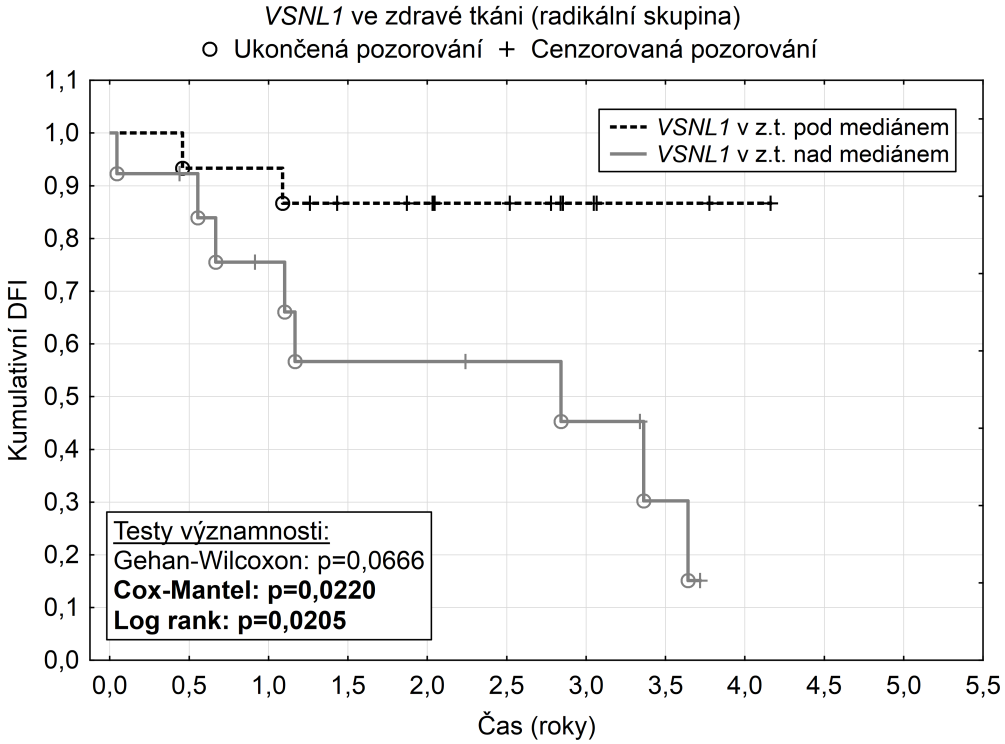
U lokalizace tumoru bylo porovnání expresních hladin všech 12 studovaných genů pomocí Man-Whitney U testu v nádorové tkáni nesignifikantní. U zdravé střevní sliznice byly popsány rozdíly v hladině *MAPK1* (p = 0,032), *LGR5* (p = 0,003), *MIER3* (p = 0,026) a *CTHRC1* (p = 0,042), kdy pacienti s tumorem lokalizovaným v tlustém střevě vykazovali vyšší hladinu těchto genů v porovnání s geny referenčními. Z pohledu relativní expresní změny byl pozorován rozdíl u genu *VCAN*, kdy pacienti s tumorem v tlustém střevě vykazovali menší nárůst exprese v nádorové tkáni oproti pacientů s tumorem v rektosigmoideu/rektu, kde byla expresní změna vyšší (p = 0,031).



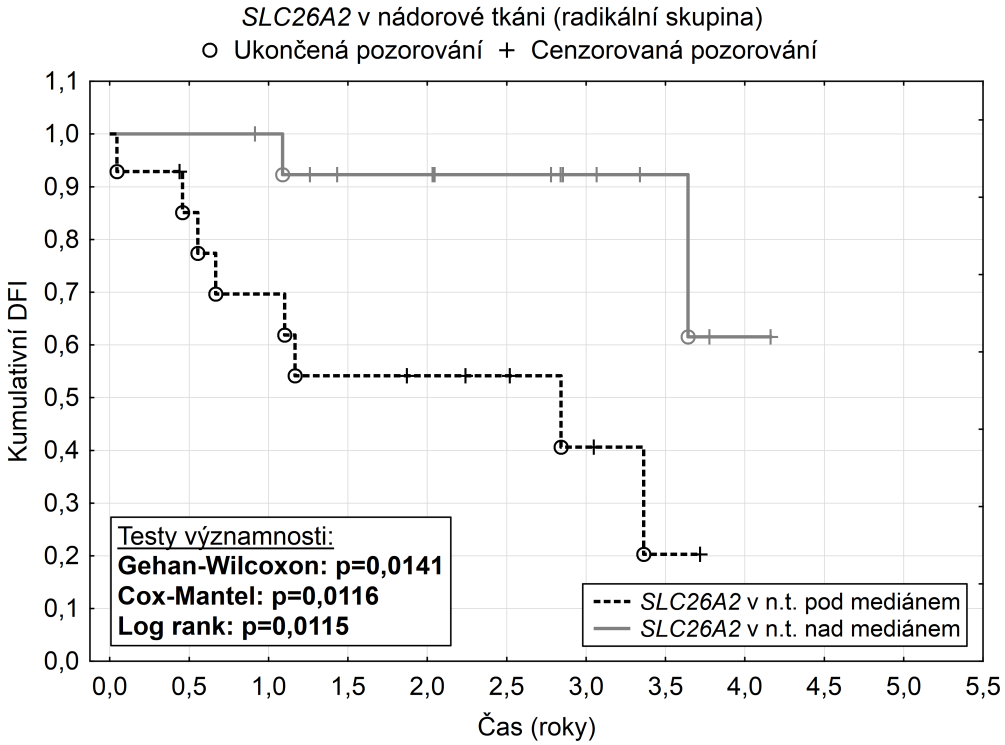
Obrázek 18 – Vliv *VSNL1* exprese v nádorové tkáni (n.t.) na OS v kompletním studovaném souboru.



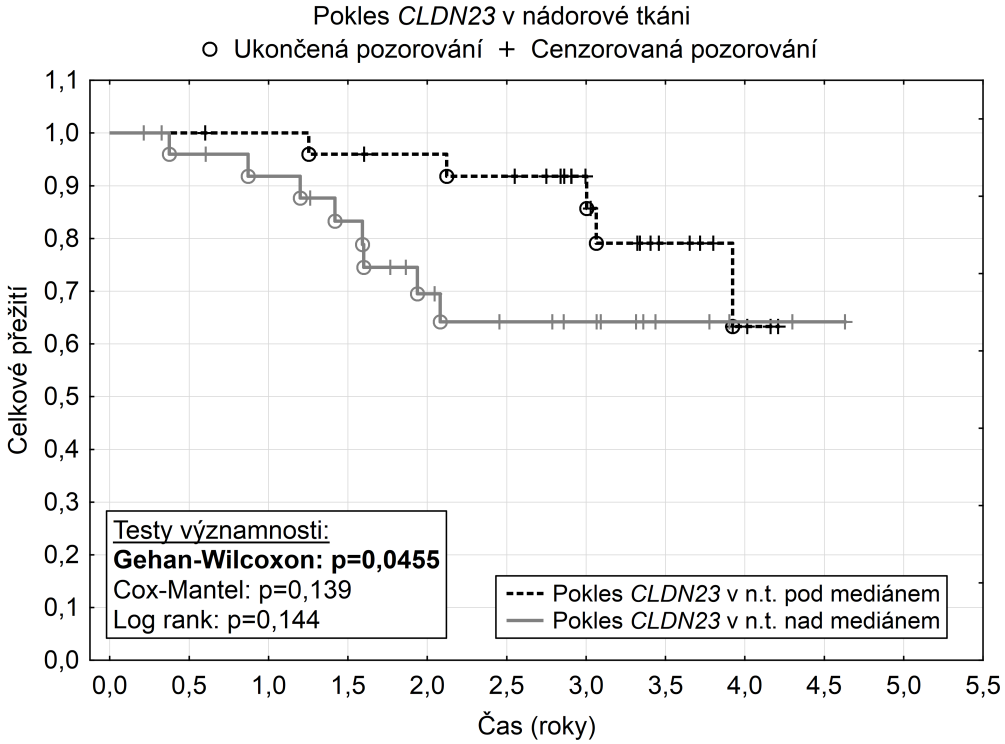
Obrázek 19 – Vliv exprese *SLC26A2* v nádorové tkáni na OS u radikální skupiny.



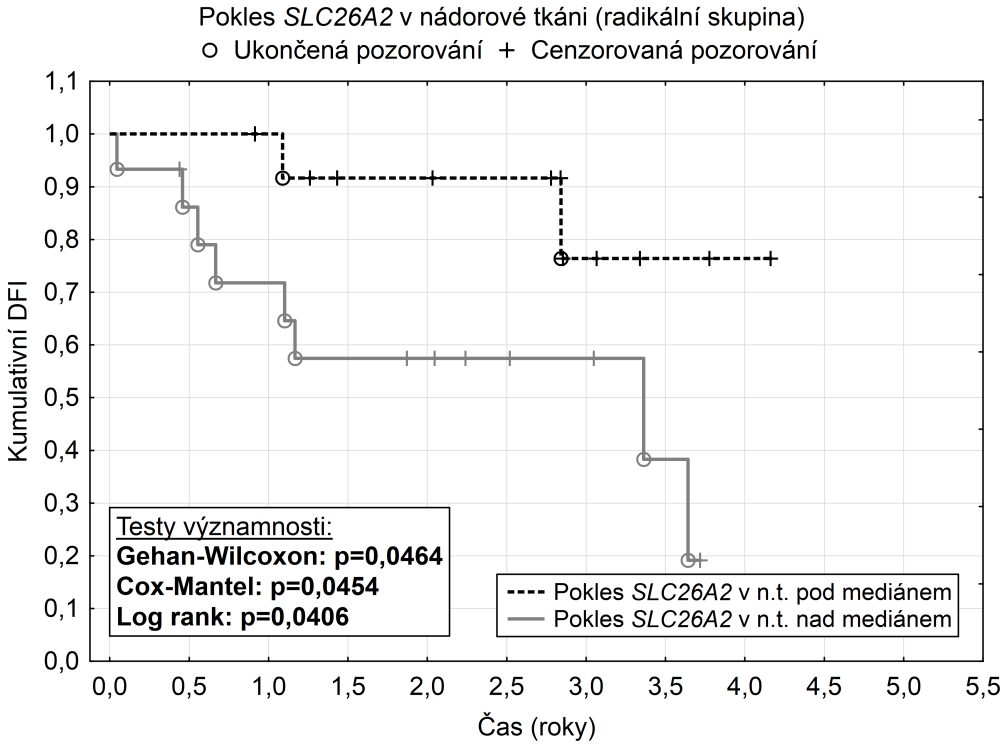
Obrázek 20 – Vliv exprese *VSNL1* ve zdravé tkáni (z.t.) na DFI u radikální skupiny.



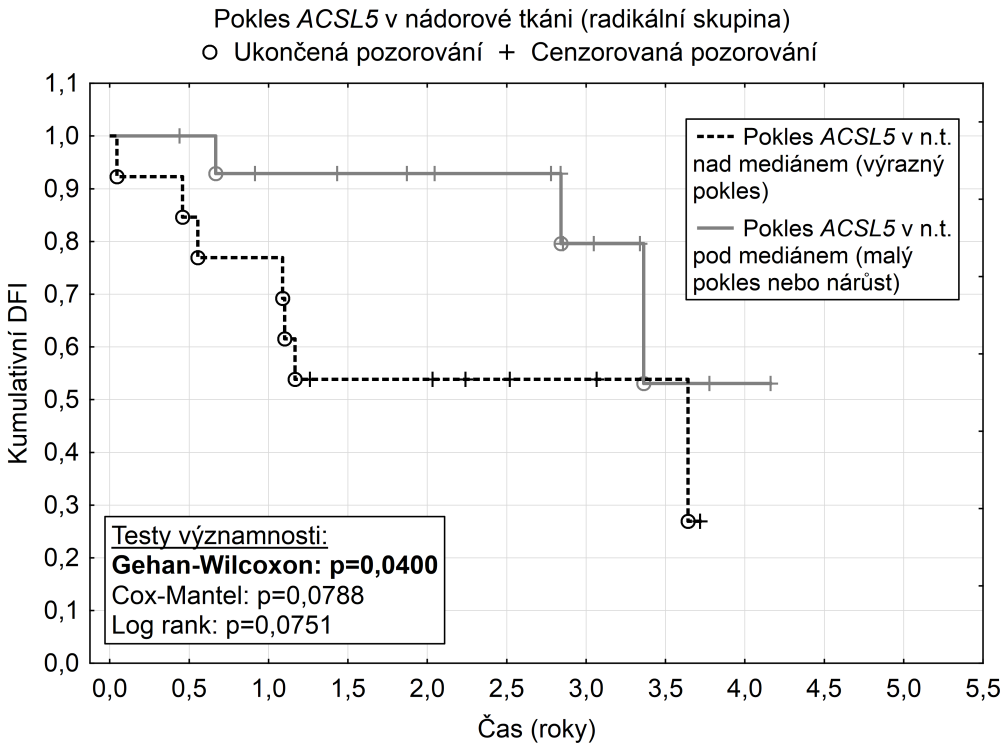
Obrázek 21 – Vliv exprese *SLC26A2* v nádorové tkáni na DFI u radikální skupiny.



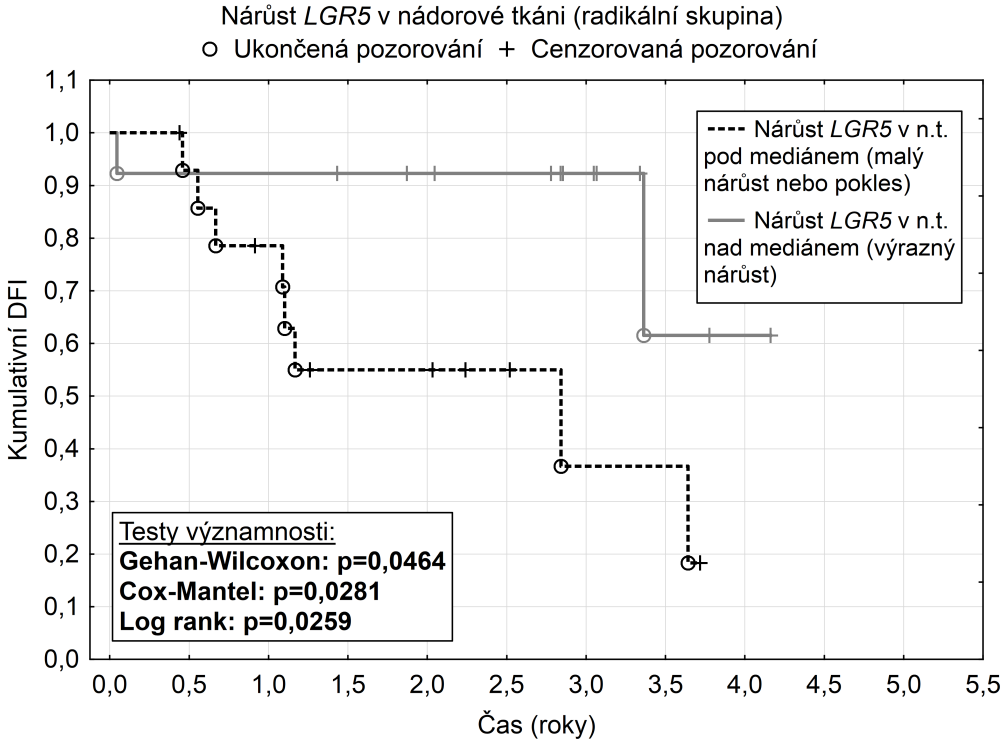
Obrázek 22 – Vliv expresní změny *CLDN23* na OS v kompletním studovaném souboru.



Obrázek 23 – Vliv expresní změny *SLC26A2* na DFI u radikální skupiny.



Obrázek 24 – Vliv expresní změny *ACSL5* na DFI u radikální skupiny.



Obrázek 25 – Vliv expresní změny *LGR5* na DFI u radikální skupiny.

5.2.2 Diskuze

Vzhledem k rozmanitosti studovaných genů budou výsledky u každého z nich diskutovány samostatně.

VSNL1

Tento gen kóduje protein pro neuronální vápníkový kanál a jeho funkcí je modulace aktivity adenylát cyklázy (Braunewell et al., 1997). *VSNL1* byl popsán jako prognostický znak u Alzheimerovy choroby (Braunewell, 2012; Tarawneh et al., 2011). V souvislosti s nádorovým onemocnění byl *VSNL1* popsán jako inhibitor proliferace u buněk odvozených od neuroblastomu, zároveň zvyšoval jejich migrační kapacitu (Xie et al., 2007). U epiteliálních nádorů byl popsán jako supresor nemalobuněčného karcinomu plic (Fu et al., 2008) a inhibitor epitelo-mezenchymálního přechodu u buněk skvamózního karcinomu (Schönrath et al., 2011).

V případě kolorektálního karcinomu vykazoval *VSNL1* sníženou expresní hladinu u buněčné kultury HCT116 po zastavení buněčného cyklu pomocí selenomethioninu (Goulet et al., 2007) a byl zkoumán jako potenciální znak pro identifikaci cirkulujících nádorových buněk v periferní krvi (Findeisen et al., 2008). V předchozích studiích byla popsána korelace mezi VSNL1 expresí a infiltrací lymfatických uzlin nádorovými buňkami (Akagi et al., 2012), v námi studovaném souboru pacientů vztah pozorován nebyl. Přínosem studie byl popis vztahu mezi vyšší expresní hladinou *VSNL1* v nádorové tkáni a prodloužením OS, čímž by bylo možné ukázat na jeho potenciální roli jako nádorového supresoru i u kolorektálního karcinomu a doplnit tak data dostupná z jiných typů nádorů (Fu et al., 2008, 2010). Opačný efekt na zkrácení DFI u pacientů s vyšší expresí *VSNL1* ve zdravé tkáni je otázkou k dalšímu výzkumu.

VCAN

Protein kódovaný genem *VCAN* je jednou z hlavních složek extracelulární hmoty a hraje podstatnou roli v regulaci buněčné migrace (Zhang et al., 2012), proliferace (Miquel-Serra et al., 2006) a adheze (Evanko et al., 2012). V minulosti byl *VCAN* spojen s různými maligními onemocněními, například s karcinomem žaludku (Zhang et al., 2012), pankreatu (Skandalis et al., 2006) a kolorekta (Tsara et al., 2002).

U námi zkoumaného souboru pacientů jsme prokázali zvýšení exprese genu *VCAN* v nádorové tkáni, což je v souladu se změnami na proteinové úrovni popsanými v literatuře (Theocharis, 2002; Tsara et al., 2002). Rozdíl ve změně exprese byl odlišný v závislosti na lokalizaci tumoru (rektum/sigmoideum oproti tlustému střevu). V naší studii změna exprese nekorelovala se žádným ze studovaných klinických parametrů.

LGR5

LGR5 protein je považován za jeden ze znaků fyziologických intestinálních a jaterních kmenových buněk (Becker et al., 2008; Huch et al., 2013). Předchozí studie ukazují na nárůst exprese *LGR5* v nádorové tkáni spojený s produkcí *LGR5* v takzvaných nádorových kmenových buňkách (Kleist et al., 2011; Uchida et al., 2010).

U souboru pacientů studovaného v předkládané studii bylo pozorováno zvýšení expresní hladiny v nádorové tkáni a statistická analýza ukázala na vztah mezi prodloužením DFI a vyšší expresí *LGR5*. Důvodem by mohl být vztah mezi expresí *LGR5* a buněčnou adhezí, kdy jsou potenciální nádorové kmenové buňky s vysokou hladinou *LGR5* pevněji vázány ve struktuře nádoru a buňky se sníženou produkcí *LGR5* jsou více schopné migrovat (Walker et al., 2011), což by vysvětlovalo kratší DFI u pacientů s nižším množstvím *LGR5* z důvodu snazšího rozsevu nádoru. Oproti tomu řada předchozích publikací popsala negativní efekt nárůstu exprese *LGR5* u pacientů s různými typy nádorů (Nakata et al., 2013; Simon et al., 2012; Wu et al., 2012). Vzhledem k rozporům

ohledně efektu *LGR5* na prognózu KRCa připravujeme ověření výsledků na nezávislém souboru pacientů.

CTHRC1

Tento gen kóduje protein s popsanou rolí při remodelaci cévního řečiště (Pyagay et al., 2005) a jeho expresní změna byla pozorována v řadě typů solidních nádorů včetně nádoru tlustého střeva (Palma et al., 2012; Tang et al., 2006). Nedávno byl *CTHRC1* popsán jako prediktor sníženého přežívání u KRCa (Tan et al., 2013). V námi studovaném souboru tento gen vykázal nejvyšší nárůst exprese v nádorové tkáni, ale jeho vztah k prognóze potvrzen nebyl, přestože byl dobře pozorovatelný trend zkrácení OS u pacientů se zvýšenou hladinou *CTHRC1* v nádoru.

SLC26A2

Tento gen patří do rodiny aniontových výměníků a kanálů a jeho hlavní funkcí je přenos SO_4^{2-} aniontů. Změna v jeho funkci je příčinou části chondrodysplastických poruch (Alper and Sharma, 2013).

In vitro byla nižší exprese *SLC26A2* spojena s vyšší proliferací buněčné linie odvozené od kolorektálního karcinomu (Yusa et al., 2010) a snížení exprese bylo pozorováno i u bioptických vzorků (Galamb et al., 2008). V našem souboru pacientů bylo detekováno výrazné snížení expresní hladiny *SLC26A2* v nádorové tkáni, které znamenalo snížení OS i DFI ve skupině radikálně léčených pacientů. Možnou příčinou vedoucí ke zkrácení OS i DFI může být nepřesné uspořádání extracelulární hmoty z důvodu aberantní sulfatace proteinů kvůli snížení množství SLC26A2 proteinu, což může usnadnit migraci nádorových buněk skrze tkáň.

CLDN23

Tento gen je členem rodiny klaudinů, která u člověka čítá 24 zástupců kódujících proteiny se čtyřmi transmembránovými doménami s rolí ve tvorbě

těsných spojů mezi sousedícími buňkami (Lal-Nag and Morin, 2009). *CLDN23* byl popsán jako gen se sníženou expresí u intestinálního typu nádoru žaludku (Katoh and Katoh, 2003). Naše studie odhalila snížení expresní hladiny i u kolorektálního karcinomu. Úroveň změny korelovala s prognózou celkového přežití. Pacienti s méně výrazným snížením expresní hladiny měli delší OS v porovnání s těmi, u nichž došlo k výraznému poklesu přepisu genu *CLDN23*. Protein CLDN23, stejně jako další členové rodiny klaudinů, hraje roli ve tvorbě těsných spojů mezi buňkami. Deregulace produkce klaudinů byla v poslední době spojena nejen s vyšší migrační schopností nádorových buněk, ale i s epitelo-mesenchymálním přechodem a nádorovými kmenovými buňkami, a jejich role v nádorové biologii je tedy pravděpodobně komplexnější, než se tušilo (Kwon, 2013).

ACSL5

Proteinový produkt tohoto genu je enzym s rolí v biosyntéze lipidů a degradaci mastných kyselin. Snížení exprese *ACSL5* bylo spojeno s karcinomem tenkého střeva (Gassler et al., 2003). U gliomů je exprese *ACSL5* považována za faktor zlepšující přežití pacientů (Mashima et al., 2009). Role ACSL5 proteinu v buněčné apoptóze je spojena s proteinem mortalinem a aktivací p53 (Klaus et al., 2014). V předchozí studii věnované KRCa bylo pomocí microarray dat zjištěno zvýšení exprese v nádorové tkáni (Yeh et al., 2006), v naší studii bylo množství *ACSL5* mRNA nižší v nádorové tkáni oproti zdravé tkáni, navíc jsme pozorovali vztah mezi snížením exprese a zkrácením DFI, kdy nejkratší DFI měli pacienti s nejvýraznějším poklesem *ACSL5*. Tyto závěry ukazují na pravděpodobnou protektivní funkci proteinu ASCL5 u KRCa, v souladu s údaji dostupnými z jiných typů nádorových onemocnění. U analýzy *ACSL5* exprese a rovněž funkce jsou problematické odlišné efekty jednotlivých sestřihových variant, kdy například *ACSL5* o plné délce má pro-apoptotickou funkci (Gassler et al., 2007).

Následující tři geny měly ve sledovaném souboru pacientů sníženou expresní hladinu v nádorové tkáni, ale statistická analýza neprokázala žádné signifikantní spojení se sledovanými klinickými parametry.

DSTN

DSTN gen kóduje aktin vazebný protein destrin, který hraje podstatnou roli v dynamice polymerizace aktinu jako aktin depolymerizační faktor (Bamburg and Wiggan, 2002). V souvislosti s nádorovým onemocněním byla role proteinu DSTN popsána během apoptózy indukované membránovým androgenovým receptorem u buněk nádoru prostaty (Papadopoulou et al., 2008) a jeho pozitivní efekt na migrační kapacitu neuroblastomových buněk (Lefranc et al., 2009). Snížení exprese *DSTN* bylo jedním z mechanismů vedoucích k zablokování migrace a invazivní schopnosti nádoru žaludku (Yu et al., 2013). V naší studii bylo pozorováno snížení mírné expresní hladiny *DSTN* v nádorové tkáni bez vztahu ke klinickým parametrům

MIER3

MIER3 je velice málo studovaným genem, až v roce 2012 byl asociován s nádorovými onemocnění jako kandidátní gen u nádorů prsu (denDekker et al., 2012) a zároveň se zařadil mezi nejčastěji mutované geny u vysoce mutovaných podtypů kolorektálního karcinomu (The Cancer Genome Atlas, 2012). Naše data ukázala sníženou expresi u KRCa bez pozorování silných vztahů ke klinickým datům.

MAPK1

Mitogen-aktivována proteinová kináza 1 je finální kinázou MAPK dráhy, která má mnoho různých rolí v buněčném životě včetně regulace proliferace, diferenciace či genové exprese (Zassadowski et al., 2012). V kolorektálním karcinomu bylo pozorováno snížení exprese v nádorové tkáni v předchozích

studiích (Wang et al., 2000), stejně tomu bylo i v našich vzorcích, vztah ke klinickým parametrům pozorován nebyl.

U dvou následujících genů nebyla v našem souboru pozorována změna v expresní hladině mezi zdravou a nádorovou tkání.

SAMD3

Funkce *SAMD3* je neznámá, tento gen byl vybrán na základě možného zvýšení expresní hladiny během vývoje KRCa (Kleivi et al., 2007) a popsané interakci proteinu SAMD3 s proteinem FANCG (Fanconi anemia, complementation group G) (Rual et al., 2005), který hraje roli v udržování genomické integrity (Fei et al., 2005). Tato interakce by mohla mít podstatnou roli během vývoje KRCa. Díky absenci expresní změny *SAMD3* jsme nemohli hodnotit jeho vztah ke klinickým parametrům.

CAPN10

U genu *CAPN10*, vápníkem aktivované cysteinová proteázy (Ono and Sorimachi, 2012), byl dříve popsán jednonukleotidový polymorfizmus spojený se susceptibilitou ke vzniku KRCa (Frances et al., 2007). V naší studii jsme se pokusili porovnat vztah mezi hladinou transkripce a klinickými parametry pacientů s KRCa, změna množství jeho mRNA mezi zdravou a nádorovou tkání ale nebyla detekována, a proto nebylo možné tento vztah hodnotit.

6 Závěry práce

V naší studii bylo prokázáno, že i často studované znaky spojované s nádorovými kmenovými buňkami, jako CD44 a CD133, mohou nést i jiný význam pro chování nádoru, než je v současnosti obecně přijímáno. Například u CD133 je možné pozorovat pozitivní i negativní prognostický význam. Naše studie byla od přechozích prací odlišná tím, že nehodnotila počet nádorových kmenových buněk či intenzitu značení CD133 protilátkou v jednotlivých buňkách, ale celkové množství CD133 pozitivních žlázek v poměru ke žlázkám negativním na CD133 barvení. Tento přístup, pokud by byl prognostický význam ověřen na validační skupině pacientů, by byl jednoduše hodnotitelný patology při rutinním vyšetření nádorové tkáně, což by mohlo přinést nové znaky podstatné pro prognózu.

Zároveň je potřeba studovat roli jednotlivých proteinů, používaných jako znaky nádorových kmenových buněk, v biologii nádoru. Pochopení jejich funkce může pomoci s vysvětlením pozorovaných protichůdných vlastností při korelaci s klinickými daty, a usnadnit tak použití těchto znaků pro zpřesnění prognózy u pacientů s kolorektálním karcinomem.

V části věnované expresní hladině vybraných genů byla na studovaném souboru pacientů identifikována expresní změna mezi zdravou a nádorovou tkání u 10 genů. Nejdůležitějšími výsledky práce je spojení expresní změny *CLDN23* a celkového přežití pacientů a spojení expresních změn *SLC26A2*, *ACSL5* a *LGR5* s bezpříznakovým přežitím u pacientů, kteří v době primární operace neměli vzdálenou metastázu. I přes poměrně malý soubor studovaných pacientů jsou výsledky studie zajímavé, jelikož ukazují nové spojitosti mezi jednotlivými geny a prognózou u pacientů s kolorektálním karcinomem.

Kromě role jako prognostického faktoru mohou objevené vztahy mezi expresí genů a DFI či OS přinést nové informace i o biologii kolorektálního karcinomu. Například vliv expresní změny *ACSL5* svědčí o metabolické změně v nádorové tkáni, u *SLC26A2* zase o změně v transportu iontů přes membránu, přičemž

o přesném mechanizmu a vlivu těchto změn na nádorové buňky je v současnosti možné pouze spekulovat.

Geny, jejichž expresní změna byla spojena s klinickými parametry, budou dále prozkoumány na rozšířeném souboru pacientů s cílem získat robustnější sadu dat. Zároveň byl vliv expresních změn použit pro konstrukci matematického modelu, který dokáže předpovídat prognózu pacientů na základě diferenciální exprese čtyř ze setu dvanácti studovaných genů s vyšší přesností, než jakou má prognóza založená pouze na jednom genu. Tento skórovací systém je v současnosti podrobován další analýze na novém souboru pacientů.

Z hlediska původních hypotéz se nám podařilo částečně potvrdit hypotézu I, kdy korelace mezi primárním nádorem a jeho metastázou byla nalezena u CD133. Hypotéza číslo II byla vyvrácena, vztah ke klinickým datům byl nalezen pouze u CD133 a to opačný, než bylo předpokládáno. Hypotéza číslo III byla potvrzena, když z dvanácti studovaných genů deset vykazovalo rozdílnou hladinu transkripce mezi zdravou a nádorovou tkání. I pro hypotézu číslo IV bylo pozorováno několik pozitivních spojení, které jsou rozebrány v předchozím textu.

Identifikace nových prognostických znaků je podstatná z pohledu zpřesnění odhadu dalšího rozvoje KRCa u jednotlivých pacientů. Prognostické znaky mají potenciál pomoci s rozdělením pacientů nejen dle patologického vyšetření, ale i dle molekulárních znaků. Cílem je rozlišit pacienty, u kterých je nezbytné použití onkologické léčby od těch, jejichž prognóza by podáním této léčby nebyla ovlivněna. Tato práce ukazuje nové znaky využitelné v prognóze KRCa, které by po jejich validaci bylo možné zařadit do prospektivních klinických studií.

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9 Seznam příloh

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Příloha I:

Pitule P, Cedikova M, Daum O, Vojtisek J, Vycital O, Hosek P, Treska V, Hes O, Kralickova M, Liska V. Immunohistochemical detection of cancer stem cell related markers CD44 and CD133 in metastatic colorectal cancer patients. Biomed Res Int. 2014;2014:432139. **(IF_{2012}=2,880)**

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Research Article

Immunohistochemical Detection of Cancer Stem Cell Related Markers CD44 and CD133 in Metastatic Colorectal Cancer Patients

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Aim. The goal of this study was to semiquantitatively detect presence of cancer stem cells markers CD44 and CD133 in immunohistochemically stained paired samples of colorectal cancer (CRC) and colorectal liver metastases (CLM). Level of staining intensity was compared to clinical and pathological characteristics of tumors with the aim to identify impact of CD44 or CD133 expression on tumor behavior. *Patients and Methods*. Formalin fixed paraffin embedded samples from 94 patients with colorectal tumor and liver metastases were collected at Sikl's Department of Pathology. Samples were stained by antibodies against CD44 and CD133. Presence and intensity of staining was assessed semiquantitatively by three trained researchers. *Results*. Patients with higher level of CD133 staining in CRC had longer disease free interval (Cox-Mantel $P = 0.0244$), whereas we found no relation between CD44 expression and overall survival or disease free interval. CD133 expression in CRC and CLM differed based on CRC grading; in case of CD44 we found differences in staining intensity in individual stages of tumor lymph node invasion. *Conclusion*. Effect of cancer stem cell markers on prognosis of colorectal cancer can vary depending on pathological classification of tumor, and we have shown that CD133, generally considered to be a negative marker, can bear also clinically positive prognostic information in group of patients with colorectal liver metastases.

1. Introduction

For a long time, tumors were considered to be unhierarchical cell mass, sometimes with several clonal populations, where all cells had almost the same potential for development, growth, or secondary tumor formation. The last two decades brought new findings and tumors have gradually become regarded as hierarchical tissues, similarly to normal tissues, with different cell populations, each having a distinct function and characteristic within the tumor. One minor population is of particular importance—small percentage of tumor cells called cancer stem cells (CSCs).

Hierarchical organization of tumors was for the first time identified in acute myeloid leukemia in 1997 [1], where cells with $CD34^{++}CD38^{-}$ phenotype were described as primitive leukemic stem cells with the potential to differentiate into the leukemic blasts. Similar subpopulation with low level differentiation was later described also in many different solid tumors, including breast [2], prostate [3], colon [4, 5], or pancreatic cancer [6]. Today, there are two main models

describing involvement of CSCs in tumor development: deterministic, according to which all tumor cells arise from CSCs, which are dividing asymmetrically and stochastic, which supposes the tumor cells to be randomly acquiring mutations and undergoing clonal evolution that can result in the formation of a clone with stem-cell properties. The latter model assumes that the cells are dividing symmetrically [7].

Cancer stem cells share many similarities with physiologically normal adult stem cells. Both of these cell types are undifferentiated with the capacity to differentiate into hierarchical sequence of other tumor or normal cells; they are capable of self-renewal and asymmetric division and they have relatively long cycling times and long-term survival [8]. In addition to this, CSCs were described to be highly resistant to chemotherapy and radiotherapy which makes them very difficult to target and eliminates them by common therapy regimens. This characteristic makes them a possible source of later recurrence of the disease or therapy-driven selection of resistant clones [9]. Several treatment regimens specifically targeting CSCs are now emerging to overcome this problem. One of these approaches is forced differentiation of CSCs combined with targeted therapy [10].

During the initial attempts to identify CSCs, tumor cells were usually separated according to the expression of a particular marker into two groups (positive and negative). The capacity of the cells to form new tumors was then assessed and compared between the two groups. Using this approach in colorectal cancer, it was described that cells expressing CD133 are more tumorigenic than CD133 negative ones [4, 5]. Later on, additional markers were described, for example, CD44 and CD166 [11], CD29, CD24, and Lgr5 [12], and ALDH1 [13]. Many of these markers are also expressed in normal colonic stem cells (e.g., Lgr5, ALDH1, or CD29), which complicates the distinction between CSCs and normal stem cells. There are also other discrepancies regarding these markers. It was described, for example, that cells with stem cell capacity also exist within the CD133 negative cell population and that CD133 negative cells can form tumors with the same frequency as CD133 positive cells [14]. Further studies are necessary to identify the characteristics of cancer stem cells more precisely, because their unbiased identification and understanding of their biology can open new options for cancer treatment.

In the presented study, we selected two putative cancer stem cell markers, CD44 and CD133, to compare their expression in matched primary colorectal tumor and colorectal liver metastases within clinically well-specified set of patients. We evaluated the relationship between markers expression in primary and secondary tumor and tested the impact of CD44 and CD133 positivity on clinical behavior of tumor, mainly on overall survival and disease free interval.

2. Methods

Assessment of positivity or negativity for CD44 and CD133 was performed semiquantitatively from immunohistochemically stained sections of matched primary and secondary tumor samples from patients with colorectal carcinoma and either synchronous or metachronous liver metastasis. Immunohistochemical staining was selected as a method of choice, because it is a commonly used technique in many pathology departments and new markers can be easily implemented to current protocols.

Table 1: Description of set of patients used for the study.

Total number	94
Gender (males/females)	57/37
Age at primary surgery (years)	
Median	61.9
Interquartile range	12.4
Age at liver surgery (years)	
Median	63.2
Interquartile range	11.9
Tumor size (T)	
T1	3
T2	3
T3	73
T4	9
Unknown	6
Lymph nodes involvement (N)	
N0	27
N1	37
N2	16
Unknown	14
M classification	
M0	45
M1	49
Grade	
G1	19
G2	53
G3	13
Unknown	9

2.1. Selection of Samples. Samples used for this study were collected from the depository of formalin fixed paraffin embedded samples of Sikl's Department of Pathology, Medical School and Teaching Hospital in Pilsen. We have selected patients who underwent surgery for primary colorectal cancer between years 1996 and 2010 and who were afterwards subjected to the surgery for colorectal cancer liver metastases at Department of Surgery, Medical School and Teaching Hospital in Pilsen.

The data required to determine overall survival and disease free interval, as well as grading and staging (TNM classification) scores, were available from the clinical information system of Teaching Hospital in Pilsen. The patients' data were anonymized by authorized medical personnel before being processed. Description of the patient sample is summarized in Table 1.

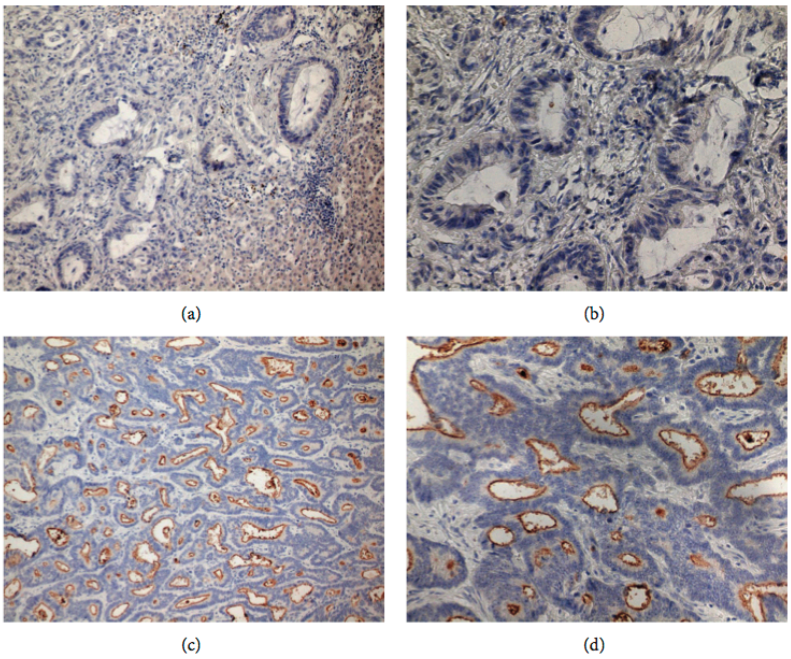

Figure 1: Expression of CD133. (a, b) Samples negative for CD133 in the lumen of tumor glands, (c, d) samples with positive CD133 staining on the apical portions of tumor cells. Magnification 200x (a, c) and 400x (b, d) of the same samples.

2.2. Immunohistochemical Staining. Tissue samples for light microscopy were fixed in 4% formaldehyde and embedded in paraffin using routine procedures. Five-micrometer thick sections were cut from the tissue blocks and stained with hematoxylin-eosin.

For immunohistochemical staining the following primary antibodies were used: CD133/1 (AC133, 1 : 100, Miltenyi Biotec, Bergisch Gladbach, Germany) and CD44 (DF1485, 1 : 100, Dako, Glostrup, Denmark). No special pretreatment was used. The primary antibodies were visualized using the supersensitive streptavidin-biotin-peroxidase complex (Biogenex, San Ramon, CA). Appropriate positive and negative control slides were employed.

2.3. Semiquantitative Analysis of Slides. A method based on previous study was used for analysis of slides [15]. All slides stained for CD44 and CD133 were analyzed independently by three trained researchers (Pavel Pitule, Miroslava Cedikova, and Jan Vojtisek). Tumors were localized using 10x objective and level of positivity on scale from 0 (negative) to 3 (highly positive) was assessed for CD44 staining. For CD133 we evaluated five microscopical fields using 40x objective and the percentage of CD133 positive tumor glands compared to all tumor glands in the view field were assessed. Positive staining of bile duct walls that should occur after every successful CD133 staining was used as an internal control of the staining process in case of liver metastasis samples. CLM slides with unstained bile ducts were excluded from the analysis. Examples of markers expression are summarized in Figures 1 and 2.

2.4. Statistical Analysis. CD44 and CD133 positivity assessments provided by the three researchers were averaged for every slide and in case of high variability of the scores the slide was reviewed by all of the three researchers. Resulting scores were on the scale from 0 to 3 for CD44 as described above and from 0 to 1 for CD133 expressing an average ratio of CD133 positive glands to all present glands.

Two overall survival (OS) times were defined for every patient, one from the time of the CRC surgery and the other from the time of the CLM surgery. Disease free interval (DFI) was calculated from the time of the CLM surgery to the time of metastases recurrence. The analyses of OS and DFI were performed using two-sample Kaplan-Meier method with Cox-Mantel test. The two samples (patient groups to be compared) were formed independently for each variable based on its median. Positive results ($P < 0.05$) were validated by Cox proportional hazards model with subsequent Chi-square test. Possible relations between tumor grading/staging and CD44/CD133 positivity were investigated using Mann-Whitney U test. Correlations between CD44/CD133 in CRC/CLM (all combinations) were explored using Spearman

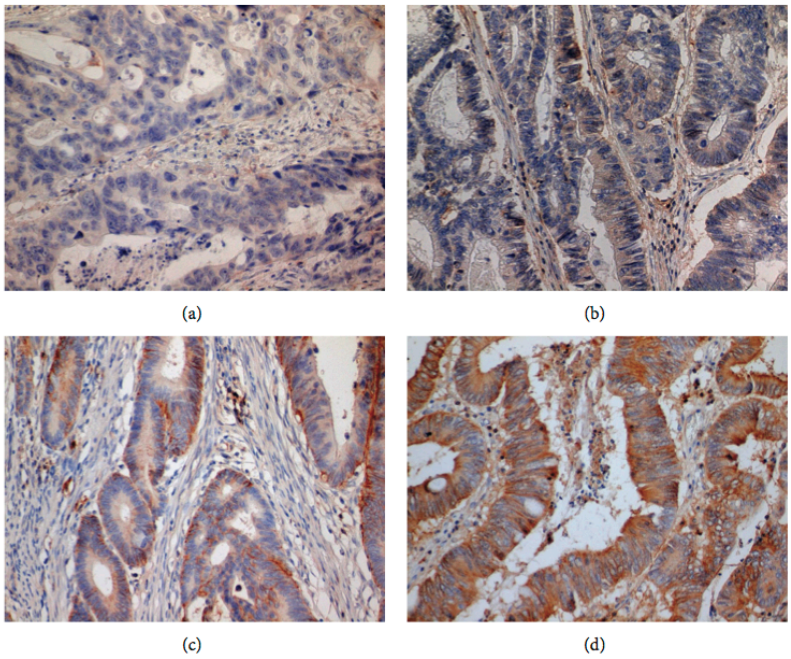

FIGURE 2: Example of samples with different levels of CD44 staining intensity. On our semiquantitative scale, samples were marked as intensity 0 (a), intensity 1 (b), intensity 2 (c), and intensity 3 (d). Magnification 400x.

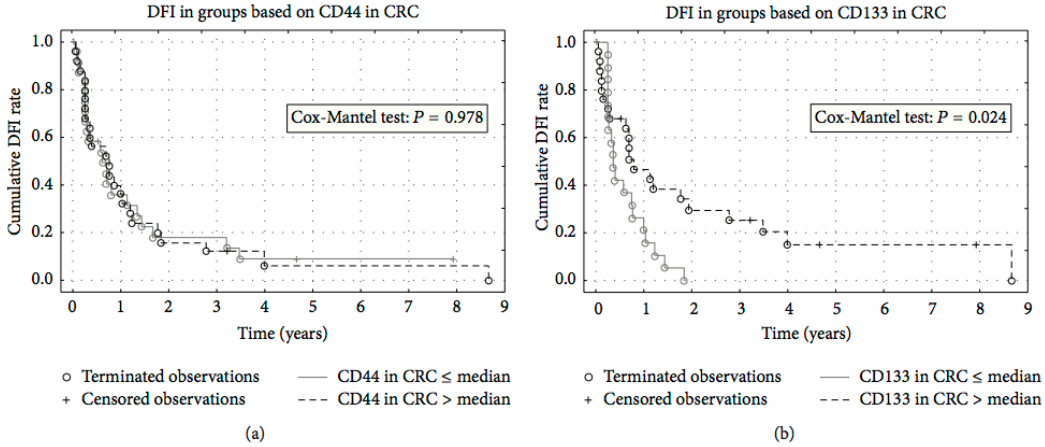

FIGURE 3: Kaplan-Meier curves comparing the levels of CD44 (a) or CD133 (b) staining intensity in primary colorectal cancer sample to the disease free interval.

rank-order method. Statistical analysis was performed using the statistical software Statistica 10.0 (StatSoft, Inc. 2011, Tulsa, OK, USA).

3. Results

We have included 94 patients with primary and secondary CRC in our study. Samples with low quality staining were excluded from analyses. OS after CLM surgery at 1, 3, and 5 years was 88%, 65%, and 35%,respectively, and DFI at 1, 3, and 5 years was 38%, 16%, and 8%, respectively.

We did not find any statistically significant effect of CD44 expression in CRC or CLM on either OS or DFI Figure (3(a)). CD133 positivity over median in primary tumor was found to be a positive prognostic factor of DFI (Cox-Mantel P = 0.0244) (Figure 3(b)). This finding was confirmed by Cox

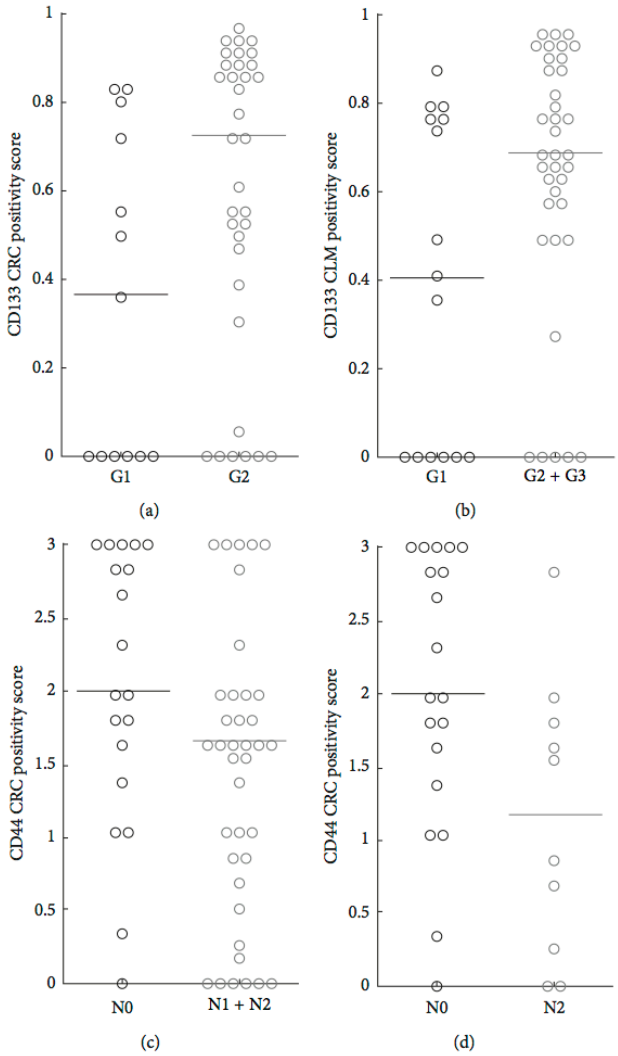

FIGURE 4: Comparison of the clinical data with the levels of markers abundance. (a) Difference of CD133 positivity in CRC based on tumor grade, (b) difference of CD133 positivity in CLM based on primary tumor grade, (c) comparison of CD44 intensity in CRC between N0 and N1 + N2 groups, and (d) comparison of CD44 intensity in CRC between N0 and N2 groups only.

proportional hazards model using the CD133 CRC score as a single independent variable (Chi-square $P = 0.0137$). CD133 positivity in CLM was not connected to any effect on OS or DFI (Cox-Mantel $P = 0.3855$). We identified differences in markers quantity based on grading, where CD133 in CRC was present in lower amount in G1 compared to G2 (Mann-Whitney U Test $P = 0.0248$) and CD133 in CLM had lower expression in G1 compared to combined G2 and G3 stage (Mann-Whitney U Test $P = 0.0470$) (Figures 4(a) and 4(b)). Comparison of studied markers with TNM classification revealed differences in CD44 in CRC depending on lymph node invasion—higher expression of CD44 was detected in N0 stage compared to combined N1 and N2 groups (Mann-Whitney U Test $P = 0.0287$) as well as N0 compared to N2 (Mann-Whitney U Test $P = 0.0212$) (Figures 4(c) and 4(d)).

Spearman correlation revealed a relationship between expression of CD133 in primary CRC and CLM (Spearman $R = 0.5466$, $P = 0.00068$).

4. Discussion

The concept of contribution of colorectal cancer stem cells to tumor development is widely accepted, but the relation of individual CSC markers expression to disease prognosis is

still not completely clear [16]. In case of CD44, various splice variants differ in function and reports for CD44 in general usually fail to find any correlation with DFI or OS [17–19]. This was the case also for our set of patients, suggesting that use of CD44 as a single prognostic marker of CRC behavior is impossible. However, we observed a difference in CD44 expression when we stratified the patients according to tumor lymph node invasion with the data showing a decrease of CD44 expression in CRC in sequence from N0 to N2. Higher invasiveness of tumors with lower expression of CD44 into the lymph nodes can be related to weaker CD44 mediated binding to extracellular matrix [17].

CD133 was used as a first marker for identification of colorectal CSC [4, 5]. Immunohistochemical analysis of CD133 expression and its relevance to clinical and pathological features of CRC depends on sample type and size. Another problem is the posttranslation modification of CD133, which can mask AC133 epitope, which is the target for most antibodies against CD133 [20]. Some studies have shown that not the presence or absence of CD133 is important for CSCs identification, but that the abundance of CD133 protein can distinguish cells with different growth capacity [21]. Presented study did not assess the role of CD133 in cancer stem cell biology, but we wanted to find out whether it can be used as a marker providing new information to patients' prognosis. Large meta-analysis of CD133 expression in colorectal cancer confirmed that overexpression can be associated with several clinicopathological factors and can be used as an independent negative prognostic factor [22]. Surprisingly, the level of CD133 positivity had the opposite effect in our very confined group of patients as the statistical analysis revealed that higher levels of CD133 were associated to longer DFI. CD133 was described to be expressed in well and moderately differentiated tumors compared to undifferentiated tumor buds, which tend to be CD133 negative [23]. In metastatic CRC, CD133 expressing cells were described to be more often in G1/G0 phase of cell cycle than in S and G2/M phases [24]. Based on this information, CD133 cells can be considered to be those with low cycling rate and also those typical for tumors with better clinical outcome. These facts could be connected to positive prognostic effect of higher CD133 expression on DFI described in presented study.

Based on the described association between longer DFI and CD133 positivity in CRC but not in CLM, it is possible to speculate that the primary tumor has more important role in disease recurrence than liver metastasis.

5. Conclusion

Our study shows that in the field of cancer stem cells markers and their role on tumor behavior there is still a large space for further research. It seems that even commonly used CSCs marker CD133 can bear both negative and positive prognostic information depending on the clinical specification of studied patients and therefore there is a need for new studies aiming at describing the effect and role of CSCs markers in well-defined sets of samples. Also testing of combination of several markers can be of particular importance. It would be of great importance to fully understand the biology of individual proteins used as markers, because it can provide a new point of view on the seemingly contradictory results from individual studies. Generally, if applied with underlying understanding mentioned above, cancer stem cell markers can bring valuable information to patients' prognosis and can help to modify diagnostic and treatment strategy.

Disclosure

Pavel Pitule and Miroslava Cedikova contributed equally to this work.

Conflict of Interests

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

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Příloha II:

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Differential Expression and Prognostic Role of Selected Genes in Colorectal Cancer Patients

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Abstract. *Aim: Colorectal cancer (CRC) is one of the most common malignant diseases. The aim of our study was to describe the expression status of 12 selected candidate genes, by comparing paired samples of healthy colon mucosa and tumour tissues and to correlate obtained data with clinical and pathological features, with the goal of revealing associations for individual gene expressions and tumour behaviour. Materials and Methods: Samples from 53 patients with CRC were analyzed. Patients were divided into two groups based on the presence or absence of distant metastases at the time of primary tumour surgery. Expression levels were assessed by quantitative real-time polymerase chain reaction. Results: We found changes in the expression of 10 out of 12 analyzed genes. Four genes were significantly up-regulated in tumour tissues: leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5; p<0.001), collagen triple helix repeat containing 1 (CTHRC1; p<0.001), visinin-like 1 (VSNL1; p<0.001) and versican (VCAN; p=0.001). Six genes were down-regulated: destrin (DSTN; p=0.004), mesoderm induction early response 1, family member 3 (MIER3; p<0.001), acyl-CoA synthetase long-chain family member 5 (ACSL5; p=0.002), mitogen-activated protein kinase 1/ERK (MAPK1; p<0.001), claudin 23 (CLDN23; p<0.001) and solute carrier family 26 (sulfate transporter), member 2 (SLC26A2; p<0.001). We recorded longer overall survival (OS) in the group of patients with higher expression of VSNL1 (p=0.032). Patients with more pronounced down-regulation of CLDN23 had shorter OS (p=0.045). In the group of patients without distant metastases, longer OS and disease-free interval (DFI) were found for patients with higher SLC26A2 expression in tumour tissues (p=0.036 and p=0.011, respectively). In the same group, lower expression of VSNL1 in healthy tissue corresponded to a longer DFI (p=0.020), smaller decrease of SLC26A2 and ACSL5 meant longer DFI (p=0.041 and p=0.040, respectively), as did greater increase of LGR5 expression (p=0.026). Conclusion: We identified differences in the expression of 10 genes in colorectal cancer tissue compared to healthy colon mucosa, and found prognostic significance for these changes which could be used for the development of a disease risk scoring system.*

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Key Words: Colorectal cancer, prognostic markers, gene expression.

The incidence of colorectal cancer (CRC) is increasing and globally, this malignant disease has the third highest incidence (after breast and lung cancer) and the fourth highest mortality rate (after lung, liver and stomach cancer) (1). In the first stages of its development, CRC can be treated by surgical intervention. In later stages, it has a high capacity to form secondary tumours, mainly in the liver and lungs (2). Therefore it is neccessary to combine surgery and chemotherapy to achieve higher efficacy of treatment.

The selection for optimal treatment is also complicated by the high heterogeneity of CRC, which can be divided into three main subtypes: type with chromosomal instability, type with microsatellite instability and type with CpG island methylator phenotype (3). Individual subtypes differ in disease prognosis and prediction, and their identification is often crucial for the effective eradication of residual disease by oncological treatment.

Currently, there are several chemotherapeutic regimens and the possibility of biological treatment also exists. The most commonly used chemotherapeutics are 5-fluorouracil,

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Table I. *Summary of genes selected for the study.*

GeneID	Symbol	Full name	mRNA RefSeq
7447	*VSNL1*	Visinin-like 1	NM_003385.4
11034	*DSTN*	Destrin (actin-depolymerizing factor)	NM_006870.3
154075	*SAMD3*	Sterile alpha motif domain containing 3	NM_001017373.2
8549	*LGR5*	Leucine-rich repeat-containing G-protein-coupled receptor 5	NM_003667.2
1462	*VCAN*	Versican	NM_004385.4
5594	*MAPK1*	Mitogen-activated protein kinase 1/ERK	NM_002745.4
1836	*SLC26A2*	Solute carrier family 26 (sulfate transporter), member 2	NM_000112.3
137075	*CLDN23*	Claudin 23	NM_194284.2
166968	*MIER3*	Mesoderm induction early response 1, family member 3	NM_152622.3
11132	*CAPN10*	Calpain 10	NM_023083.3
51703	*ACSL5*	Acyl-CoA synthetase long-chain family member 5	NM_016234.3
115908	*CTHRC1*	Collagen triple helix repeat containing 1	NM_138455.2

oxaliplatin and irinotecan, usually in various combinations (4). Biological treatment is targeted against the epidermal growth factor receptor (EGFR) or vascular endothelial growth factor receptor (VEGFR). Two categories of agents acting against EGFR exist: small tyrosine kinase inhibitors (gefitinib, erlotinib) and monoclonal antibodies (panitumumab and cetuximab) (5). The most complicated step in the treatment is the selection for the appropriate agent for each patient individually.

At present, only a few predictive and prognostic markers are used in CRC therapy and several others are in the phase of experimental validation. Two of these predictive markers are related to the use of biological treatment, the expression status of the EGFR and the mutational status of the EGFR proximal effector, the small G-protein Kirsten rat sarcoma viral oncogene homolog (KRAS) (6, 7). Only patients who have tumours positive for EGFR expression and have a wild-type allele for the *KRAS* gene can be treated by anti-EGFR treatment, but there is still a substantial proportion of patients who meet the elegibility criteria but lack treatment benefit (8). Other markers are being studied, namely the mutational status of other *RAS* family genes, v-raf murine sarcoma viral oncogene homolog B (*BRAF*) and phosphatidylinositol-4,5-bisphosphate 3-kinase (*PI3K*) or the expression of phosphatase and tensin homolog (PTEN) protein (negative regulator of the PI3K pathway) (9, 10). Markers predicting the efficacy of classical chemotherapy are also studied, for example the excision repair cross-complementing rodent repair deficiency, complementation group 1 (*ERCC1*) polymorphisms in the oxaliplatin treatment (11) or DNA topoisomerase 1 (TOP1) expression level in irinotecan treatment (12). Markers with prognostic function are of particular interest because on their potential to inform about disease agressiveness and for their possible contribution to follow-up optimization.

Despite the number of studied genes, there is still a need to identify for novel markers whose mutation or expression status would provide additional information that would be useful for more precise patient selection, with the final goal of individualized medicine. Therefore, here, we have selected potentially interesting candidate genes according to their known or possible function in diverse aspects of tumour progression, *i.e.* angiogenesis, metabolism or cell adhesion, and tried to relate their altered expression with clinical behaviour of primary CRC.

Materials and Methods

Selection of studied genes. The selection of studied genes was based on a search through the dataset from the high-throughput studies focused on colorectal cancer (13, 14). Our aim was to find genes whose expression change was already identified in the large-scale analysis, but was never confirmed by different approaches. Our search finally narrowed down 12 candidate genes whose names and identificators are summarised in Table I.

Two of the genes - leucine-rich repeat-containing G protein-coupled receptor 5 (*LGR5*) and mitogen-activated protein kinase 1/ERK (*MAPK1*) - were selected as control genes with known and verified expression change in CRC. Selection of reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and polymerase (RNA) II (DNA directed) polypeptide A (*POLR2A*) was based on the data from our collaborators (15) and our own laboratory practice.

Primer design. Quantitative real-time polymerase chain reaction (PCR) primers for the selected genes were designed using Primer-3 software (16) with focus on having similar annealing temperatures and lengths of the PCR product. Primer sequences are listed in Table II. Primers were synthetised by Sigma Aldrich Company (St. Louis, MO USA) using their custom oligo synthesis service.

Collection of tissue samples. Samples for the analysis were collected during surgery for the colorectal tumour. Within 20 min after the removal of the tumour tissue from the patient, small samples of

Table II. *List of primer sequences, annealing temperatures and amplicon lengths. tm, Melting temperature; a.l., amplicon length.*

Name	Sequence 5'-3'	tm	a.l.	Name	Sequence 5'-3'	tm	a.l.
F_hVSNL1	agaactgttgagttttatcattttcg	59	90	*F_hMAPK1*	ccgtgacctcaagccttc	59	72
R_hVSNL1	caggggccagtttgctatt	60		*R_hMAPK1*	gccaggccaaagtcacag	60	
F_hDSTN	cctggcatcttggaaatcat	59	99	*F_hSLC26A2*	ggttggcagcactgtaacct	60	64
R_hDSTN	aaagcagattacaatgtagccctaa	59		*R_hSLC26A2*	cacttgaaagaagcccatcg	60	
F_hSAMD3	catgcaaacagaagcagctc	59	96	*F_hCLDN23*	ttgcatcaatataattattgggtttt	59	66
R_hSAMD3	ttttcagctggatagaaagatgg	59		*R_hCLDN23*	agtttgcatggcaaggagtt	59	
F_hLGR5	aatcccctgcccagtctc	60	74	*F_hCAPN10*	tgccagagggaggatgtg	60	73
R_hLGR5	cccttgggaatgtatgtcaga	59		*R_hCAPN10*	gctcgtaggacccatggac	60	
F_hVCAN	gcacctgtgtgccaggata	60	70	*F_hACSL5*	ttcctgtctcttgcataaaggtt	59	95
R_hVCAN	cagggattagagtgacattcatca	60		*R_hACSL5*	ccaattcggagatgatccac	60	
F_hMIER3	ttgaggaaggaaataatgattggt	60	113	*F_hCTHRC1*	ccaaggggaagcaaaagg	60	74
R_hMIER3	caccaaagtaactggtcttcgtt	59		*R_hCTHRC1*	cccttgtaagcacattccatta	59	

tumour and healthy mucosa (anatomically the most distant tissue which was macroscopically healthy, usually in the range of 15-20 cm from the tumour location) were collected. Samples were immediatelly frozen in cryotubes (Thermo Fisher Scientific, Waltham, MA, USA) and stored at –80°C.

Isolation of RNA and quality validation. RNA was isolated from frozen samples using the standard protocol for the RT Trisol Reagent (Molecular Research Center, Cincinnati, OH, USA). The tissue was removed from the cryotube and placed in liquid nitrogen in a mortar. The tissue was pulverized and transferred into an Eppendorf tube with 1ml of chilled Trisol RT and total RNA was isolated according to the manufacturer's protocol. Total isolated RNA was dissolved in nuclease-free water (Ambion, Carslbad, CA, USA). RNA concentration was assessed by absorbance measurement using the Infinite M200 (Tecan, Männedorf, Switzerland) in the NanoQuant setting. Only samples with a 230 nm/260 nm ratio >1.7 and samples with proper bands without degradation on agarose gel electrophoresis were used for further analysis. Selected samples were additionally analysed by measurement of RNA integrity number (RIN) using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Reverse transcription (DNase, PCR control). cDNA was synthetised using 500 ng of total RNA in 20 µl reaction by RevertAid First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA) according to the manufacturer's protocol. Before reverse transcription, isolated RNA was treated for 5 min at room temperature with DNaseI (Top-Bio, Vestec, Czech Republic) to remove potential traces of genomic DNA. For priming of reverse trancription, we used a combination of oligo$(dT)_{18}$ and random hexamer primers each at 2.5 µM final concentration. The quality of cDNA and possible contamination by genomic DNA was assessed by control PCR reaction (*GAPDH* amplification, 40 cycles) and agarose gel electrophoresis.

Quantitative real-time PCR. For the quantitative PCR we used Power SYBR Green PCR master mix (Life Technologies, Carslbad, CA, USA). cDNA was diluted to final concentration of 0.5 ng/µl and 4 µl of cDNA were used in each reaction. Optimal cycling parameters and annealing temperatures were assessed by the measurement of sensitivity, specificity and efficiency of individual quantitative PCR reactions. After this initial analysis, genes were divided into two groups based on their ideal annealing temperature (58°C for *CLDN23, SLC26A2, VSNL1, CAPN10, VCAN* and *MAPK1*; 60°C for *LGR5, DSTN, MIER3, ACSL5, CTHRC1* and *SAMD3*). The instrument used for the analysis was 7500 Fast (Life Technologies). Cycling parameters were: initial hold at 50°C for 20 s and initial denaturation at 95°C for 10 min followed by 42 cycles consisting of denaturation at 95°C for 15 s and annealing and polymeration at 58°C (60°C) for one minute. Results were analysed by the 7500 instrument software and basic statistical analysis was carried out using the REST2009 software (Qiagen, Hildesheim, Germany).

Collection of clinical information. Clinical data were retrieved from the patient's records. We focused on the pathological examination of the samples (pTNM classification, grading and histological type of tumour) and on the patient's data (gender, age, date of diagnosis and surgical intervention and date of the last follow-up examination, recurrence, death). All data were anonymised.

Statistical analysis. Since the normality of the measured expression levels is not certain, nor can it be reliably tested given the limited sample size, non-parametric statistical methods, which do not require any assumption regarding the distribution shape, were preferred during the analysis.

First the differences in the candidate genes expressions between tumorous and healthy tissue were tested for significance using the Wilcoxon signed-rank test.

Then a series of two-sample survival analyses was performed in order to investigate possible relations between the expression data (healthy tissue expression level, tumorous tissue expression level and tumorous/healthy tissue expression ratio for each candidate gene) and patient survival (calculated from the day of surgery). The two patient groups to be compared were formed independently for each variable based on its median value. In cases where patients were later removed from the analysis due to missing data or when only a subset of patients was analyzed, the median value used to divide patients into two groups was kept at its original value. The relation between the expression variables and overall survival (OS) time was analyzed for all the patients and also in the subsets of

Table III. *Clinical and pathological description of the analysed group.*

	Group A (palliative) n=25	Group B (adjuvant) n=28	*p*-Value	Test
Gender (males/females)	17/8	19/9	1	Fisher's exact
Age at diagnosis (years)			0.485	Mann-Whitney *U*
Median	65	63.,5		
Interquartile range	7	12		
Tumour size			0.989	Pearson Chi-square
T2	1	1		
T3	19	21		
T4	5	6		
Lymph node involvment			0.681	Pearson Chi-square
N0	7	11		
N1	11	10		
N2	7	7		
M Classification			<0.001	Fisher's exact
M0	0	28		
M1	25	0		
Grade			0.101	Pearson Chi-square
G1	4	3		
G2	20	18		
G3	1	7		
Primary tumour localization			0.025	Fisher's exact
Colon	9	19		
Rectosigmoideum or rectum	16	8		

adjuvantly and palliatively treated patients separately. In the group of patients treated adjuvantly, the correspondence between the expression data and the disease-free interval (DFI) after surgery was also investigated. The date of disease recurrence was determined as the average of the date of the last negative and the first positive examination if the interval between the examinations was 180 days or less. In cases of a longer examination interval, the recurrence date was set 90 days before the first positive examination based on previous methodology (17). During each analysis, Kaplan-Meier curves were plotted for the two patient groups based upon the variable of interest and the significance of the survival (or DFI) difference was tested by Gehan-Wilcoxon, Cox-Mantel and "log-rank" test.

To examine the possible relation between tumour localisation and candidate gene expression, the patients were first divided into two groups according to tumour localization (first group with the CRC of the colon, second group with CRC of the rectum and rectosigmoid). The candidate gene expression levels (in both tumour and healthy tissue) and expression ratios were then tested for significant differences between the groups using Mann-Whitney *U*-test.

Correlations of expression variables were investigated using Spearman's correlation coefficient with appropriate significance test.

All the analyses were performed in STATISTICA (StatSoft, Tulsa, OK, USA).

Results

In the present study, we analysed the expression of 12 genes in a sample set of 53 patients. Patients were divided into two groups, palliative and adjuvant (25 and 28 patients, respectivelly), depending on the presence or absence of distant macrometastases at the time of surgery. Clinical and pathological description of both groups is summarised in Table III. Both groups differed clinically in overall survival, with patients of the adjuvant group having longer survival than those in the palliative group.

Different RNA expression between tumour tissue and healthy mucosa. In the set of 12 selected genes, 10 exhibited a difference in expression level between tumour and normal tissue in the group of all patients. Four (*LGR5, CTHRC1, VSNL1* and *VCAN*) were up-regulated in tumour, six (*DSTN, MIER3, ACSL5, MAPK1, CLDN23* and *SLC26A2*) were down-regulated (Figure 1). Expression differences in both subgroups were similar to those obtained for the whole set of patients, except for *DSTN*, the expression of which was not different in the palliative group, but was down-regulated in the adjuvant group.

Correlation between candidate gene expressions and clinical data. Dependence of clinical characteristics on relative expression levels of candidate genes was statistically analyzed by the tests described above. We did not find any significant result at a *p*-value of 0.05 for correlation with age, individual parameters of TNM classification and tumour grading.

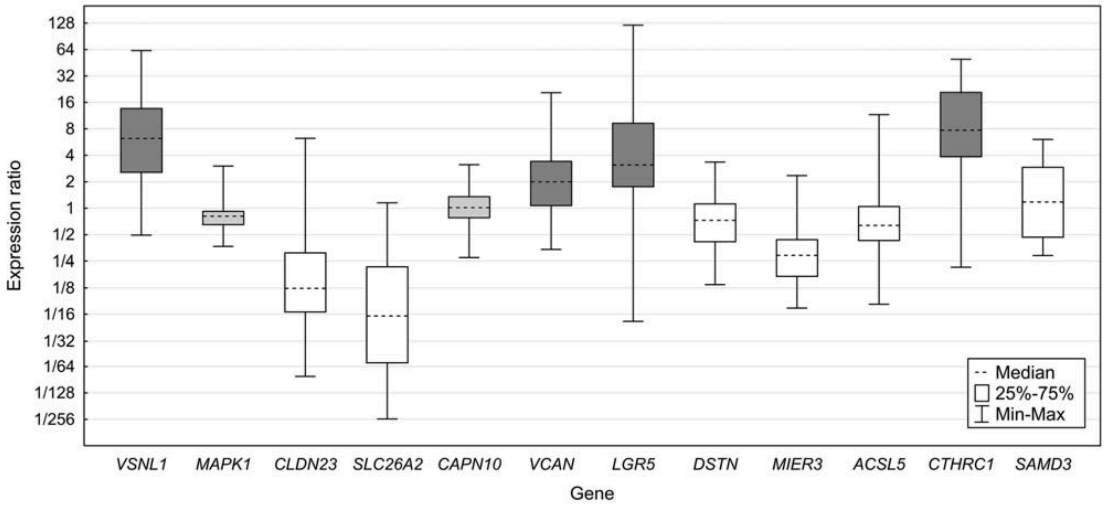


Figure 1. *Expression of candidate genes in tumour tissue relative to healthy tissue. Genes in dark grey were up-regulated in tumour tissue, genes in light grey did not change in expression and genes in white were down-regulated in tumour tissue.*

When investigating the dependence of overall survival and disease-free survival on relative gene expression, in the overall group of patients, we found correlation of *VSNL1* expression with OS (significantly longer OS was observed among patients with *VSNL1* expression level in tumour tissues above the median value; Cox-Mantel p=0.033 and log rank p=0.032) (Figure 2A).

In the adjuvant group, patients with *SLC26A2* expression above the tumour tissue median had a longer OS than those with an expression level below the median (Cox-Mantel p=0.036) (Figure 2B). In the case of DFI, patients with *VSNL1* expression in the healthy tissue below the median had a longer DFI than patients with higher expression (Cox-Mantel p=0.022, log-rank p=0.020) (Figure 2C). The opposite trend was observed for *SLC26A2* (Cox-Mantel p=0.012, Wilcoxon p=0.014 and log-rank p=0.011) (Figure 2D).

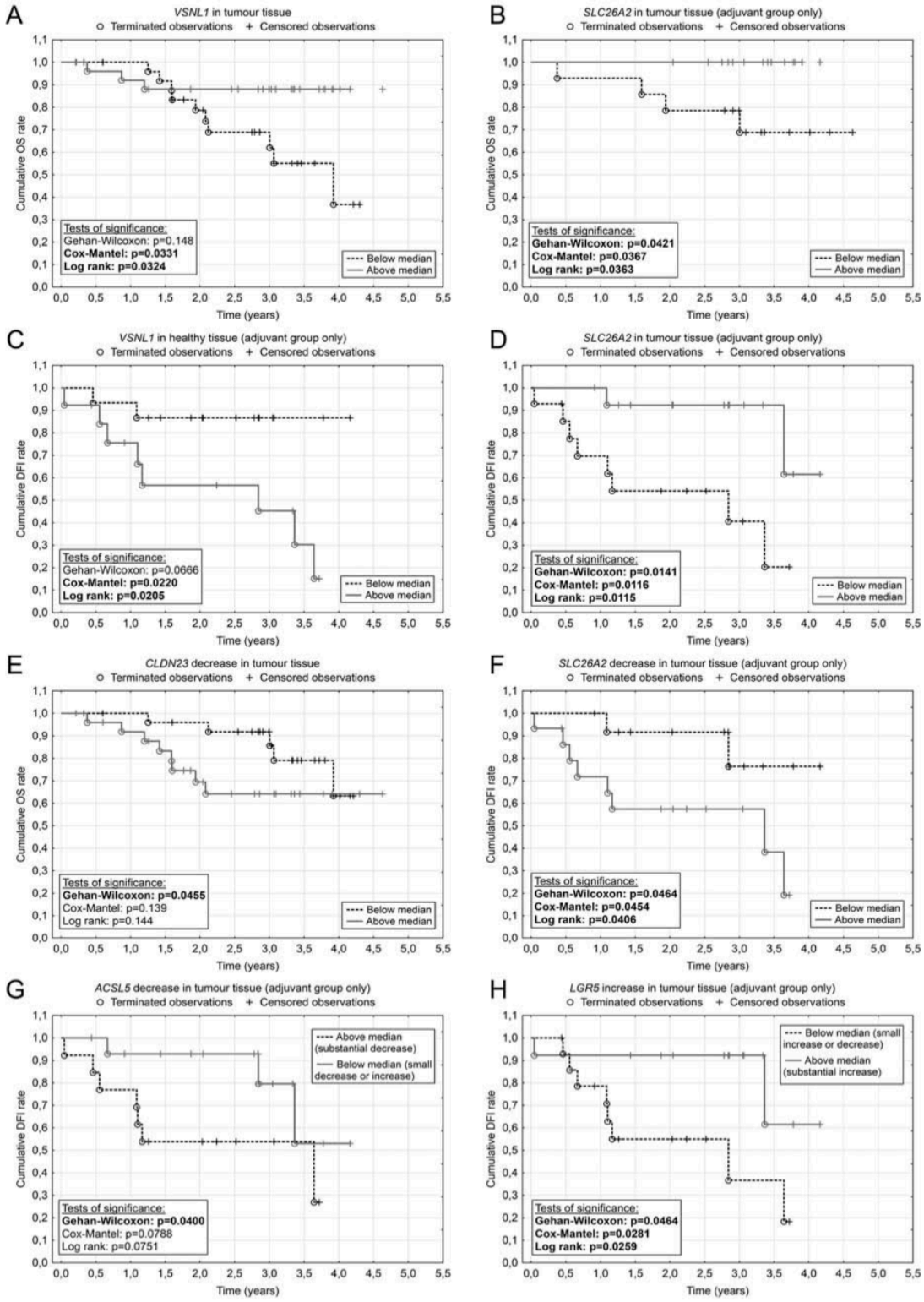
Then we analyzed the OS and DFI of the patients with respect to the difference in candidate gene expression between healthy and tumour tissues. In the overall group of patients, we found that the greater the down-regulation of *CLDN23* expression in tumour, the worst the outcome for the patient in terms of shorter OS (Wilcoxon p=0.045) (Figure 2E). In the adjuvant group, a smaller decrease of *SLC26A2* and *ACSL5* expression in tumour led to a longer DFI (Wilcoxon p=0.046, Cox-Mantel p=0.045 and log-rank p=0.041 for *SLC26A2*, and Wilcoxon p=0.040 for *ACSL5*) (Figure 2F and 2G) as did a greater increase of *LGR5* expression (Wilcoxon p=0.046, Cox-Mantel p=0.028 and log-rank p=0.026) (Figure 2H).

Impact of tumour location on candidate gene expression. Patients were divided into two groups according to the location of the primary CRC and gene expression was analysed using the Mann-Whitney *U*-test. The groups did not exhibit any significant difference in candidate gene expression in tumour tissue, but we found differential expression of *MAPK1* (p=0.032), *LGR5* (p=0.003), *MIER3* (p=0.026) and *CTHRC1* (p=0.042) in healthy mucosa. All of these genes were expressed more highly in the group of patients with tumours localised in the colon. Regarding the difference in expression, we found a significant difference in *VCAN* expression change between patients with colon localisation (smaller increase) and those with rectosigmoid/rectum-localised tumours (greater increase) (p=0.031).

Discussion

The relationship between the change of gene expression and potential functional effects is not usually straightforward. In the following paragraphs, we briefly summarise present knowledge about the individual genes analyzed here and discuss our results with respect to current literature, focusing on possible explanations of the role of these expression changes on the clinical behaviour of colorectal tumours. Because of the heterogeneity of the selected genes, we discuss individual genes separately.

VSNL1. This gene encodes neuronal calcium sensor protein which can modulate the activity of adenylate cyclase (18) and


A
VSNL1 in tumour tissue
o Terminated observations + Censored observations
Cumulative OS rate
Tests of significance:
Gehan-Wilcoxon: p=0.148
Cox-Mantel: p=0.0331
Log rank: p=0.0324
Below median
Above median
Time (years)
B
SLC26A2 in tumour tissue (adjuvant group only)
o Terminated observations + Censored observations
Cumulative OS rate
Tests of significance:
Gehan-Wilcoxon: p=0.0421
Cox-Mantel: p=0.0367
Log rank: p=0.0363
Below median
Above median
Time (years)
C
VSNL1 in healthy tissue (adjuvant group only)
o Terminated observations + Censored observations
Cumulative DFI rate
Tests of significance:
Gehan-Wilcoxon: p=0.0666
Cox-Mantel: p=0.0220
Log rank: p=0.0205
Below median
Above median
Time (years)
D
SLC26A2 in tumour tissue (adjuvant group only)
o Terminated observations + Censored observations
Cumulative DFI rate
Tests of significance:
Gehan-Wilcoxon: p=0.0141
Cox-Mantel: p=0.0116
Log rank: p=0.0115
Below median
Above median
Time (years)
E
CLDN23 decrease in tumour tissue
o Terminated observations + Censored observations
Cumulative OS rate
Tests of significance:
Gehan-Wilcoxon: p=0.0455
Cox-Mantel: p=0.139
Log rank: p=0.144
Below median
Above median
Time (years)
F
SLC26A2 decrease in tumour tissue (adjuvant group only)
o Terminated observations + Censored observations
Cumulative DFI rate
Tests of significance:
Gehan-Wilcoxon: p=0.0464
Cox-Mantel: p=0.0454
Log rank: p=0.0406
Below median
Above median
Time (years)
G
ACSL5 decrease in tumour tissue (adjuvant group only)
o Terminated observations + Censored observations
Cumulative DFI rate
Above median (substantial decrease)
Below median (small decrease or increase)
Tests of significance:
Gehan-Wilcoxon: p=0.0400
Cox-Mantel: p=0.0788
Log rank: p=0.0751
Time (years)
H
LGR5 increase in tumour tissue (adjuvant group only)
o Terminated observations + Censored observations
Cumulative DFI rate
Below median (small increase or decrease)
Above median (substantial increase)
Tests of significance:
Gehan-Wilcoxon: p=0.0464
Cox-Mantel: p=0.0281
Log rank: p=0.0259
Time (years)

has been described to be a prognostic marker in Alzheimer's disease (19, 20). VSNL1 is also considered to be a potentiator of invasiveness and inhibitor of proliferation of neuroblastoma cells (21), a tumour suppressor in non-small cell lung carcinomas (22), and an inhibitor of epithelial-to-mesenchymal transition in squamous carcinoma cells (23).

In colon cancer, *VSNL1* was found to be down-regulated after selenomethionine-induced growth arrest of the colon cancer cell line HCT116 (24) and it was described to be a putative marker for identification of circulating tumour cells in peripheral blood (25). We did not observe the correlation of *VSNL1* overexpression with lymph node metastasis that was described earlier (26), but we confirmed overexpression of *VSNL1* in tumour tissue compared to healthy mucosa, and, more importantly, we found a relation between higher expression of *VSNL1* in tumour tissue and longer survival of patients, hence our results should support the possible function of *VSNL1* as tumour suppressor as reported for other types of tumour (22, 27). The opposite effect of *VSNL1* expression in healthy tissue on DFI is the subject of further investigation.

VCAN. Versican protein, encoded by the *VCAN* gene, is one of the major components of the extracellular matrix (ECM) with various functions in the regulation of cell migration (28), proliferation (29) and cell adhesion (30). *VCAN* was shown to play a role in many types of cancer, including gastric (28), pancreatic (31) and colorectal (32).

We found an up-regulation of *VCAN* mRNA in tumour, which is in agreement with previously described changes of expression on the protein level, which was accompanied by altered post-translation modifications (32, 33). The level of up-regulation found here was different depending on the tumour location (colon *versus* rectosigmoid/rectum).

LGR5. Leucine-rich repeat-containing G-protein-coupled receptor 5 protein is a wingless-type MMTV integration site family (WNT) signaling target and a marker of intestinal (34) and liver stem cells (35). Expression of *LGR5* is increased in intestinal tumours, and can be found in cancer cells with stem cell properties (36, 37).

In our study, we observed an up-regulation of *LGR5* expression in tumour tissue, and a greater increase of expression in tumour tissue compared to healthy tissue correlated significantly with a longer DFI in adjuvantly treated patients, which is contradictory to previously published data, where overexpression in malignant tissue was shown to have adverse effects on disease outcome in CRC and other types of tumours (38, 39, 40). This finding needs to be further evaluated.

CTHRC1. *CTHRC1* exhibited the highest up-regulation in tumour tissue among the genes we analyzed. *CTHRC1* gene encodes a protein involved in vascular remodeling (41) and an expression change has been described in many types of solid tumours (42), including CRC (43). Recently, the expression status of *CTHRC1* was identified as being a predictor of poor prognosis in CRC patients (44). We confirmed the expression change, but we were not able to significantly prove the prognostic role of *CTHRC1* expression at a p-value below 0.05. Nevertheless, we identified a trend for shorter survival of patients with higher *CTHRC1* expression levels in tumour tissues.

SLC26A2. In mammals, there are 11 genes of the SLC26 family, which function as anion exchangers or channels. The main role of SLC26A2 is to transport SO_4^{2-} anions and its altered function was related to several types of chondrodysplasias (45).

Lower expresion of *SLC26A2* was related to enhanced proliferation of colon cancer cells *in vitro* (46). A decrease of expression was also observed in bioptic samples (47), and we detected a severe down-regulation of *SLC26A2* in tumour tissue. Based on these data, we can conclude that *SLC26A2* down-regulation is also important for tumour propagation *in vivo*. The expression level of *SLC26A2* was associated with the decrease of OS and DFI in the adjuvantly treated group of patients. A probable effect of down-regulation of *SLC26A2* is disorganisation of the ECM by improper sulfation of ECM proteins, therefore helping tumour cells to migrate through the tissue.

CLDN23. This gene belongs to the claudin family of 24 genes encoding proteins with four transmembrane domains involved in formation of tight junctions among adjacent cells (48). *CLDN23* itself was described to be down-regulated in intestinal-type gastric cancer (49), but there are no studies focused on this gene in a larger set of patients with colon cancer.

←

Figure 2. *Kaplan-Meier curves of statistically significant associations between gene expression and disease-free survival (DFI)/overall survival (OS). Relation of the gene expression in the tumour (TT) and healthy (HT) tissue to the DFI and OS (A-D). Relation of gene expression change to the DFI and OS (E–H). Patients were divided by the median of the expression change. In the case of ACSL5, one group consisted of patients with substantial decrease of ACSL5 expression and the second group consisted of patients with slight decrease or increase of ACSL5 expression. Similarly, for LGR5, we grouped patients with small increase or decrease of expression and compared them with patients with a substantial increase in LGR5 mRNA. A: Visinin-like 1 (VSNL1); B: Solute carrier family 26 (sulfate transporter), member 2 (SLC26A2); C: Visinin-like 1 (VSNL1); D: Solute carrier family 26 (sulfate transporter), member 2 (SLC26A2); E: Claudin 23 (CLDN23); F: Solute carrier family 26 (sulfate transporter), member 2 (SLC26A2); G: Acyl-CoA synthetase long-chain family member 5 (ACSL5); H: Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5).*

In our set of patients, *CLDN23* was down-regulated in tumour tissue and we found that the level of down-regulation correlates with the OS: a smaller difference of *CLDN23* expression between tumour and normal tissue was associated with a better prognosis. This effect can be explained by the fact that for the migration of tumour cells, it is neccessary to break the bonds between adjacent cells and strong down-regulation of tight junction proteins, including CLDN23, is one such crucial step in this proscess. Therefore, patients with very low *CLDN23* expression would be more prone to progression of CRC.

ACSL5. This gene encodes an enzyme implicated in lipid biosynthesis and fatty acid degradation. *ACSL5* down-regulation was associated with small intestine carcinoma (50), but *ACSL5* was found to be up-regulated in CRC by microarray analysis (51). In gliomas, it can be considered as a cancer survival factor (52). The function of ACSL5 is probably dependent on the particular splice variant, since the full-length ACSL5 is pro-apoptotic (53). Our analysis found *ACSL5* to be down-regulated in tumour tissue, and we also identified that the greater the decrease, the shorter the DFI observed in the adjuvantly-treated group of patients, assuming a protective function for ACSL5.

DSTN, MIER3 and *MAPK1*. These three genes were found to be down-regulated in tumour tissues, but in our set of samples, we did not identify any correlation with clinical characteristics.

The product of the *DSTN* gene is the actin-binding protein destrin, also known as actin-depolymerizing factor, which has an important function in regulation of actin dynamics (54). Regarding the role of DSTN in cancer development and progression, there are several pieces of information about its role in membrane androgen receptor-induced apoptosis of prostate cancer cells (55), and its positive regulation of the migration of neuroblastoma cells (56); the down-regulation of DSTN also blocks migration and invasive capacity of gastric cancer (57). We found a slight down-regulation in tumour tissue, but this change did not correlate with any clinical parameter.

MIER3 is a practically unresearched gene, but in 2012, it was shown that it probably plays a role in tumourigenesis because it was described as a candidate breast cancer susceptibility gene (58) and also a highly mutated gene in hypermutated colorectal tumours (59). Our data suggest down-regulation in CRC, but without strong correlations to clinical data.

Mitogen-activated protein kinase 1 is the final kinase in the MAPK pathway, which has a pleiotropic role like regulation of proliferation, differentiation and gene transcription (60). In CRC, down-regulation of MAPK1 was shown in a previous study (61), as well as in our set of samples.

CAPN10 and SAMD3. The function of sterile alpha motif domain-containing 3 is unknown; this gene was selected because of its possible up-regulation during CRC development (13). This gene is interesting regarding its observed interaction with Fanconi anemia, complementation group G (FANCG) (62), which plays a role in the maintanence of genomic integrity (63), but we did not observe any change of its expression. This was also the case with CAPN10, a member of the calcium activated cystein proteases (64). Particular single nucleotide polymorphisms in the *CAPN10* gene were shown to be associated with type 2 diabetes mellitus (65), and another was also associated with CRC susceptibility (66).

Conclusion

We have described significant expression changes of 10 studied genes in the CRC tissue compared to healthy mucosa. We also identified associations between different gene expressions and overall and disease-free survival, which could provide with useful information for disease prognosis and patient follow-up. We identified several putative positive prognostic factors, such as ACSL5 and CLDN23, in tumour tissues. Our results will serve for the development of a disease risk scoring system based on the expression of these selected genes. This system will be validated and evaluated on a new group of patients with CRC. The most promising genes (mainly up-regulation of SLC26A2 and down-regulation of *CLDN23* and *ACSL5*) will be further studied individualy on a larger set of patients.

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Post-Treatment Recovery of Suboptimal DNA Repair Capacity and Gene Expression Levels in Colorectal Cancer Patients

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DNA repair in blood cells was observed to be suboptimal in cancer patients at diagnosis, including colorectal cancer (CRC). To explore the causality of this phenomenon, we studied the dynamics of DNA repair from diagnosis to 1 yr follow-up, and with respect to CRC treatment. Systemic CRC therapy is targeted to DNA damage induction and DNA repair is thus of interest. CRC patients were blood-sampled three times in 6-mo intervals, starting at the diagnosis, and compared to healthy controls. DNA repair was characterized by mRNA levels of 40 repair genes, by capacity of nucleotide excision repair (NER), and by levels of DNA strand breaks (SBs). NER and base excision repair genes were significantly under-expressed ($P < 0.016$) in patients at diagnosis compared to controls, in accordance with reduced NER function ($P = 0.008$) and increased SBs ($P = 0.015$). Six months later, there was an increase of NER capacity, but not of gene expression levels, in treated patients only. A year from diagnosis, gene expression profiles and NER capacity were significantly modified in all patients and were no longer different from those measured in controls. All patients were free of relapse at the last sampling, so we were unable to clarify the impact of DNA repair parameters on treatment response. However, we identified a panel of blood DNA repair-related markers discerning acute stage of the disease from the remission period. In conclusion, our results support a model in which DNA repair is altered as a result of cancer.

Key words: Colorectal cancer; DNA instability; DNA repair; biomarker; anti-cancer therapy; follow-up study

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequently occurring malignancies in Western countries. The genetic basis of hereditary forms (~6% of all cases) is relatively well explored while for sporadic forms (~75% of all cases) there is still a lack of knowledge about the genetic–epigenetic–environmental triggers of the disease. More importantly, CRC has high mortality worldwide due to the insufficient treatment efficacy and a lack of predictive markers [1]. Only approximately half of CRC patients will be cured using currently available therapies [2]. A commonly used conventional regimen for CRC treatment is based on the synergistic action of two DNA-damaging agents: 5-fluorouracil (5FU) and oxaliplatin. In response to this treatment, cells activate a range of resistance-promoting mechanisms including the DNA repair pathways [3]. Some of the optimal biomarkers might be thus found within the DNA repair network.

Abbreviations: 5FU, 5-fluorouracil; BPDE, (+)-anti-Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide; BER, base excision repair; CRC, colorectal cancer; CV, coefficient of variation; DDR, DNA damage response; HR, homologous recombination; MMR, mismatch repair; NER, nucleotide excision repair; PBMCs, peripheral blood mononuclear cells; PCA, principal component analysis; SBs, strand breaks.

Alessio Naccarati and Pavel Vodicka contributed equally to this work.

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The present study represents a continuation of our previous report, where we showed that peripheral blood mononuclear cells (PBMCs) collected from CRC patients at the time of diagnosis exhibited elevated genetic instability. Specifically, they had increased levels of DNA strand breaks (SBs) and low DNA repair capacity accompanied by altered expression of repair genes [4]. The suboptimal DNA repair capacity in PBMCs of cancer patients is now well documented and observed in multiple cancer types [4]. Thus, there is a large body of evidence obtained in case–control studies supporting the association between cancer and a decrease in the ability of blood cells to protect against DNA damage. However, a sampling of the patients at one time point does not bear information about the causality of this phenomenon and so it remains to be established whether low DNA repair is one of the susceptibility factors for sporadic CRC, or it is a consequential effect of the disease. On the other hand, it is known that DNA repair capacity detectable in PBMCs is significantly related to the repair capacity of colorectal tissue [5,6]. These observations give more confidence for using blood as a surrogate for cancer-target tissue to study DNA repair involvement in CRC. Subsequently, a few recent studies provided evidence that tumor tissue maintains comparable base excision repair (BER) capacity and slightly elevated capacity of nucleotide excision repair (NER) as compared to adjacent healthy colorectal mucosa, although there is a differential mRNA expression of DNA repair genes [6–8]. Impaired excision repair, thus, may not contribute to the malignant transformation of the colon, but rather might be involved in the treatment response of the patients.

Conventional therapy of CRC, based on the mutagenic properties of anticancer drugs, is expected to be more harmful to fast growing tumor cells than to normal cells. However, details of the effects of radiotherapy and chemotherapy on the cellular and molecular functions of the tumor and normal cells are still unexplored. Almost all DNA repair pathways are presumably involved in the cellular response to CRC treatment. 5FU-mediated DNA lesions are recognized by the BER and the mismatch repair (MMR) systems [9]. Oxaliplatin binds to nucleobases forming intra- and inter-strand crosslinks. The former are eliminated mainly by the NER pathway while the latter require NER together with cross-link repair activity, translesion synthesis, and homologous recombination repair (HR) [10]. Understanding the involvement of DNA repair processes in the response of cancer cells to antineoplastic drugs is crucial for the design of improved therapy regimens and for the prediction of therapeutic response in CRC.

The present exploratory study was aimed to address several questions: (i) Do variations in DNA repair contribute to the risk of developing sporadic CRC, or are they rather a consequence of the systemic disease? (ii) Is the DNA repair response to CRC treatment measurable at the mRNA/functional level? (iii) Is it induced or suppressed by the systemic genotoxic exposure mediated by chemotherapy? In an attempt to answer these questions, we have designed a prospective study in which sporadic CRC patients were blood-sampled at the diagnosis (i.e., active disease), 6 mo, and 1 yr later (i.e., covering the tumor resection, administration of chemotherapy, either neoadjuvant or adjuvant, and remission) and were compared with an healthy population. The dynamics of DNA repair over a 1 yr period and with respect to ongoing CRC treatment were analyzed. All main repair pathways including excision repair (BER, NER, and MMR), repair of double-strand breaks (HR and non-homologous end joining), and DNA damage response (DDR) were characterized at gene expression levels. NER, the most deregulated pathway in patients observed by us, was also studied using a functional assay and through measuring SBs accumulation.

MATERIALS AND METHODS

Study Population

Incident CRC cases were recruited in the Czech Republic between 2008 and 2010. Patients were newly diagnosed and histologically confirmed for CRC. Eighty-three patients were initially recruited for the study but only 39 (47%) attended all the three planned blood samplings. Reasons for dropout were: (i) 7 (8%) patients died before third sampling, (ii) 10 (12%) patients moved and were treated in other hospitals or interrupted therapy for unspecified reasons, (iii) 19 (23%) patients missed one out of the three samplings or did not attend regular hospital follow-up at agreed times, or the material provided did not meet the quality standards required, and (iv) 8 (10%) patients were excluded because they were found not to be primary cancer cases. No other selection of patients was performed. Forty-seven controls were recruited among individuals who had never been diagnosed with any cancer and did not manifest any relevant systemic disease, nor had any known significant exposure to potentially harmful chemicals. Study subjects were all of Caucasian origin. They signed a written informed consent with the study in accordance with the Helsinki declaration. The Ethics committees of participating hospitals approved the study. Trained personnel interviewed the study subjects using a structured questionnaire for lifestyle habits, body mass index, diabetes, and family/personal history of cancer. Expression analysis and functional assays could not be performed on all patients, due to various reasons, that is, viability of PBMCs or quality of RNA. For each analysis, the actual number of examined cases is therefore specified.

Study Design

Blood samples were collected from patients three times: at diagnosis, that is, before tumor resection and

administrating any therapy (T0); 6 mo after tumor resection, that is, approximately 6 mo from administration of chemotherapy (T1); and after 1 yr, during the regular follow-up appointment (T2). At T1 all patients had completed the planned chemotherapy, except for seven patients who received chemotherapy for additional 1 or 2 mo. In eight patients, no therapy was administered. At T2, all patients were free of relapse.

Blood Processing

Blood samples were drawn into heparin and EDTA vacutainers, and kept at 4°C until processed within 3 h. PBMCs from EDTA tubes were isolated on Ficoll–Paque PLUS (GE Healthcare Life Sciences, Prague, Czech Republic) and stored in TRIzol (Invitrogen, Carlsbad, CA) at −80°C until RNA extraction. PBMCs from heparin tubes were isolated on Histopaque-1077 (Sigma–Aldrich, St. Louis, MO), counted, suspended in full medium (RPMI 1640, 2 mM L-glutamine, 10% FBS, 0.2% penicillin/streptomycin, 1.5% phytohemagglutinin) and incubated at 37°C for 20 h to stimulate mitosis. PBMCs were then processed for DNA damage and DNA repair analysis.

DNA Strand Breaks

The level of SBs in DNA was evaluated by the alkaline comet assay [11]. Experimental conditions were as follows: lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% TritonX-100, 10% DMSO, pH 10, ≥1 h, 4°C), alkali treatment (300 mM NaOH, 1 mM EDTA, pH 13, 20 min, 4°C), electrophoresis (300 mM NaOH, 1 mM EDTA, pH 13, 20 min, 4°C, 1.3 V/cm), and neutralization (0.4 M Tris–HCl, pH 7.5, 2 × 10 min). Data are reported as tail DNA%, determined as an average of 100 randomly selected comets from two parallel slides per experimental point. Repeatibility of the assay was checked by repeated measuring of 12 randomly chosen samples and obtained values were in agreement (Spearman's $R = 0.76$, $P = 0.028$).

Nucleotide Excision Repair Capacity

NER capacity was analyzed as the level of SBs generated by the incision of (+)-anti-Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE)-adducts in cultured PBMCs of study participants. The detailed protocol is described in Slyskova et al. [11]. Briefly, PBMCs were treated with 1 μM of BPDE for 30 min at 37°C. BPDE was washed out and PBMCs were further cultured and harvested immediately after the treatment and at 1, 2, and 4 h after the treatment. Untreated PBMCs were cultured in parallel. For each time point, the SBs level of the untreated PBMCs was subtracted. The NER capacity was expressed as the difference between the level of SBs measured immediately after the BPDE treatment, and the highest level of SBs detected within 4 h of culturing. Data are presented as tail DNA%. Repeatibility of the assay was checked by repeated measuring of 16 samples and obtained results were in agreement (Spearman's $R = 0.61$, $P = 0.012$).

Gene Expressions

A panel of 40 genes (Supplementary Table S1) were selected from the list of all known DNA repair genes [12]. Total RNA was isolated using TRIzol (Invitrogen). RNA integrity was between 8.0 and 10.0 units. cDNA was synthesized from 0.5 μg of RNA using the RevertAid™ First strand cDNA synthesis kit (Fermentas, Ontario, Canada). cDNA was preamplified and qPCR was performed using the BioMark™ HD System (Fluidigm) and using FAM-MGB assays (Primer Design, Southampton, UK) as described previously [6]. *TOP1* was the reference gene selected by Normfinder using GenEx Enterprise software (MultiD, Goteborg, Sweden). Data were converted to relative quantities and transformed to $\log_2$ scale. The repeatibility of gene expression assays was calculated by mixed ANOVA, comparing the values obtained in two different experiments in 2 days for each assay. All assays showed high degree of precision. The results are reported in Supplementary Table S1 for each assay separately.

Data Analysis

Statistical analysis was conducted using R environment version 2.15 (open tool). Gene expression data were pre-processed with GenEx Enterprise. DNA damage and DNA repair capacity were consistent with the Gaussian distribution, and so were the gene expression data when log transformed. Student's *t*-test, paired *t*-test, ANOVA and Pearson's rank correlation coefficient were used to compare means and calculate bivariate correlations. Statistical tests were performed at 5% level of statistical significance. Gene expression data are presented as fold-changes relative to the reference samples, calculated using linear models and the empirical Bayes method as implemented in the "limma" package in the Bioconductor suite (open tool). *P*-values were adjusted according to the Benjamini–Hochberg method. Principal Component Analysis (PCA), Dynamic PCA, Hierarchical Clustering (HCL), and Kohonen self-organizing map analysis (SOM) of size 2 × 1, with parameters: 0.1 learning rate, 2 neighbors, and 3000 iterations were used to analyze gene expression profiles between patients and controls and between different samplings in patients. The *k*-means algorithm was used to classify genes into clusters based on their expression profiles over the three time points, considering the patients clinical characteristics.

RESULTS

Study Population

Patients and controls did not differ significantly for any recorded confounders, except for family history of cancer, which was more prevalent among patients ($P = 0.01$). The clinical, biological, and lifestyle

characteristics of the study population, as well as an overview of the administered therapies, are summarized in Table 1. All patients underwent surgical resection of the tumor. Eighteen patients (46%) received neoadjuvant therapy prior to resection. Neoadjuvant therapy included the following regimens: two patients received radiotherapy (6 MeV X-rays, 45–46 Gy total dose in 23–25 fractions), three patients received preoperative chemotherapy without radiotherapy (FOLFOX or capecitabine), and 13 patients received a combined regimen. Twenty-two patients (56%) received adjuvant systemic therapy; nine of them were given 5FU-based therapy, while 13 received a combination of 5FU and oxaliplatin. Adjuvant therapy was given over a period of 1–8 mo. Tumor relapse after the treatment was not detected in any of the patients. Eight patients did not receive any systemic treatment.

Case–Control Study

DNA repair genes expression

Twenty-seven CRC cases and 38 controls were analyzed for the expression of 40 DNA repair genes, sampled at diagnosis and before any treatment intervention (T0). Patients had different expression levels of BER and NER genes compared to controls: four out of nine BER genes (*APEX1*, *NTHL1*, *PARP1*, and *MPG*) and 10 out of 19 NER genes (*RPA1*, *RPA2*, *RPA3*, *CDK7*, *DDB1*, *DDB2*, *XPA*, *XPD*, *ERCC8*, and *RAD23B*) had significantly decreased levels in patients. Only *LIG3* (BER) was increased in patients as compared to controls. Fold-changes and *P* values are reported in Supplementary Table S2. The dendogram in Figure 1, performed by HCL, discriminates between patients and controls by expression profiles of all 40 repair genes. The same discrimination was observed for the NER genes only (data not shown).

NER capacity and SBs

Functional assays were performed for 31 CRC cases and 38 controls. NER capacity was significantly lower in the CRC patients analyzed at T0 compared to the controls (mean ± SD; 9.9 ± 7.5 and 15.5 ± 13.6, respectively; *t*-test $P = 0.008$; Figure 2A), and the patients had significantly higher levels of SBs than

Table 1. Study Population Characteristics

Characteristics	Category	CRC patients ($n = 39$)	Controls ($n = 47$)	*P*-value
Sex	Female	15	24	0.25
	Male	24	23	
Age (years)	Mean ± SD	64.5 ± 10.5	64.2 ± 13.7	0.94
	Median	65	62.5	
	Range	32–81	33–87	
Body mass index	Mean ± SD	27.6 ± 4.4	26.1 ± 3.6	0.14
	Median	27.1	25.1	
	Range	17.6–37.3	20.7–34.7	
Smoking status	Non-smoker	27	38	0.72
	Smoker	7	8	
Alcohol consumption	No	11	17	0.74
	Yes	22	29	
Diabetes	No	26	43	0.08
	Yes	7	3	
Cancer in family	No	13	31	**0.01**
	Yes	21	15	
CRC in family	No	28	36	0.55
	Yes	3	6	
Tumor localization	Colon + rectosigmoideum	19	—	
	Rectum	20	—	
TNM staging	I	6	—	
	II	20	—	
	III	4	—	
	IV	9	—	
Grade	I	2	—	
	II	25	—	
	III	12	—	
Therapy	None	8	—	
	Neoadjuvant	18	—	
	Adjuvant	22	—	
	Neoadjuvant + adjuvant	11	—	
Regimen of adjuvant therapy	5FU	9	—	
	5FU + oxaliplatin	13	—	

Significant differences are shown in bold.

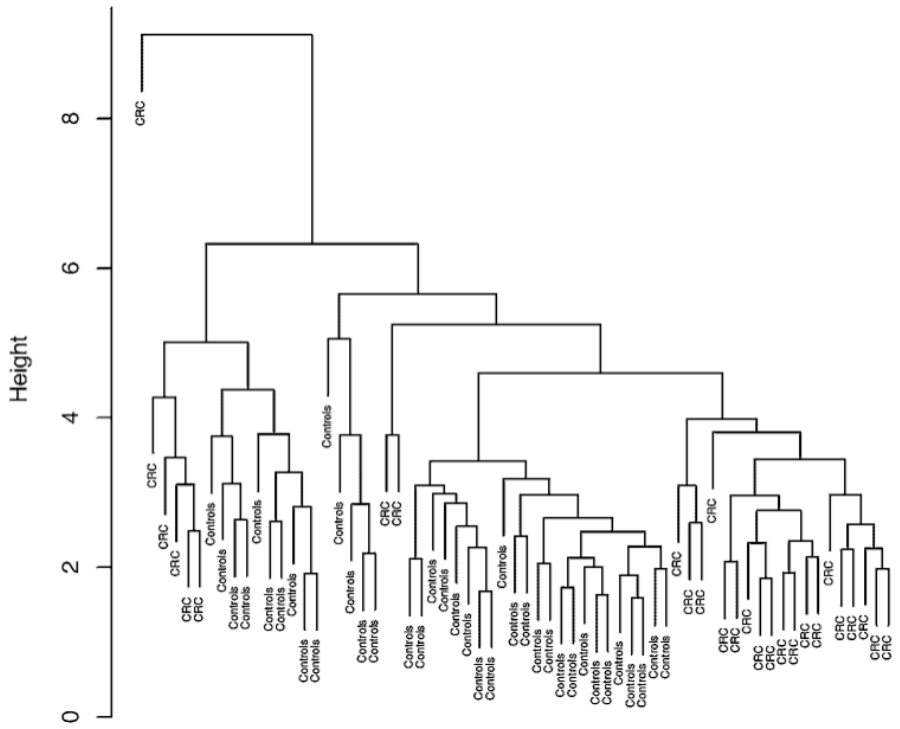

Figure 1. Hierarchical clustering of CRC patients sampled at the diagnosis (T0) and healthy controls according to the expression profiles of 40 DNA repair genes.

the controls (25.6 ± 21.4 and 13.9 ± 13.8, *t*-test $P = 0.015$; Figure 2B).

Longitudinal Study

Changes in DNA repair genes expression during 1 yr of follow-up

Twenty-seven patients analyzed at T0 were sampled two more times with at 6-mo intervals (T1 and T2). Expression of the majority of the studied genes did not change appreciably between T0 and T1 but substantially changed at T2. PCA of all 40 studied genes revealed similar expression patterns of samples obtained at T0 and T1 while the expression at T2 had a significantly different pattern and the samples were organized in a distinct cluster together with controls (Figure 3). To identify differentially expressed genes that best discriminate both clusters, dynamic PCA in combination with Kohenen SOM was used. Six genes (BER: *LIG3*, NER: *RPA3*, *CDK7*, *DDB2*, HR: *NBN*, and DDR: *CHEK2*) were found to be responsible for the aggregation of patients into two distinct clusters (Figure 4).

DNA repair genes expression over time in relation to therapy

The *k*-means algorithm was used to correlate the gene expression levels over time to treatment (untreated vs. treated patients) and to the different regimens of adjuvant therapy (5FU vs. 5FU + oxaliplatin). Four main clusters were generated based on the *Cq* delta values representing the differences between T1–T0 and T2–T1 (Supplementary Figure 1). A trend line for the four clusters was constructed and each cluster was also represented by a heatmap showing for each gene the expression values over time. The heatmap revealed great variability in the expression dynamics of the studied genes, particularly in the T1–T2 interval. The expression of the majority of the genes did not differ between treated and untreated patients, except for three genes. The BER gene *MUTYH* mRNA levels increased in adjuvantly treated patients but was reduced in untreated. *POLB* (BER) and *XPB* (NER) had constant expression over time in treated patients, but were upregulated from T0 to T2 in untreated patients (Supplementary Figure 1). No differences were observed between therapy regimens of 5FU + oxaliplatin versus 5FU only.

Changes in NER capacity and SBs during 1 yr of follow-up

NER capacity and SBs were compared between the three samplings in a group of 27 patients. NER capacity increased significantly between T0 and T1, and did not change further between T1 and T2 (T0: 9.9 ± 7.5, T1: 15.8 ± 13.2, T2: 11.2 ± 6.9, ANOVA $P = 0.002$; Figure 2A). NER capacity at T1 and T2 did

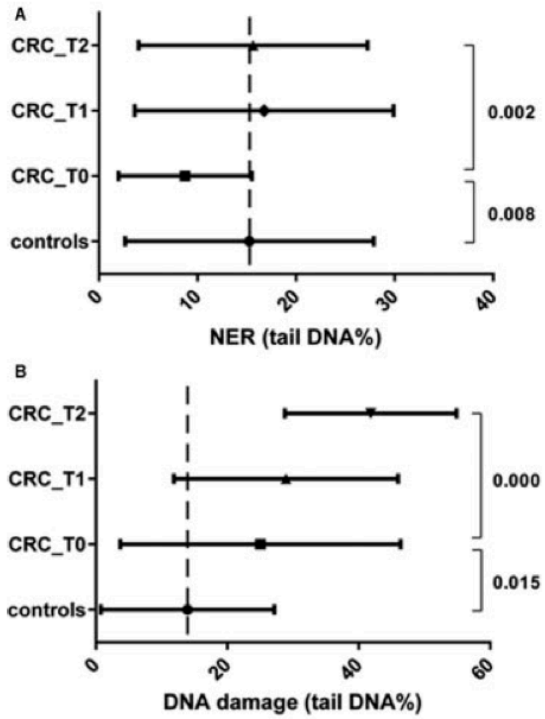

Figure 2. NER capacity (A) and SBs (B) in CRC patients measured in three consecutive samplings at 6-mo intervals starting from the diagnosis (T0, T1, and T2) and of healthy controls. Figure shows mean ± SD and ANOVA *P* values.

not differ from that in the controls (*t*-test $P = 0.87$ and $P = 0.10$, respectively). SBs increased gradually, with the largest rise being observed between T1 and T2 (T0: 25.6 ± 21.4, T1: 29.2 ± 17.1, T2: 41.8 ± 12.8, ANOVA $P < 0.001$; Figure 2B). At all the three time points, SBs level was significantly higher in cases than in the controls (*t*-test $P < 0.001$). *P* values for comparisons between each sampling in patients computed by paired *t*-test are reported in Supplementary Table S3.

NER capacity and SBs over time in relation to therapy

NER capacity increased over time in adjuvantly treated patients (ANOVA $P = 0.01$), while the trend in untreated patients was not significant (ANOVA $P = 0.08$; Figure 5A). This trend was significant also for patients receiving neoadjuvant treatment (ANOVA $P = 0.02$). No differences in NER capacity were seen after stratification for treatment regimen: 5FU (ANOVA $P = 0.07$) and 5FU + oxaliplatin (ANOVA $P = 0.19$). SBs increased over time in all patients irrespectively of the treatment. Figure 5B displays SBs values over time in untreated (ANOVA $P < 0.001$) and adjuvantly treated patients (ANOVA $P = 0.05$), and the same trend of increasing SBs was observed for neoadjuvantly treated patients (ANOVA $P = 0.04$). By stratifying adjuvantly treated patients, it was found that the degree of SBs was not affected by 5FU treatment (ANOVA $P = 0.78$), but increased with the 5FU + oxaliplatin regimen (T0: 19.0 ± 22.4, T1: 28.6 ± 21.6, T2: 42.4 ± 14.1, ANOVA $P = 0.047$). Paired *t*-test *P* values for comparisons between each sampling in patients stratified for different treatments are reported in Supplementary Table S3.

DISCUSSION

The present study was focused on characterizing differences in DNA repair between CRC patients and cancer-free population. Subsequently, defined impairment of DNA repair in cancer patients was

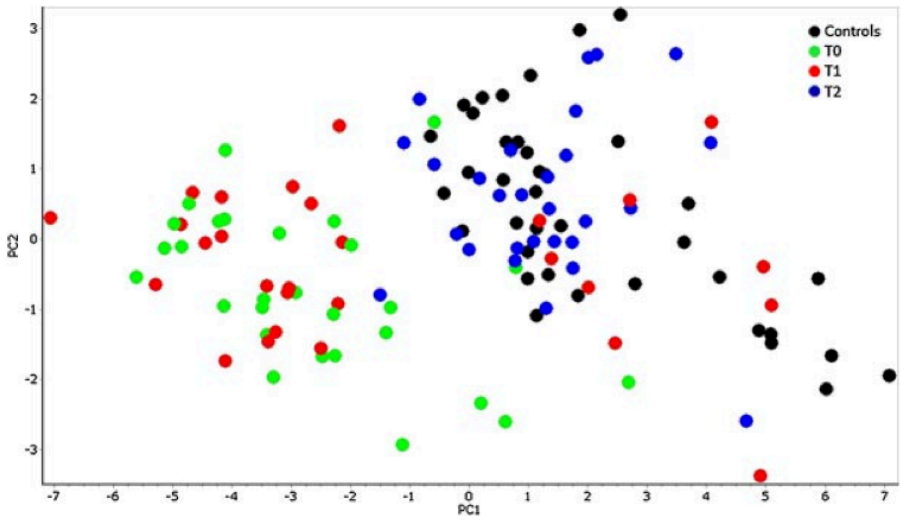

Figure 3. Principal component analysis of gene expression levels of 40 DNA repair genes analyzed in CRC patients at three time points (T0, T1, and T2) and in control population.

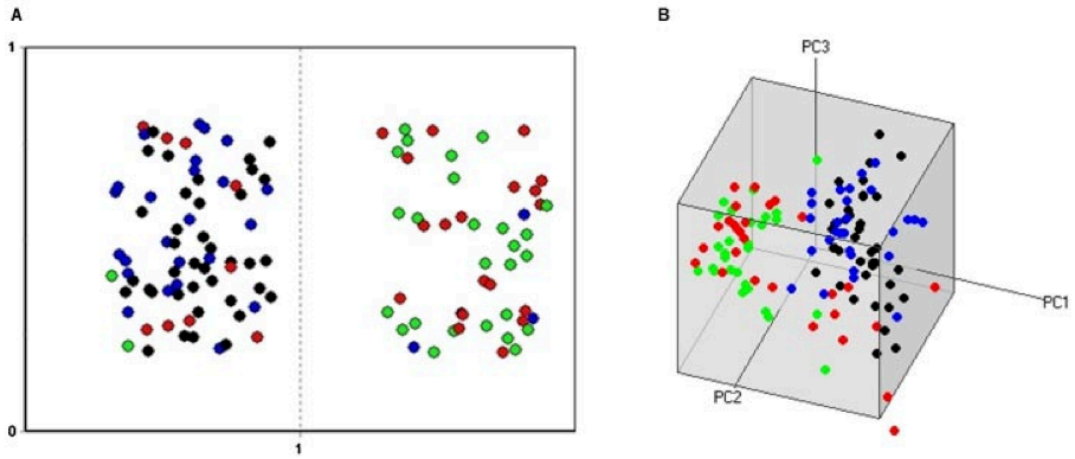

Figure 4. Identification of the most significant genes responsible for division of samples into two separate clusters. The color coding is the identical with the one in Figure 3. Kohenen self-organizing map analysis (A) and three-dimensional principal component analysis (B) based on the mean-centered differential gene expression profiles of *LIG3*, *RPA3*, *CDK7*, *DDB2*, *NBN*, and *CHEK2*.

followed-up during a post-treatment period, with samplings at 6 mo after tumor resection and chemotherapy administration, and an additional sample 6 mo later. This study should reflect changes in the DNA repair proficiency in patients under the genotoxic stress of the anti-cancer treatment. We have characterized DNA repair processes in blood samples of CRC patients. PBMCs are considered to be representatives of the general condition of the organism and we have shown that their DNA repair capacity reflects that of colonic mucosa [6]. Therefore, PBMCs represent potential surrogates of CRC target tissue, with the great advantage that if repeated biopsies are difficult to obtain, repeated blood samplings are feasible and much better suited for therapy monitoring.

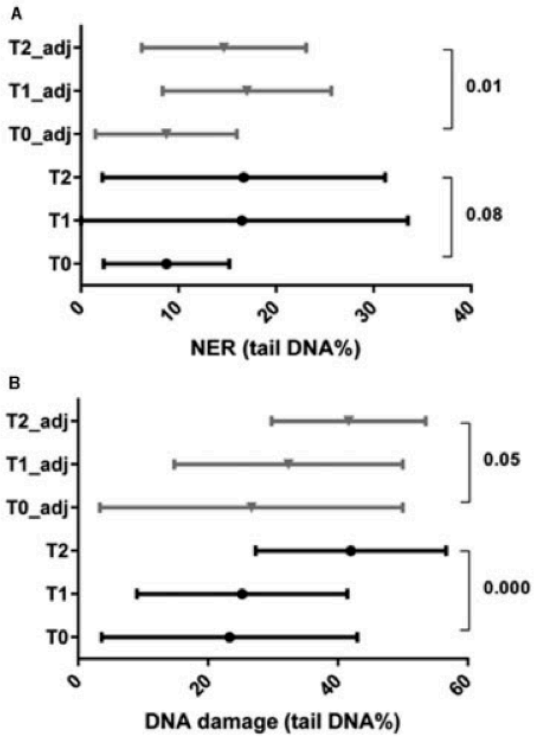

Figure 5. Subgroups of untreated patients and adjuvantly treated patients compared for changes in NER capacity (A) and DNA SBs levels (B) as analyzed in three consecutive samplings obtained at 6-mo intervals (T0, T1, and T2). Figure shows mean ± SD and ANOVA *P* values.

DNA Repair in Patients Versus Controls

In the present case–control study, we have identified genes that were differentially expressed between CRC patients at diagnosis and controls. Several BER and the majority of the studied NER genes were underexpressed in CRC patients. We evaluated NER also on functional level to determine if the reduced mRNA expression levels were concomitant with reduced functional capacity of the pathway. Decreased NER capacity in patients was confirmed and was accompanied by increased levels of SBs. These findings are consistent with our previous observations of CRC patients sampled at diagnosis having lower NER capacity and higher DNA damage than controls [4]. A large body of evidence is now available on impaired NER in patients diagnosed with different types of malignancies, as reviewed by us [4] and by [13]. However, these are case-control studies with no further indication on causality of this phenomenon in relation to cancer onset.

DNA Repair in Patients' Follow-Up

We designed a longitudinal study to follow up the CRC patients from diagnosis to 1 yr in three different

samplings obtained 6 mo apart. Interestingly, while expression profiles at diagnosis and 6 mo later were substantially similar for the 40 analyzed genes, significant changes in mRNA levels were observed at the third sampling obtained 12 mo after diagnosis. At that time point, patients were considered "cured" and there were no cases in the study group that relapsed. We can hypothesize that the expression profiles of DNA repair genes reflected the disease activity. Indeed, patients' expression profiles a year from diagnosis were comparable to those of the healthy subjects. NER capacity showed a similar tendency. From reduced level in patients at the time of diagnosis, NER capacity increased during the post-treatment period between diagnosis and 1 yr later, eventually matching the level of the healthy subjects. Different results were obtained for the comparison of SBs levels overtime in patients. SBs were twofold higher in patients at diagnosis compared to controls, and their level further increased reaching threefold difference a year from diagnosis. This accumulation of SBs was independent of the presence of the tumor in the body as well as treatment with DNA-damage inducing drugs. Possibly some other clinical or biological parameters that we have not controlled for may be responsible for this late effect.

The dynamics of DNA repair capacity were previously studied in healthy individuals by comparing BER and NER activities in six repeated samplings over a 5-mo period [14]. The values positively correlated and the coefficient of variation (CV) was 27% for BER and 49% for NER, respectively. Intra-individual variability of NER capacity was evaluated also by us in a pilot study of 16 healthy subjects sampled twice 6 mo apart. Values of two independent samplings significantly positively correlated and the CV of 20% was much lower than the inter-individual variability observed in our recent study on 340 healthy individuals (CV 84% for BER and 90% for NER; unpublished data). Similar observations were also reported in the study by Vogel et al [15]. DNA repair capacity thus seems to be a characteristic parameter for each individual (but having a rather high variability across the general population). Here, we showed that this hypothesis based on healthy population is not applicable in cancer patients. In fact, in the present study we showed that cancer patients undergoing anti-cancer treatment displayed significant changes in DNA repair overtime, between the diagnosis and the recovery period 1 yr later.

Only a few studies have examined DNA repair prospectively to elucidate the role of this process in cancer development. The expression levels of DNA repair genes *OGG1*, *NEIL1*, *MUTYH*, *ERCC1*, and *XPD* were not associated with subsequent risk of getting lung cancer, and it was proposed that mRNA levels should be regarded as a biomarker of exposure to oxidative stress rather than a marker of inborn DNA repair capacity [16,17]. Quite the opposite was concluded by Paz-Elizur et al. [18], who analyzed OGG1 activity in lung cancer patients at 1 yr follow-up starting at diagnosis and reported a lack of an effect of the tumor on OGG1 activity, suggesting the inherent characteristic of this parameter. Our study, in contrary to previous ones, used a multivariate approach to search for biomarkers that best characterize an individuals' DNA repair status. It is becoming imperative to classify diseases not on the basis of a single biomarker, but on the basis of a set of molecular markers [19]. This is expected to be more robust and reliable than using any single biomarker because of their generally high variability. Changes in individual parameters will not necessarily result in a significant change of the entire pattern. Using this approach, we have seen that a decreased DNA repair status was strongly related to disease at diagnosis, but after the follow-up was no longer detectable. We have characterized a set of DNA repair-related parameters, which blood levels could distinguish between a condition of acute CRC versus disease in remission. The mRNA levels of six DNA repair genes (*LIG3*, *RPA3*, *CDK7*, *DDB2*, *NBN*, and *CHEK2*) together with NER capacity, if verified on a larger and independent population, might represent a panel of CRC related biomarkers.

DNA Repair in Relation to Therapy

NER capacity was increased upon tumor resection in all patients, but this trend was statistically significant only in patients administered to chemotherapy. In this context it is noteworthy that the *P* values observed for the increase in DNA repair in untreated patients were of borderline significance. An increase in the probability of these findings by enlarging the study group cannot be ruled out. Therefore, it is difficult at present to clearly distinguish whether the enhancement of NER capacity is a consequence of surgical tumor elimination, a defense reaction of normal cells against a systemic treatment, or both. Mutagenic activity of radio- and chemotherapeutics poses a challenge to not only the tumor, but also normal cells, that need to adjust their cellular functions in order to protect their genetic integrity. Indeed, several studies have shown that DNA repair processes are induced by genotoxic stress [20–23]. In our study group, an increase in the NER capacity at the end of treatment was not accompanied by an increase in the NER genes transcription, except for *XPF* and *XPG*. These two might be the main activators of NER function. In fact, endonucleases are critical components of NER and their knockdown dramatically reduces NER activity [24]. In this context, we cannot neglect the role of post-transcriptional [25,26] and post-translational [27–30] modifications in DNA repair regulation. At the mRNA level, only 3 out of 40 genes showed differential behavior between treated and untreated subjects, and those were involved again in the BER and NER pathways. BER gene *MUTYH* (MutY Homolog *E. coli*) increased its

expression over time in treated patients and remained stable in untreated patients. On the contrary, *POLB* (Polymerase Beta) involved in BER and NER gene *XPB* (Xeroderma Pigmentosum B) showed increased expression over time in untreated patients only. Although these differences did not seem to have any effect on the immediate response to therapy, it cannot be excluded that they may play some role in long-term survival. Above genes have been observed to be implicated in CRC development. Aberrant MUTYH glycosylase has been linked to one type of CRC [31], and *POLB* is mutated in about 50% of human cancers [32], including CRC [33]. Also, therapeutic downregulation of *POLB* activity was recently considered in order to meet better treatment response [34], and its mRNA levels have been proposed to be a prognostic indicator in CRC treatment [35].

So far, few studies have examined DNA repair in relation to anti-cancer therapy response and survival, but they usually sampled patients only once before therapy. Jewell et al. studied melanoma patients and reported that higher mRNA levels of DNA repair genes in biopsies were associated with a higher risk of relapse [36]. Wang et al. [37] found that elevated DNA repair activity in peripheral lymphocytes correlated with shorter survival in lung cancer. Similar tendencies were reported by Asakawa et al. [38] in breast cancer biopsies in which high DNA damage response was linked with poor response to neoadjuvant therapy. We were unable to analyze DNA repair parameters in relation to the patients' response to therapy at endpoint since no post-treatment tumor relapse was observed in the study group. Considering the small size of the study population, further stratifications for specific treatments would not reach sufficient statistical power to draw strong conclusions.

In summary, we described the dynamics of DNA repair in blood cells of CRC patients in a time frame of 1 yr. DNA repair downregulation in the presence of active disease, as reflected by quantitative PCR and functional assays, was centered on two excision repair pathways—BER and NER. One year after the diagnosis and successful treatment, the downregulation was not detectable anymore, and the patients exhibited a molecular pattern of DNA repair similar to that of healthy controls. DNA repair markers evaluated in blood cells can be used to distinguish between an acute and a post-treatment cancer-free condition, thus reflecting the disease activity in CRC patients.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Příloha IV:

Liska V, Vycital O, Daum O, Novak P, Treska V, Bruha J, Pitule P, Holubec L. Infiltration of colorectal carcinoma by S100+ dendritic cells and CD57+ lymphocytes as independent prognostic factors after radical surgical treatment. Anticancer Res. 2012 May;32(5):2129-32. **(IF_{2012}=1,713)**

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Infiltration of Colorectal Carcinoma by S100+ Dendritic Cells and CD57+ Lymphocytes as Independent Prognostic Factors after Radical Surgical Treatment

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Abstract. *Background: S100+ dendritic cells and CD57+ lymphocytes are factors reflecting the immune system´s ability to suppress the progress of tumor growth. CD57+ cells include natural killer cells and late stages of T-effector lymphocytes. We evaluated the relationship between the known clinical and histological factors and tumor markers as well as the presence of S100+ and CD57+ cells in the tissue of colorectal carcinoma with the aim of detecting patients at high risk of short overall survival (OS) or short disease-free interval (DFI) after radical surgical treatment and we further analyzed whether S100+ and CD57+ positivity could bring on new information regarding the treatment regimen. Materials and Methods: Data of 150 patients (97 males and 53 females) that underwent an elective radical surgical procedure for colorectal cancer were studied. The influence on DFI and on OS of the following parameters was evaluated: grading, staging and positivity for S100 and CD57 by immunohistochemical staining. We also analyzed the relation of preoperative serum levels of the tumor markers Carcinoembryonic Antigen (CEA), Cancer Antigen 19-9 (CA19-9), Cancer Antigen 72-4 (CA72-4), Thymidine kinase (TK), Tissue-Specific Polypeptide Antigen (TPS) and Tissue Polypeptide Antigen (TPA) in relation to S100 and CD57 positivity/negativity for the same patients. Results: OS at 1, 3 and 5 years was 92.2%, 76.5% and 70.2%; the DFI at 1, 3 and 5 years was 85.3%, 64.3% and 49.4%. CD57 positivity in the tumor mass was proven as a positive prognostic factor for OS. Risk of short OS was 2.5-fold higher in patients with low tumor infiltration by CD57+ lymphocytes. The combination of N2 stage for lymph nodes and the absence of CD57+ cells was proven to be the strongest negative prognostic factor for OS. No significant influence of CD57 positivity on DFI appeared. There was no significant influence of S100 positivity on OS or DFI; nor was there any statistical dependence of CD57 and S100 positivity or negativity on preoperative serum levels of CEA, CA19-9, CA72-4, TK, TPS or TPA. Both studied factors were shown to be statistically independent factors. Conclusion: The present study showed infiltration of colorectal cancer tissue by CD57+ cells as being an important independent positive prognostic factor for OS.*

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Key Words: S100+ dendritic cells, CD57+ lymphocytes, colorectal cancer, prognosis.

S100+ dendritic cells (DC) and CD57+ lymphocytes are factors reflecting the immune system's ability to suppress the progress of tumor growth. Atreya and Neurath demonstrated their role in supression of the progress of colorectal carcinoma (1). CD57 is a glycoprotein with cell adhesion function also called human natural killer-1 or LEU7 (2). CD57+ cells include natural killer (NK) and late stages of T-effector lymphocytes. NK cells as a part of the cellular innate immunity are able to destroy malignant cells (2). NK cells mediate lysis of malignant cells but the mechanism of detection of cancer cells is different from that of the CD8+ T-lymphocytes (3); NK cells do not recognize specific tumor-associated antigens of cancer cells as CD8+ T-lymphocytes do. NK cells lyse cancer cells that are opsonized by surface antibodies or after stimulation by other signals such as cytokines, produced by antigen-presenting cells (4). A lower preoperative number of NK cells was associated with increased postoperative recurrence of colorectal cancer (5). The S100 antigen protein is present in many types of cells: cells of neural crest, chondrocytes, adipocytes, myoepithilial cells, macrophages, Langerhans cells and dendritic cells. In particular dendritic cells (DC) (antigen-presenting cells) play a key role in activating naive T-lymphocytes and NK cells. DC gather antigens in their surrounding tissues, process them and migrate to the secondary lymphoid organs to present antigens on major histocompatibility complexes class I or class II to CD8+ cytotoxic lymphocytes or CD4+ helper T-lymphocytes (2, 6). In the majority of the available studies, increased

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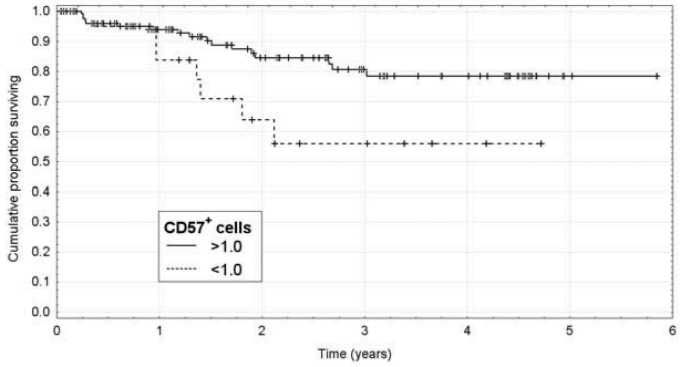

Figure 1. *CD57 positivity as a statistically significant prognostic factor for OS (cut-off 1 cell, p-value=0.0350).*

numbers of immune cells infiltrating the tumors correlate with an improved prognosis for cancer patients (7), but the role of certain lymphocytes remains unclear (8). Here we evaluated the relationship between the known clinical, histological factors and tumor markers that are used in standard clinical follow-up of patients and the presence of $S100^+$ and $CD57^+$ cells in the tissue of colorectal carcinoma (CRC) with the aim of detecting patients with a high risk of short overall survival (OS) or short disease-free interval (DFI), after radical surgical treatment and to analyze whether $S100^+$ and $CD57^+$ positivity could bring new information on the treatment regimen, independently of standard clinical examinations.

Materials and Methods

Data from 150 patiens (97 males and 53 females) in a patient cohort that underwent an elective radical surgical procedure at the Department of Surgery of the Teaching Hospital and Medical School in Pilsen between 2004-2007. The influence on DFI and on OS of following parameters was evaluated: grading, staging and positivity of S100 and CD57 by immunohistochemical staining.

Histological and immunohistochemical analysis. Tissue for light microscopy was fixed in 4% formaldehyde and was embedded in paraffin. Five micrometer-thick sections were cut from the tissue blocks and stained with hematoxylin-eosin. Three different sections of each tumor were examined.

For the immunohistochemical investigations the following primary antibodies were used: CD57 (clone NK1, ready-to-use; Ventana, Rocklin, CA, USA), and S100 (polyclonal, 1:1000; Dako, Glostrup, Denmark). Microwave pretreatment was used in both cases. The primary antibodies were visualized using the supersensitive streptavidin-biotin-peroxidase complex (Biogenex, San Ramon, CA, USA). The appropriate positive and negative control slides were employed. The number of DCs and NK cells was evaluated in five high power microscopical fields and was expressed as the number of immunopositive cells per high power microscopical field.

Oncomarkers. All the blood samples for assessment of tumor markers were obtained under standard conditions from the cubital vein during

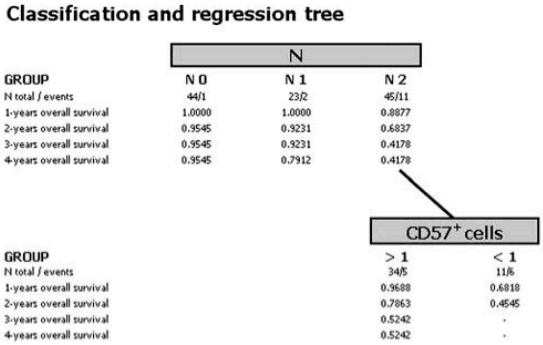

Figure 2. *Classification and regression tree diagram presenting the strongest negative prognostic factor combination for OS.*

the morning hours. The serum for the assessment of routine tumor markers was acquired through centrifugation and was stored at –20°C until laboratory analysis. Tumor markers were assessed at the Department of Nuclear Medicine, Faculty Hospital Pilsen with commercial laboratory kits, in accordance with the manufacturers' recommendations. The following tumor markers were assessed: CEA (ng/ml, Immunotech, Czech Republic), carbohydrate antigen 19-9 (CA19-9, IU/l, Shering-CIS BioInternational, France), cytokeratins: tissue-specific polypeptide antigen (TPS, kIU/l, IDL, Sweden), tissue polypeptide antigen (TPA, kIU/l, DiaSorin, Italy). Thymidine kinase (TK, IU/L) was measured by radioenzyme analysis (REA) using the Immunotech (Prague, Czech Republic) assay kits.

Statistical analysis. Statistical analysis was processed by the statistical software Statistica 9.0 (StatSoft, CA, USA). The relationships between the variables were described by the Spearman rank correlation coefficients. The analyses of OS and DFI were performed by Kaplan-Meier survival functions. The influence of given covariates (clinical and histopathological factors or tumor markers) was tested by the log-rank test and the Wilcoxon test. The Cox regression the hazard model, hazard ratio (HR) and the 95% confidence interval (CI) for HR were computed for the evaluation of given clinical and histopathological factors and tumor markers to OS or DFI. Multivariate analysis was

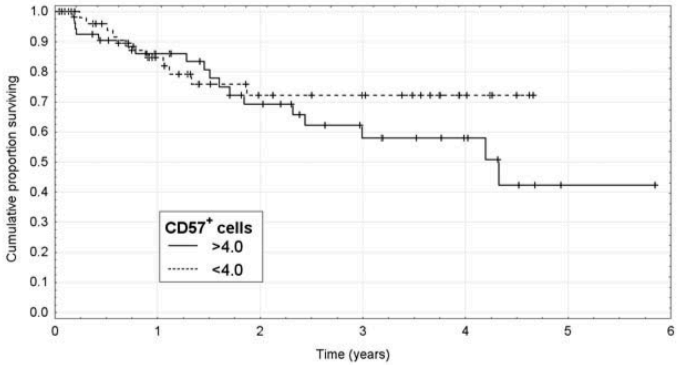

Figure 3. *CD57 positivity was not proven as being a statistically significant prognostic factor for DFI (cut-off 4 cells, p-value=0.3415).*

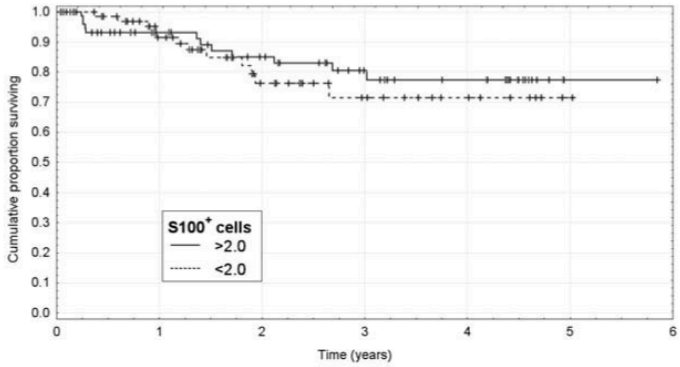

Figure 4. *S100 positivity was not proven as being a statistically significant prognostic factor for OS (cut-off 2 cells, p-value=0.5877).*

performed by the use of classification and regression trees (CART). The Cox regression hazard model (stepwise regression) was applied in order to find the predictors in CART.

Results

There were 93 males (mean age=65.27 years, median=65.94 years) and 57 females (mean=68.07 years median=67.72 years) in studied patients cohort. No statistically significant differences were proven regarding the age between males and females. OS at 1, 3 and 5 years was 92.2%, 76.5% and 70.2%; the resulting DFI at 1, 3 and 5 years was 85.3%, 64.3% and 49.4%. CD57 positivity of cells in the mass of tumor was a statistically significant positive prognostic factor (cut-off 1 cell, *p*-value=0.0350, Figure 1) of OS. Risk of short OS was 2.5-fold higher in patients with low tumor infiltration by CD57$^+$ lymphocytes. CART showed that the combination of N2 stage of lymph node disease and the absence of CD57$^+$ cells was the strongest negative prognostic factor of OS (Figure 2). No statistically significant influence of CD57 positivity on DFI appeared (Figure 3).

There was no statistically significant influence of S100 positivity on OS nor on DFI (Figures 4 and 5). There was no statistical dependency or correlation of CD57 and S100 positivity or negativity on preoperative serum levels of CEA, CA19-9, CA72-4, TK, TPS or TPA. Both studied factors were found to be statistically independent factors.

Discussion

The role of the immunological response in controlling the growth and relapse of CRC remains controversial and contemporary studies have not answered all the questions about the prognosis of patients after radical surgical treatment of CRC (9, 10, 11, 14). We analyzed a large cohort of patients with CRC aiming at detecting the relation between these types of immune cells and the prognosis of patients after radical CRC surgery. This aim was stimulated by some dilemmas in the decision for

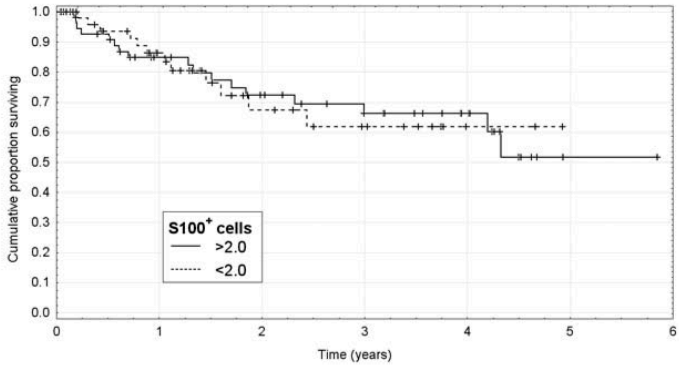

Figure 5. *S100 positivity was not proven as being a statistically significant prognostic factor for DFI (cut-off 2 cells, p-value=0.9173).*

surgical and oncological treatment, when early recurrence depreciates the effort of radical surgery due to a high risk of complications and the long duration of the decreased quality of life of the patients (3, 12). Our results support the hypothesis that the immunological response observed in the tumor tissue can influence the behavior of CRC and subsequently affect the prognosis of patients (9). Tumor infiltration by NK cells seems to be a promising positive prognostic factor reflecting the decreased risk of patients for poor OS. Future work should focus on the molecular-biological background of tumor infiltration by lymphocytes to provide understanding over their pathophysiological functions (1). The tumor markers inform us of the negative prognosis of patients (early recurrence (DFI) or for the poor prognosis for long OS (13). On the other hand $CD57^+$ positivity was demonstrated as being a positive prognostic factor for radically operated patients with CRC. This information is more important for clinicians than a negative factor would be, since it could modify their decision about adjuvant oncological treatment or follow-up regimen. The present study demonstrated the positive influence of tumor-infiltrating $CD57^+$ cells on the prognosis of CRC. The results could lead upon intensifying the follow-up strategy for the patients with higher risk of early CRC reccurence.

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The role of ABC transporters in progression and clinical outcome of colorectal cancer

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Worldwide, colorectal cancer (CRC) is the third most common cancer, with the highest mortality rates occurring in Central Europe. The use of chemotherapy to treat CRC is limited by the inter-individual variability in drug response and the development of cancer cell resistance. ATP-binding cassette (ABC) transporters play a crucial role in the development of resistance by the efflux of anticancer agents outside of cancer cells. The aim of this study was to explore transcript levels of all human ABCs in tumours and non-neoplastic control tissues from CRC patients collected before the first line of treatment by 5-fluorouracil (5-FU)-containing regimen. The prognostic potential of ABCs was evaluated by the correlation of transcript levels with clinical factors. Relations between transcript levels of ABCs in tumours and chemotherapy efficacy were also addressed. The transcript profile of all known human ABCs was assessed using real-time polymerase chain reaction with a relative standard curve. The majority of the studied ABCs were down-regulated or unchanged between tumours and control tissues. ABCA12, ABCA13, ABCB6, ABCC1, ABCC2 and ABCE1 were up-regulated in tumours versus control tissues. Transcript levels of ABCA12, ABCC7 and ABCC8 increased in direction from colon to rectum. Additionally, transcript levels of ABCB9, ABCB11, ABCG5 and ABCG8 followed the reverse significant trend, i.e. a decrease in direction from colon to rectum. The transcript level of ABCC10 in tumours correlated with the grade ($P = 0.01$). Transcript levels of ABCC6, ABCC11, ABCF1 and ABCF2 were significantly lower in non-responders to palliative chemotherapy in comparison with responders. The disease-free interval of patients treated by adjuvant chemotherapy was significantly shorter in patients with low transcript levels of ABCA7, ABCA13, ABCB4, ABCC11 and ABCD4. In conclusion, ABCC11 may be a promising candidate marker for a validation study on 5-FU therapy outcome.

Introduction

Worldwide, colorectal cancer (CRC) is the third most common cancer, with an estimated 1 233 700 newly diagnosed cases and 608 700 deaths/year in 2008. For this type, Central Europe has repeatedly recorded the highest mortality rates (1).

Although inherited susceptibility underlies roughly 35% of variance in CRC risk (2), germ line mutations in genes with high penetrance account for <6% of cases (3). There is growing evidence that lifestyle and environmental factors contribute to the CRC development (4).

Systemic chemotherapy is an important component of the complex treatment of CRC, with regimens based on 5-fluorouracil (5-FU), irinotecan and/or oxaliplatin used as standard. These drugs are often used in combination with biological therapy in patients with advanced disease. Generally, the use of chemotherapy to treat cancers is limited by the inter-individual variability in drug response and the development of cancer cell resistance. Xenobiotics, including anticancer drugs, are extensively metabolised in the body by Phase I (activation enzymes, e.g. cytochromes P450) and Phase II (conjugation enzymes as glutathione *S*-transferases, GSTs, or glucuronide transferases, UGTs) enzymes. Phase III of biotransformation represented by ATP-binding cassette (ABC) transporters presents another important component of xenobiotic metabolism (5). ABC transporters play a crucial role in the development of resistance by the efflux of anticancer agents outside of cancer cells (6). In fact, the expression of ABCs may significantly vary among individuals and thus affects the efficacy of drug treatment (7).

The human ABC transporters are encoded by a large transporter gene superfamily, which is composed of 49 members grouped into seven subfamilies (A–G) according to the sequence homology. ABC proteins facilitate translocation of heterogeneous substrates including metabolic products, lipids and sterols, peptides and proteins, saccharides, amino acids, inorganic and organic ions, metals and drugs across the cell membrane. To transport these substrates across extracellular and intracellular membranes against a concentration gradient, ABCs use energy acquired by the hydrolysis of ATP (8). Genetic variation in these genes causes a variety of genetic diseases and disorders (9). The structure of ABC transporters consists of two types of domains: ATP-binding domains, also known as nucleotide-binding folds (NBFs) and transmembrane domains (TMDs). The NBFs contain typical motifs: Walker A and Walker B and so-called ABC-signature sequence. The TMDs are formed by 6–11 alpha helices and provide the substrate specificity (10). Although all share a similar NBF, their domains are organised in different ways, with different numbers and localisations of TMDs (11). Some of the ABCs behave as full transporters with two TMDs, each with six transmembrane segments, and two NBFs. Others are half transporters with only one TMD and become functional after dimerisation or oligomerisation (12).

The ABCA subfamily contains 12 members acting as full transporters (13; supplementary Table IS, available at *Mutagenesis* Online). ABCA5 mRNA was detected in poorly differentiated colon adenocarcinoma cell lines G-112 but not in normal colon. In contrast, ABCA2 mRNA was detected in well-differentiated colon adenocarcinoma cell lines CX-1. Thus, induction of ABCA5 may correlate with the differentiation status of human colon tumours and contribute to the tumour development (14).

The ABCB subfamily consists of 11 members (supplementary Table IIS, available at *Mutagenesis* Online). Four of them are full transporters (ABCB1, ABCB4, ABCB5 and ABCB11) and the rest is composed of half transporters. P-glycoprotein, a product of *ABCB1* gene, is localised in epithelial cells of the small and large intestine as a part of the barrier that protects cells against xenobiotics from diet, bacterial toxins, drugs and other biologically active compounds (15). ABCB1 confers MDR phenotype to cancer cells (16). ABCB1 substrates are generally amphipathic lipid-soluble compounds including anticancer drugs as *vinca* alkaloids, anthracyclines, taxanes, epipodophyllotoxins, camptothecins or anthracenes (17). Single-nucleotide polymorphisms (SNPs) in *ABCB1* have been shown to influence its phenotype (expression, protein function and drug response) in context with numerous diseases [(11,18,19); supplementary Table VS, available at *Mutagenesis* Online]. ABCB1 is highly expressed in the apical membrane of enterocytes, where it pumps xenobiotics back into the intestinal lumen. No relationship was seen between ABCB1 protein expression, genotype and long-term prognosis of patients treated by 5-FU and leucovorin (20). Hypoxia is generally associated with chemoresistance. Ding *et al.* (21) suggested that hypoxia induced the expression of hypoxia-inducible factor, alpha subunit and ABCB1 in colon carcinoma. Complementary DNA (cDNA) microarray analysis of cell sublines derived from K562 leukaemia, MCF7 breast cancer and S1-colon cancer with acquired resistance against daunorubicin, doxorubicin, vincristine, etoposide and mitoxantrone has shown that ABCB1, ABCB4 and ABCG2 were up-regulated in several resistant sublines (22). ABCB2 and ABCB3 expressions were significantly associated with major histocompatibility complex class I antigen (MHCI) expression in 336 sporadic CRC tumours examined by immunohistochemistry. Thus, the expression of key components of the antigen-processing machinery represented by ABCB2 and ABCB3 is linked with the density of tumour-infiltrating lymphocytes (TILs), which are positive prognostic factors in CRC *in vivo* (23).

The ABCC family contains 13 members; nine of them are the multidrug resistance-associated proteins (MRPs; ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, ABCC11 and ABCC12, supplementary Table IIIS, available at *Mutagenesis* Online). *In vitro* ABCC transporters can collectively confer resistance to anticancer drugs and their conjugated metabolites, platinum compounds, folate antimetabolites, nucleoside and nucleotide analogues (24). ABCC1 and ABCC2 have been shown to act synergistically with several Phase II enzymes including GSTs and UGTs and this synergy conferred resistance to several drugs (25,26). ABCC1 exports drug conjugates with glutathione and unconjugated drugs together with free glutathione (17). By contrast, ABCC2 transports a wide range of unconjugated organic anions, including e.g. methotrexate, irinotecan or ampicillin (27). ABCC2 haplotype predicted variability in pharmacokinetics of irinotecan in Japanese metastatic CRC patients treated by FOLFIRI (regimen containing 5-FU and irinotecan) (28). mRNA expression of ABCC2 was significantly associated with resistance to cisplatin but not 5-FU (29). 5-FU significantly suppressed ATP7B and the organic cation transporter SLC22A2 and increased ABCC2 mRNA expression (5.8-fold) in human cells from colon adenocarcinoma (LS180). Overexpression of ABCC2, ABCC3 and ABCG2 was demonstrated to be beneficial for the efficacy of oxaliplatin in Madin-Darby canine kidney II cells (MDCKII). Thus, the authors predicted that the modulation of expression of transporters of both drugs could favour the synergic action of the FOLFOX (5-FU/leucovorin/oxaliplatin) combination (30). ABCC5 protein confers resistance to 5-FU by the efflux of monophosphate metabolites. The overexpression of ABCC5 in CRC may contribute to the resistance to 5-FU (31). In cell lines, celecoxib induced ABCC4 and ABCC5 expression at both mRNA and protein levels, but no induction of ABCC1 and ABCC2 was found. This observation suggested that the low response rate observed in clinical trials using combinations of celecoxib with 5-FU or irinotecan may reflex celecoxib-mediated induction of efflux of drugs from cancer cells through the up-regulation of ABCs (32). Interestingly, patients treated with oxaliplatin- and 5-FU-based regimen for metastatic CRC (n = 40) had a significantly shorter progression-free survival in the case where their circulating tumour cells (CTCs) expressed alcohol dehydrogenase (ALDH) 1, survivin and ABCC5. Thus, detection of survivin+/ABCC5+ CTCs from peripheral blood may help in identifying metastatic CRC patients resistant to standard oxaliplatin- and/or 5-FU-based regimen (33). ABCC11 shares the highest structural similarity with ABCC4 and ABCC5. ABCC11 was also shown to confer resistance to 5-FU and certain fluoropyrimidines (supplementary Table IIIS, available at *Mutagenesis* Online). The rs17822931 SNP in *ABCC11* affects enzyme function that determines earwax type (34).

The ABCD subfamily consists of four half transporters localised in peroxisomes, providing thus peroxisomal transport of long-chain fatty acids (supplementary Table IVS, available at *Mutagenesis* Online). ABCE and ABCF subfamilies contain genes that are clearly derived from ABC transporters and have ATP-binding domains but not TMDs (35, supplementary Table IVS, available at *Mutagenesis* Online). There are currently no data available on the role of ABCD and ABCF subfamilies in CRC.

The six known members of ABCG subfamily are half transporters that have a reversed structure. N-terminus contains NBF and TMD is at the C-terminus (supplementary Table IVS, available at *Mutagenesis* Online). ABCG2 is a multidrug resistance enzyme that can export both unmodified drugs and drug conjugates, including mitoxantrone, bisantrene, epipodophyllotoxins (e.g. etoposide), camptothecins (topotecan and irinotecan) or flavopiridol (17). Patients harbouring the −19572 to 19569delCTCA mutation in *ABCG2* had a significantly lower relative extent of conversion of irinotecan to its SN-38 metabolite than patients carrying the ancestral genotype without this deletion (P = 0.019; 36). Dietrich *et al.* (37) examined 29 colon adenomas from 21 patients and 8 adenomas from C57BL/6 $Apc^{Min/+}$ mice. ABCG2 protein was significantly down-regulated in both human and mice colon adenomas. ABCG2 confers resistance to a narrower range of anticancer agents than ABCB1 and ABCC1 or ABCC2, e.g. anthracyclines, mitoxantrone and irinotecan. ABCG2 transports the glucuronide conjugate of SN-38, the active metabolite of irinotecan, but with ∼7-fold lower affinity than unconjugated

SN-38 (38). Dye-efflux effective side population (SP) of SW480 colon cancer cells has shown a higher resistance to 5-FU and irinotecan, higher ABCB1 and ABCG2 expressions and activation of the Wnt signalling pathway when compared to non-SP containing cells. Silencing the Wnt signalling pathway may thus present a promising strategy for targeting chemotherapy-resistant CRC cells (39). The ABCG5 and ABCG8 form heterodimers and overexpression of putative stem cell marker EpCAM with ABCG5 within the buds of colorectal tumours is frequently observed and associated with poor prognosis (40).

The current state of knowledge shows that ABCs are functionally important for anticancer drug disposition and efficacy. However, apart from functional data obtained using cell and animal models, there is a limited amount of information on the importance of ABCs for the prognosis of individual patients and the prediction of the chemotherapy outcome. Some of the published studies in patients suggested that ABCs may influence not only cancer initiation but also its progression, invasion and metastasis. The aim of our study is to: (i) explore transcript levels of ABCs in tumour and control tissues from CRC patients treated by substrates of ABCs, (ii) evaluate prognostic potential of ABCs by the correlation of transcript levels with clinical factors such as tumour size, lymphatic node involvement and grading and (iii) elucidate relations between transcript levels of ABCs in tumours and chemotherapy efficacy. In our pilot study presented here, we have addressed these points in a complex way by an assessment of the transcript profile of all known human ABCs. This is because some of the drugs under study are transported by numerous and in some cases unknown transporters. The selection and validation of candidate genetic markers identified in the pilot study are the final goal of our current research.

Materials and methods

Materials

Phenol, chloroform, agarose and chemicals for preparation of buffers were purchased from Sigma–Aldrich (Prague, Czech Republic). Deoxynucleotides (dATP, dCTP, dGTP and dTTP) for polymerase chain reaction (PCR), molecular weight standard for electrophoresis ΦX174DNA-HaeIII digest and Lambda DNA EcoRI + HindIII Marker were products of New England Biolabs Inc (Ipswich, MA, USA) and Fermentas Inc (Vilnius, Lithuania), respectively. Taq-Purple DNA polymerase and Combi PPP Master Mix for PCR were supplied by Top-Bio s.r.o. (Prague, Czech Republic).

Subjects

Tissue samples of primary tumour of human colorectal carcinoma and paired distant unaffected mucosa were collected from CRC patients diagnosed and treated at the Departments of Surgery and Oncology, at the Teaching Hospital and Medical School in Pilsen and Charles University in Prague, both in the Czech Republic during the period 2008–10. Native tissue samples were taken during surgery, macrodissected, snap frozen in liquid nitrogen and stored at −80°C until total RNA isolation. The control mucosa samples were taken from the macroscopically unaffected resection margins of colon tissues. The resection margins were microscopically evaluated and only samples free of malignant cells were further analysed. Corresponding tumour tissue samples were verified by the experienced pathologist. Only histologically verified patients and their samples were included in this study. The following data on patients were retrieved from medical records: age, gender, date of diagnosis, pTNM stage according to Union for International Cancer Control (41), histological type and grade of the tumour, type of chemotherapy and treatment response.

The following eligibility criteria were applied to the recruitment of patients into the study: (i) patients who were subject to surgery for CRC, (ii) no prior chemotherapy before surgery (in order to eliminate its influence on transcript levels), (iii) patients who received only first-line chemotherapy in either palliative (Group A) or adjuvant (Group B) setting and (iv) patients who received regimens based on 5-FU, leucovorin and/or oxaliplatin, e.g. FOLFOX.

Response to the treatment was evaluated by Response Evaluation Criteria In Solid Tumors (RECIST) criteria (42) based on routinely used imaging techniques for assessment of tumour mass (by computer tomography, with or without positron emission tomography, magnetic resonance or ultrasonography).

Increase in tumour mass or the appearance of new lesions in patients with palliative treatment (Group A) indicated progression and thus no response to the treatment. Response to the treatment was defined as a decrease of the number or volume of metastases or stabilisation of the disease.

In patients treated by adjuvant therapy after radical surgical resection R0 (Group B), disease-free interval (DFI) served as the treatment outcome for analyses. DFI was defined as the time elapsed between radical surgical R0 resection and disease recurrence. CRC patients treated by 5-FU and/or oxaliplatin were monitored for adverse symptoms of treatment and toxicity was scored according to the National Cancer Institute Common Toxicity Criteria, Version 2, during the whole period of treatment. Grade 3 or 4 toxicity was followed as an important factor of toxicity of the administered regimens.

All patients were informed about the study aims, methods and potential risks and signed consensually an informed consent form in agreement with the requirements of the Ethical Commission of the Medical Faculty and Teaching Hospital in Pilsen, Czech Republic.

Isolation of total RNA and cDNA preparation

Methods were published (19,43) and are presented in the supplementary Material, available at *Mutagenesis* Online.

Quantitative real-time PCR

The diluted amplified cDNA was used for quantitative real-time polymerase chain reaction (qRTPCR) by the relative standard curve method performed in 7500 and ViiA7 Real-Time PCR System using TaqMan Gene Expression Assays (Life Technologies). The following criteria were applied to the selection of TaqMan Gene Expression Assays: (i) exon–exon boundaries span where possible, in order to minimise signals from traces of contaminating DNA, (ii) as short as possible amplicons and (iii) location near to 3′-end of the transcript to reduce possible influence of RNA quality on qRTPCR. The list of reference genes, genes of interest and respective assays is given in supplementary Table VIS, available at *Mutagenesis* Online. Cycling parameters of qRTPCR were initial hold at 50°C for 2 min and initial denaturation at 95°C for 10 min, followed by 50 cycles consisting of denaturation at 95°C for 15 sec and annealing at 60°C for 60 sec (except ABCC9, ABCG4 and ABCG5 where 90 sec and ABCB1 where 58°C for 90 sec were used). Each reaction contained 2.5 µl of 20 times diluted preamplified cDNA, 5 µl of TaqMan Gene Expression Master Mix and 0.5 µl of TaqMan Gene Expression Assay in a final reaction volume of 10 µl. Fluorescence was acquired after each extension step. Each sample was assessed in duplicate and the mean value was used for further analyses. Samples with >15% variation between duplicates were reanalysed. The non-template control contained water instead of cDNA. Negative cDNA synthesis controls (RNA transcribed without reverse transcriptase) were also employed to reveal possible carry-over contamination. One sample of normal colon mucosa was used as calibrator for preparation of standard curves for each gene to assess reaction efficiency. For the real-time PCR reactions, preamplified cDNA of the calibrator diluted 1:4 was used as the highest template concentration point for construction of the standard curve. The rest of the standard curve points were prepared by five subsequent serial five times dilutions. The resulting standard curve was used for calculation of PCR efficiency (E) according to the following formula: $E = 10^{-1/\text{slope}} - 1$ (supplementary Table VIS, available at *Mutagenesis* Online). Reference genes were selected on the basis of our pilot study, where the TaqMan Array Plates (Life Technologies) were used to compare the stability of 24 different potential reference genes chosen from the literature in a set of 10 pairs of human CRC tumours and distant unaffected mucosa samples. Stability of reference genes was analysed by geNorm version 3.5 of March 2007 (44) and NormFinder version 19 of June 2009 (45) software programs. According to both programs, EIF2B1, MRPL19, PSMC4, POLR2A, PPIA and HPRT1 were selected as the most stable genes for normalisation of transcript levels of target genes. The qRTPCR study design adhered to the MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments; 46).

Statistical analyses

Transcript levels were analysed by 7500 and ViiA7 System Software. Acquired average C_T values were further processed by relative expression software tool (REST) 2009 Software (47; Qiagen, Hildesheim, Germany). REST is routinely used for the determination of differences between different types of sample and control groups and considers both normalisation to numerous reference genes and reaction efficiency. For statistical analyses of correlation of transcript levels of all established genes with clinical data, the non-parametric tests as Mann–Whitney, Kruskall–Wallis and Spearman rank tests were used according to the

distribution of data. The mean, median, SD, variance, minimum, maximum, quantiles, frequencies and other basic statistical measurements were computed in the given groups and subgroups. The relations between the parameters were computed by Spearman correlation coefficient. The DFI analyses were performed by Kaplan–Meier Survival estimates and Cox regression hazard model. In the first step, each of the investigated covariates were divided into four groups using quartiles of the investigated variable. For these groups, the Kaplan–Meier survival functions were computed. All possible cut-offs were tested and the 'optimal cut-off' was defined as the highest statistical significance in Kaplan–Meier (log-rank test). For these cut-offs, the hazard ratios (HRs) were then computed. In order to reduce the chance of Type I and II errors, only the cut-offs, where the DFI divided by quartiles had a similar direction to the DFI, were used. *P*-values are always departures from two-sided tests. A *P*-value of <0.05 was considered statistically significant. Statistical analyses of clinical data were performed using SPSS v15.0 software (SPSS Inc, Chicago, IL, USA).

Results

Fifty-one patients meeting the eligibility criteria were recruited into the study (26 patients in the palliative Group A and 25 patients in the adjuvant Group B). The clinical and pathological characteristics of the patients are listed in Table I. Patients in both groups did not significantly differ in terms of gender, age at diagnosis, tumour size, lymph node involvement and grading. On the other hand, patients significantly differed in metastatic spread of the disease (all patients in the palliative group were M1 versus M0 in all adjuvant patients) and subsequent treatment (the palliative group was treated predominantly by more aggressive FOLFOX regimen). Tumour localisation also significantly differed between both groups (rectum prevailed in the palliative group versus adjuvant group, $P = 0.008$). The median of follow-up was 20 months. Neither the median overall survival nor the median DFI was reached. Therefore, the importance of transcript levels of ABCs for overall survival was not evaluated. DFI was evaluated as a measure of short-term survival; 72% of censors occurred in the course of follow-up.

Differences in transcript levels of ABCs between tumour and control tissues

A large number of ABC transcripts were down-regulated in tumour versus control mucosa tissues (REST 2009 software, Table II). Among these, ABCA1, ABCA4, ABCA5, ABCA6, ABCA8, ABCA9, ABCA10, ABCB1, ABCB4, ABCB5, ABCB11, ABCC3, ABCC5, ABCC6, ABCC7, ABCC8, ABCC13, ABCD2, ABCD3, ABCD4, ABCG1 and ABCG2 transcripts were down-regulated at a very high level of significance ($P \leq 0.001$). Contrastingly, ABCA12, ABCA13, ABCB6, ABCC1, ABCC2 and ABCE1 were up-regulated in tumours versus control tissues to a high extent (Table II). The remaining ABCs did not show significant deregulation in compared tissues. Transcript level of ABCC12 was below the limit of detection in all samples.

Correlations between transcript levels of ABCs in tissues and clinical data

Relative transcript levels of ABCs were first normalised to six reference genes. Average C_T value of EIF2B1, MRPL19, PSMC4, POLR2A, PPIA and HPRT1 transcript levels was calculated for each sample and designated as ENDO value.

Table I. Clinical characteristics of studied groups

	Group A (palliative), $n = 26$	Group B (adjuvant), $n = 25$	P[a]
Gender (males/females)	19/7	16/9	0.555 (Fisher's exact test)
Age at diagnosis (years), median ± SD	64.5 ± 9.7	63.0 ± 8.5	0.676 (ANOVA)
Tumour size (T)			0.798
T2	2	2	
T3	19	20	
T4	5	4	
Lymphnodes involvement (N)			0.856
N0	7	7	
N1	11	11	
N2	8	7	
M classification			**<0.001**
M0	0	25	
M1	26	0	
Grade			0.361
G1	4	2	
G2	21	18	
G3	1	5	
Primary tumour localisation			**0.008**
Colon	8	17	
Rectosigmoideum	6	6	
Rectum	12	2	
Chemotherapy			**0.006** (Fisher's exact test)
5-FU ± leucovorin[b]	6	12	
FOLFOX	20[c]	13	
Grade 3 or 4 toxicity	1	3	
Post-operative radiotherapy	3	4	0.477
Chemotherapy outcome			NA
Responders/non-responders	13/13	NA	
Relapse/remission	NA	7/18	

Footnotes:
[a]Analysed by Pearson chi-square test. The use of Fisher's exact test or analysis of variance (ANOVA) is specified in brackets; NA—not applicable.
[b]Ten patients received deGrammonte regimen, six patients received Xeloda (capecitabin) and four patients received 5-FU and leucovorin (FUFA) combination.
[c]Twelve patients also received Avastin in combination. Significant differences between groups in bold.

Table II. Differences in transcript levels of ABCs between tumour and control tissues

Gene	All patients, $n = 51$[a]	Tumour versus control
ABCA1	0.001	Down-regulation
ABCA2	NS	No change
ABCA3	0.017	Down-regulation
ABCA4	0.001	Down-regulation
ABCA5	<0.001	Down-regulation
ABCA6	<0.001	Down-regulation
ABCA7	NS	No change
ABCA8	<0.001	Down-regulation
ABCA9	<0.001	Down-regulation
ABCA10	<0.001	Down-regulation
ABCA12	<0.001	Up-regulation
ABCA13	0.002	Up-regulation
ABCB1	<0.001	Down-regulation
ABCB2	NS	No change
ABCB3	NS	No change
ABCB4	<0.001	Down-regulation
ABCB5	<0.001	Down-regulation
ABCB6	0.004	Up-regulation
ABCB7	NS	No change
ABCB8	NS	No change
ABCB9	NS	No change
ABCB10	0.003	Down-regulation
ABCB11	<0.001	Down-regulation
ABCC1	<0.001	Up-regulation
ABCC2	0.001	Up-regulation
ABCC3	<0.001	Down-regulation
ABCC4	0.008	Down-regulation
ABCC5	<0.001	Down-regulation
ABCC6	<0.001	Down-regulation
ABCC7	0.001	Down-regulation
ABCC8	<0.001	Down-regulation
ABCC9	NS	No change
ABCC10	NS	No change
ABCC11	NS	No change
ABCC13	<0.001	Down-regulation
ABCD1	NS	No change
ABCD2	<0.001	Down-regulation
ABCD3	<0.001	Down-regulation
ABCD4	<0.001	Down-regulation
ABCE1	<0.001	Up-regulation
ABCF1	NS	No change
ABCF2	NS	No change
ABCF3	NS	No change
ABCG1	<0.001	Down-regulation
ABCG2	<0.001	Down-regulation
ABCG4	NS	No change
ABCG5	NS	No change
ABCG8	NS	No change

[a]Significantly deregulated genes by REST software (*P*-value displayed); NS—non significant.

Then, the ratio of C_T values for ABC gene/ENDO was calculated and used for non-parametric tests as described in Materials and methods. Thus, the higher ABC/ENDO ratios signify lower relative ABC transcript levels. Clinical characteristics presented in Table I were correlated with transcript levels of ABC genes in tumour tissues. A significant inverse correlation between ABCC10 and tumour grade was found; the higher the grade, i.e. the more aggressive the tumour, the lower the ABCC10 transcript level (Table III). Transcript levels of ABCA12, ABCC7 and ABCC8 in tumour tissues had the following significant trend in tumour localisation: colon < rectosigmoideum < rectum. Interestingly, all three of the above listed transcripts followed exactly the same trend in control tissues as in tumours (Table III). However, ABCB9, ABCB11, ABCG5 and ABCG8 followed the opposite significant trend, i.e. a decrease in transcript levels in the direction from colon to rectum (Table III).

Relations between transcript levels of ABCs in tumours and chemotherapy outcome

In the group of patients with metastatic CRC, the response to the palliative chemotherapy significantly correlated with the transcript levels of ABCC6, ABCC11, ABCF1 and ABCF2 (Table III). In the group of patients with radically R0 removed tumours, the DFI after adjuvant chemotherapy was influenced by transcript levels of several ABCs. Patients with a low transcript level of ABCA7 (cut-off $\geq$ 1.25) had a significantly shorter DFI (P = 0.033, log-rank test) and a higher HR of progression than patients with a high transcript level (HR = 5.09, 95% HR confidence limits = 0.97–6.7; Figure 1A). Similarly, patients with low transcript levels of ABCA13 (cut-off $\geq$ 1.58), ABCB4 (cut-off $\geq$ 1.48), ABCC11 (cut-off $\geq$ 1.68) and ABCD4 (cut-off $\geq$ 1.19) had a shorter DFI and a higher HR of progression (P = 0.011, HR = 5.75, 95% HR confidence limits = 1.25–26.37; P = 0.003, HR = 15.83, 95% HR confidence limits = 1.72–145.92; P = 0.016, HR = 8.81, 95% HR confidence limits = 1.05–74.16; P = 0.023, HR = 5.51, 95% HR confidence limits = 1.07–28.48, respectively, Figure 1B–E) than patients with high transcript levels. ABCC11 was thus a significant predictor of chemotherapy outcome in both groups of patients.

Discussion

The efflux of endogenous substrates and also xenobiotics including anticancer drugs out of the cells presents the principal activity of ABCs. Thus, ABCs could be important for both CRC onset and CRC treatment outcome. A number of monogenic disorders have been associated with ABCs, e.g. mutations in *ABCC7* gene with the onset of cystic fibrosis and thus, the analysis of mutations in ABCs is routinely used for genetic testing (9). Although some of the studies observed significant associations between polymorphisms in ABCs and the cancer risk, including CRC, there has been no validated marker among all known 49 human ABCs for CRC prognosis and therapy outcome prediction until now. Based on the results of our pilot study, we propose ABC10 and ABCC11 as such markers and targets for validation studies focused on their confirmation or rejection.

Despite numerous published studies in animals and cell models showing the functional impact of ABCs on multidrug resistance, no convincing evidence of a clinical utility of ABCs as markers for the prediction of chemotherapy outcome in cancer patients has been published to date. The majority of published studies deal with associations of SNPs with therapy outcome or toxicity. However, there is limited knowledge about the association between genotype and phenotype. The reason may be the complexity of ABCs, which are promiscuous and overlap in spectra of transported substrates. The lack of information about gene and protein expressions and activity of ABCs in homogenous groups of patients treated by the same spectra of drugs further complicates the evaluation of their importance. CRC seems to be a suitable disease for the pharmacogenomic profiling of ABCs because it is routinely treated by prototypical substrate of certain ABCs, 5-FU. Therefore, we have conducted a pilot study aimed at the evaluation of transcript profiles of all human ABCs in two

Table III. Significant correlations of transcript levels of ABCs with clinical data

Grade	G1 (n = 6)[a]	G2 (n = 38)[a]	G3 (n = 7)[a]	P[b]
ABCC10	1.14 ± 0.04	1.17 ± 0.04	1.23 ± 0.05	0.010
Primary localisation—tumour tissue	Colon (n = 24)[a]	Rectosigmoideum (n = 12)[a]	Rectum (n = 14)[a]	P[b]
ABCA12	1.65 ± 0.22	1.50 ± 0.10	1.46 ± 0.17	0.020
ABCC7	1.14 ± 0.18	1.07 ± 0.10	1.02 ± 0.04	0.017
ABCC8	1.78 ± 0.15	1.77 ± 0.14	1.67 ± 0.15	0.042
Primary localisation—control tissue				
ABCA12	1.76 ± 0.14	1.64 ± 0.10	1.59 ± 0.10	0.001
ABCC7	1.04 ± 0.08	1.01 ± 0.04	0.98 ± 0.04	0.005
ABCC8	1.52 ± 0.08	1.50 ± 0.05	1.44 ± 0.04	0.002
ABCB9	1.25 ± 0.03	1.27 ± 0.04	1.29 ± 0.03	0.005
ABCB11	1.29 ± 0.11	1.32 ± 0.05	1.37 ± 0.05	0.004
ABCG5	1.52 ± 0.10	1.62 ± 0.07	1.65 ± 0.09	<0.001
ABCG8	1.47 ± 0.11	1.58 ± 0.07	1.60 ± 0.10	<0.001
Response in palliative group	Responders (n = 13)[a]	Non-responders (n = 13)[a]	P[b]	
ABCC6	1.23 ± 0.05	1.28 ± 0.06	0.033	
ABCC11	1.57 ± 0.06	1.69 ± 0.06	<0.001	
ABCF1	1.06 ± 0.03	1.08 ± 0.03	0.044	
ABCF2	1.09 ± 0.03	1.12 ± 0.03	0.015	

[a]Mean ± SD.
[b]Significant differences analysed by independent Kruskal–Wallis test.

groups of CRC patients strongly differing in prognosis. Patients who underwent the radical surgical resection of the tumour have better prognosis than patients with the metastatic disease. Both groups were treated by chemotherapy regimens based on 5-FU and the short-term evaluation of response to this treatment was available. We hypothesised that a strong effect of potential marker(s) should be clearly visible in both groups. As far as we are aware, this is the first complex study of the role of ABC transporters in the tumour development and treatment conducted on CRC patients.

The majority of the studied ABCs were down-regulated or unchanged between tumours and non-neoplastic control tissues in this study. ABCA12, ABCA13, ABCB6, ABCC1, ABCC2 and ABCE1 were up-regulated in tumours versus control tissues. There are several studies on the assessment of transcript or protein levels of the selected ABCs (usually ABCB1, ABCG2 and members of ABCC subfamily) in the literature of CRC. Our data comply with the previous reports with regard to the down-regulation of ABCB1 (20) and ABCC3 as well as the up-regulation of ABCC2 in colorectal tumours (29). In addition, we found the up-regulation of ABCC1 that had not been previously detected (29). Moreover, our results confirmed the previously observed down-regulation of ABCG2. ABCG2 mRNA showed a 6-fold decrease in colorectal tumours in comparison with control tissues (48). A ubiquitous expression of ABCE1 mRNA in normal and tumour colon tissues was found. Among 21 peptides with the HLA-A2-binding motifs, two ABCE1-derived peptides were recognised by the colon cancer-reactive cytotoxic T lymphocytes in a dose-dependent manner. The authors suggested that ABCE1 and its peptides could be target molecules in specific immunotherapy for HLA-A2-positive CRC patients (49). Our observation of significant up-regulation of ABCE1 in tumour tissue of CRC patients, if confirmed by independent study, may be relevant for targeted therapy of CRC. There are no literature data on transcript or protein levels of other ABCs in tumour and non-neoplastic tissues from a series of CRC patients.

The analysis of the tumour localisation has identified highly interesting trends. Transcript levels of certain ABCs increased or declined in direction from colon to rectum in a significant trend. Firstly, we noticed this effect in the tumour tissues. To discern this effect from possible deregulation of mRNA expression by the tumour pathophysiology, we analysed the tissue distribution of transcripts of ABCs also in non-neoplastic control tissues. The same ABCs as in tumours, namely ABCA12, ABCC7 and ABCC8, were found differentially distributed in control tissues maintaining the same trend towards localisation (increase from colon to rectum). Additionally, ABCB9, ABCB11, ABCG5 and ABCG8 followed the opposite significant trend, i.e. a decrease in transcript levels in direction from colon to rectum. Significant changes in gene and protein expression along the anterior–posterior intestinal axis were observed for solute carrier transporters in mice (50). Moreover, mRNA expression levels of murine Abcb2, Abcb3, Abcb9, Abcc3, Abcc6, Abcd1, Abcg5 and Abcg8 displayed significantly differential gene expression along the duodenum, jejunum, ileum and colon (51). In human samples, the expression of mRNA for ABCB1 and ABCC2 was highest in jejunum and decreased towards more distal regions, whereas ABCC1 was equally distributed in all intestinal regions (52). We did not confirm the results of Gutmann *et al.* (53) who found that mRNA expression of ABCG2 was maximal in the duodenum and decreased continuously down to the rectum, perhaps due to the fact that we had no access to samples from duodenum. Moreover, human ABCs identified by us to be differentially distributed in colorectal tissues have not been studied in this regard yet. A possible relevance of the observed gradient in expression of ABCs for efficacy of CRC chemotherapy shall be further studied.

The majority of clinically associated ABCs were either down-regulated or unchanged in tumours versus control tissues. In terms of chemotherapy outcome, the induction of mRNA levels of ABCs and subsequent enhancement of their effect cannot be ruled out. In fact, the ABCG2 mRNA content in hepatic metastases was found to be higher after an

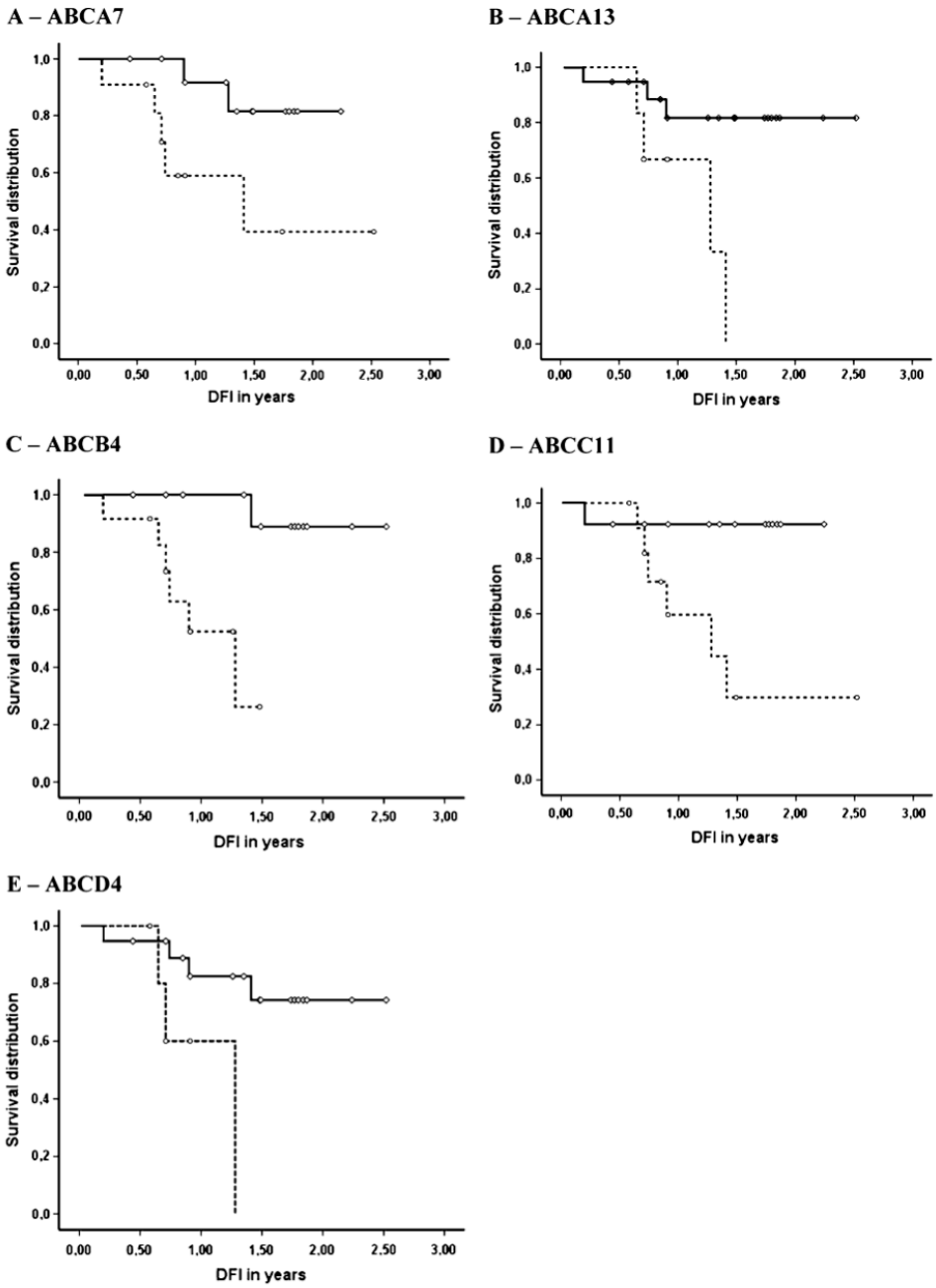

Fig. 1. Kaplan–Meier survival plots presenting the relation between DFI and transcript levels of ABCs. Survival curves are shown for two groups of patients divided by cut-off (for evaluation of cut-off, see Materials and methods). Individual figures illustrate Kaplan–Meier survival plots of patients with respect to transcript levels of ABCA7 (**A**, cut-off 1.25), ABCA13 (**B**, 1.58), ABCB4 (**C**, 1.48), ABCC11 (**D**, 1.68) and ABCD4 (**E**, 1.19). Dashed lines represent the group with higher normalised values than cut-off (meaning low transcript levels of ABCs) and solid lines represent the group with lower or equal values than cut-off (high transcript levels of ABCs).

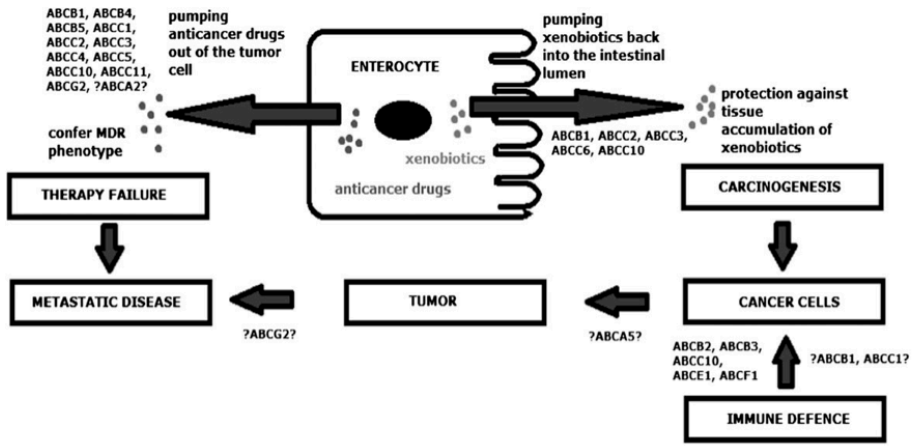

Fig. 2. Proposed involvement of ABCs in CRC development, progression and treatment outcome.

irinotecan-based chemotherapy than in irinotecan-naïve metastases (54). However, the access to post-operative liver metastases in the studied group of patients was limited and such analysis will be the subject of the validation study. In this context, it seems quite interesting that, according to recent study, synchronous or metachronous liver metastases could be treated differently on the basis of different gene expression signatures (55).

The transcript level of ABCC10 in tumours correlated with the grade in our study. There are no data on ABCC10 role in CRC in the literature. The lower ABCB1 expression was found in poorly differentiated CRC tumours ($P < 0.05$; 20) in line with the study on cell lines, where ABCB1 had a higher expression in well-differentiated colon cancer cells, when compared with the poorly differentiated ones (14). An increasing expression of another member of ABCC subfamily, ABCC3 with higher grade, i.e. the opposite trend to our observation with ABCC10, was found in pancreatic tumours (56). No further data on the association of other ABCs with grading in CRC are currently available. Zhang *et al.* (57) indicated that ABCB1 expression significantly correlated with tumour localisation ($P = 0.039$) and gender ($P = 0.043$). The intensity of immunohistochemical ABCG2 protein staining was higher in tumours of CRC patients with lymph node positive than in negative disease (30 versus 6.7%, respectively; $P < 0.025$). Thus, it seemed that ABCG2 expression may be important for progression and metastasis of CRC (58). Our pilot study, however, did not confirm any of the above published associations.

In the group of patients treated by palliative chemotherapy, the response was analysed using RECIST criteria. Patients were divided into two groups (responders versus non-responders). Transcript levels of ABCC6, ABCC11, ABCF1 and ABCF2 were significantly higher in responders in comparison with non-responders. In the group of patients treated by the adjuvant chemotherapy after the radical surgical resection of tumour, the relation between transcript levels of ABCs and DFI was evaluated. DFI was significantly shorter and HR of progression higher in patients with low transcript levels of ABCA7, ABCA13, ABCB4, ABCC11 and ABCD4, suggesting that these genes play a role in the therapy outcome of patients treated by the adjuvant chemotherapy. Thus, ABCC11 was up-regulated in the chemotherapy-naïve tumours from patients who subsequently achieved a good response to the chemotherapy in both palliative and adjuvant groups. ABCC11 was shown to be a resistance factor for fluoropyrimidines (34). Oguri *et al.* (59) discovered that expression of the ABCC11 is induced by 5-FU and that ABCC11 is directly involved in 5-FU resistance by the efflux transport of the active metabolite FdUMP in human small-cell lung cancer cell lines. We hypothesise that the high ABCC11 expression in tumours before chemotherapy observed in both groups of CRC patients studied by us may limit further induction of ABCC11 by 5-FU treatment. However, to confirm or reject our idea, we need to analyse post-chemotherapy samples from the same patients or run a larger validation study.

It is also interesting that the most studied ABCs in the area of chemoresistance, ABCB1, ABCC1-5 and ABCG2 did not predict 5-FU therapy outcome in our CRC patients. We cannot add more evidence to the results of the recent study reporting that ABCC1 in CRC tissues could predict the reduced folate level after leucovorin administration and determine the efficacy of treatment by leucovorin (60). Schmidt *et al.* (61) observed up-regulation of ABCC6 in 5-FU-resistant colon tumour cell lines. Quite recently, ABCC3, ABCC4 and ABCC5 were found up-regulated in 5-FU-resistant pancreatic carcinoma cells (62). Nevertheless, results obtained using rather uniform cell line models cultured in the presence of a drug for a long time may not reflect the real situation in such a heterogeneous entity as colorectal tumour or a series of such tumours. As regards, observation in cancer patients, a significant de-regulation of gene expression in a number of ABCs was found in breast cancer patients ($n = 19$) receiving preoperative (neoadjuvant) chemotherapy regimens including 5-FU. ABCA1, ABCA12, ABCB6, ABCC5, ABCC11 and ABCC13 were down-regulated in patients with complete pathological response, in comparison with patients with residual disease. In contrast, ABCB2, ABCC7 and ABCF2 were up-regulated in patients with complete pathological response (63). In breast cancer patients treated with endocrine therapy, ABCF2 protein immunopositive tumours had a longer disease-free survival when the tumours were oestrogen receptor negative or

progesterone receptor negative (P = 0.002 and 0.005, respectively; 64). Thus, the up-regulation of ABCF2 observed by us in CRC patients corresponds to available data on breast and cervical cancers. It seems obvious that ABCs have tumour-specific expression profiles (65) and thus also different roles in the mechanism of chemoresistance of the cancer cell (summarised in Figure 2). Thus, further studies are needed to evaluate the importance of the results obtained by this study.

The study of transcript levels rather than protein expression may be seen as the major limitation of our study. The power of protein analysis is obvious. However, the use of immunohistochemical analysis is semi-quantitative and reflects only protein level and not its activity. In addition, the issue of availability, specificity and selectivity of the antibodies further limits protein analysis. The correlations between transcript and protein levels of human ABCs are mostly unknown and thus, we shall consider transcript levels as independent markers. For the assessment of transcript levels, we have used highly sensitive methods based on real-time PCR, which is superior to relative or semi-quantitative approaches. The small sample size and absence of correction for multiple testing could be seen as another limitation of our pilot study. Thus, all results should be interpreted with caution and need to be confirmed by both retrospective and prospective validation studies on a larger and independent series of CRC patients.

In conclusion, our pilot study brings to light new and interesting data. Transcript levels of all known human ABC transporter genes were assessed in a series of tumour and control tissue samples from well-characterised CRC patients. Several genes were found deregulated in tumours compared with control tissues and a gradient of transcript levels along the superior–inferior axis of the large intestine was observed for the first time. The comparison of outcomes of patients treated by the 5-FU-based regimens suggested that ABCC11 may present a promising candidate marker. The validation of candidate genetic markers identified in the pilot study will be the next goal of our current research.

Supplementary data

Supplementary Table IS–VIS and Material are available at *Mutagenesis* Online.

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REVIEW

Assessing colorectal cancer heterogeneity: one step closer to tailored medicine

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Summary
Many advances in understanding colorectal cancer heterogeneity and its impact on the variability of treatment efficacy have been achieved in recent years. New methods have also been introduced in colorectal cancer diagnosis and early detection, including molecular biology techniques as well as newly developed or improved imaging techniques. We are currently aware of some aspects of colorectal cancer heterogeneity, such as alterations in the epidermal growth factor receptor signalling or the different behaviours of tumours belonging to different genetic and epigenetic subtypes. In the future, greater attention should also be focused on other signalling circuits with the goal to treat patients individually, based on the characteristics of their tumours. This so-called personalised medicine will bring more benefits to patients, without unnecessary adverse side effects. Therefore, all new information regarding colorectal cancer biology brings us one step closer to accomplishing this goal.

Key words: colorectal cancer; chromosomal instability; microsatellite instability; CpG island methylator phenotype; EGFR pathway; circulating tumour cells; stool DNA

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant diseases worldwide. According to the most recent data from the GLOBOCAN project, colorectal cancer, with its more than 1.2 million new cases per year, is the third most common type of tumour and results in 600 thousand deaths per year, ranking it fourth in terms of mortality (Ferlay et al. 2010).

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The biggest problem is the high ability of colorectal carcinoma to form secondary tumours, particularly in the liver and lung. Based on the different studies, 20% of patients have synchronous metastasis at the time of primary tumour identification, and more than 30% of patients develop metachronous metastasis during disease progression (Mejia et al. 2012).

Another complication connected to colorectal carcinoma is the high heterogeneity of the genetic and epigenetic changes among the individual tumours. In the past, all colorectal cancers were treated as the same tumour, and the only division was based on the hereditability of this malignancy; there were hereditary and sporadic CRCs. With the development of molecular biology and spreading of its methods into clinical medicine, it has become apparent that the division of CRCs into these two groups is insufficient. Based on the major genetic and epigenetic changes, we started to recognise three main subtypes of CRC displaying different clinicopathological features

(Walther et al. 2009). Recently, the complexity of these subtypes was further increased by the presence or absence of specific mutations in signalling pathways that can modify the response of tumours to a particular chemotherapy or monoclonal antibody treatment. Continual development of CRC is described on the Fig. 1.

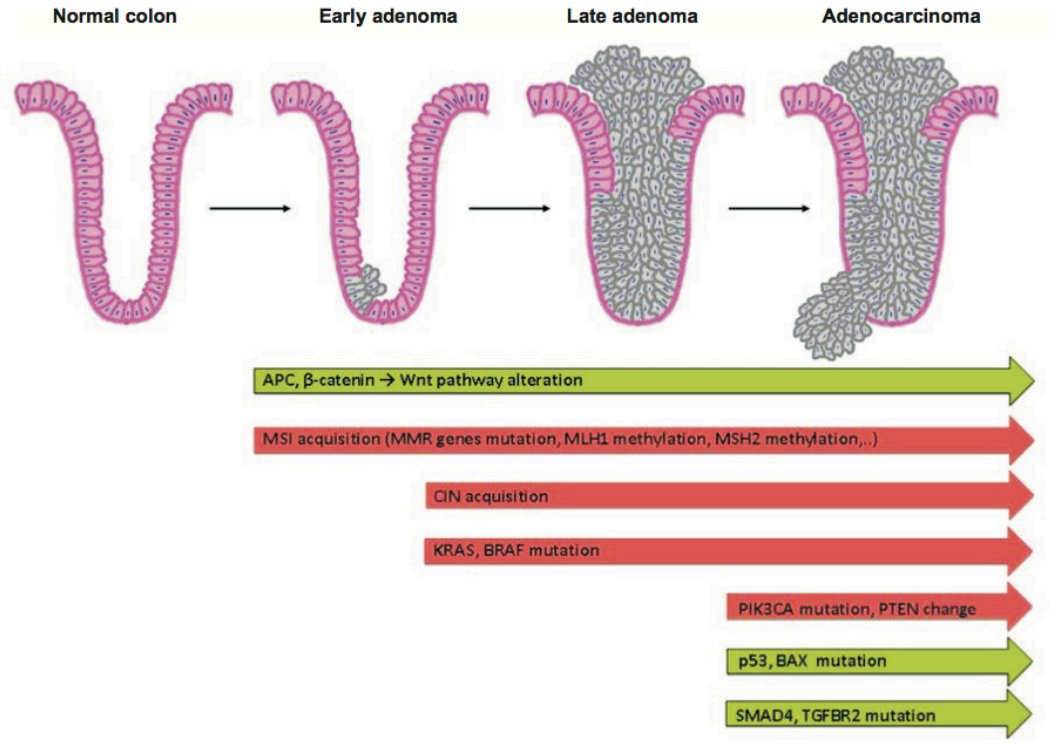

Fig. 1. **Continual development of colorectal cancer.** Cells of the colon crypt accumulate mutations and start to proliferate. In the green arrows you can see inactivation of antioncogenes, in the red arrows are mentioned most important changes in oncogenes.

Currently, surgical intervention still has an irreplaceable role in CRC treatment because it potentially removes the entire volume of the primary or secondary tumour without respect to its molecular-biological characteristics (Mulsow et al. 2011, Kosinski et al. 2012). The subsequent oncological treatment for the eradication of micrometastatic disease or circulating tumour cells is highly variable. The most common chemotherapeutics used in CRC management are based on 5-fluorouracil (5-FU) (often in combination with leucovorin), the active derivatives of platin (oxaliplatin) and irinotecan (an inhibitor of the nuclear enzyme Topoisomerase I) (Ismaili 2011). The large spectrum of chemotherapeutics was recently enriched with the possibility of biological treatment with the monoclonal antibodies (moAb) cetuximab and panitumumab, which target and block the functioning of the epidermal growth factor receptor (EGFR), thereby stopping the signalling cascade important for the growth and division of cancer cells (Wu et al. 2008, Markman et al. 2010, Garett and Eng 2011, Köhne et al. 2012). Unfortunately, none of these treatment types are universal for all CRC patients, and treatment responses vary dramatically. Additional information regarding CRC subtype, the presence of mutations and their roles in CRC development and maintenance is therefore crucial for the identification of the best course of treatment for the individual patient and for the application of "individualised medicine".

GENETIC AND EPIGENETIC SUBTYPES OF CRC

There are three subtypes of CRC based on major genetic or epigenetic changes: tumours with chromosomal

instability (CIN), those with microsatellite instability (MSI) and tumours with a CpG island methylator phenotype (CIMP) (Markowitz and Bertagnolli 2009, Perea et al. 2011, Armaghany et al. 2012). The most common subtype is CRC with chromosomal instability, which can be found in 80–85% of CRCs (Grady and Carethers 2008). MSI-positive tumours comprise 10–15% of CRCs (Malesci et al. 2007), and based on the number of altered markers, we can further distinguish the following three subtypes: MSI-high, MSI-low and microsatellite-stable (MSS). The frequency of CIMP varies between 12 to 25% of CRCs (Samowitz et al. 2005a), and we can further divide this subtype into CIMP-high and CIMP-low tumours, depending on the amount of methylated markers (Zlobec et al. 2011). The individual subtypes have different prognostic and predictive impacts on patients and are often grouped with specific mutations, which can further alter the prognosis and/or response to the selected treatment. In addition, these subtypes are not mutually exclusive, and often CRCs present with characteristics of more than one genetic and epigenetic subtype. Relatively common combinations include MSI+ and CIMP+ (Kang 2011) or MSI+ and CIN+ (Sinicrope et al. 2006). From a clinical point of view, determination of the main subtype can be helpful for the selection of appropriate chemotherapeutic treatment and for an accurate disease prognosis (Walther et al. 2009).

Chromosomal instability

The chromosomal instability pathway is the most common mechanism leading to CRC development. It can be described as global changes in the chromosome number (aneuploidy) accompanied with a loss of heterozygosity. The loss of part or all of a chromosome leads to a physical disappearance of 25–30% of alleles (Lengauer et al. 1998). There exist several different mechanisms for CIN development, including defects in chromosomal segregation (Wang et al. 2004), centrosome abnormalities (associated with aberrant expression of the genes for Aurora and Polo-like kinases) (Ganem et al. 2009, Lassmann et al. 2009, Han et al. 2012) or telomere dysfunction (O'Hagan et al. 2002, Murnane 2006, 2012). Along with karyotypic changes, specific mutations are found in the genomes of CIN-positive CRCs. It is not clear whether these mutations are products of CIN or if CIN is the product of particular mutations. These mutations affect pathways with important roles in CRC pathogenesis. The most frequently mutated tumour suppressor genes are APC, whose protein product is a major regulator of Wnt/β-catenin signalling and the cytoskeleton (Phelps et al. 2009), the TP53 gene, which is a regulator of transcription and cellular stress response (Zuckerman et al. 2009) and three genes located on the long arm of chromosome 18 (SMAD4, SMAD2 and DCC), which are frequently affected by allelic loss of this region (this loss is typical in greater than 70% of CRCs) (Fearon and Vogelstein 1990). Among the oncogenes, the most commonly mutated genes are CTNNB1, encoding the β-catenin protein, which has a very important role in CRC tumourigenesis (White et al. 2012), and KRAS and PIK3CA, which both play roles in cell survival and proliferation (Samuels and Waldman 2010). The presence of these mutations on the CIN-positive background is referred to as the chromosomal instability pathway of colorectal cancer development (Tejpar and Van Cutsem 2002). Despite an enormous effort to connect individual mutations with prognostic outcomes, none are currently in use as prognostic factors in clinical practice (Pino and Chung 2010). Generally, CIN-positive tumours are associated with less favourable outcomes than are MSI-positive CRCs (Popat and Houlston 2005, Walther et al. 2008). Revealing the pathways that lead to CIN development has aided in the identification of potential chemotherapeutic targets. The benefits of blocking the function of proteins such as the Aurora and Polo-like kinases or two proteins associated with chromosomal segregation (Eg5 and CENP-E) by small-molecule inhibitors are now being examined in preclinical and early clinical trials (data collected from www.clinicaltrials.com).

Microsatellite instability

Colorectal cancer with microsatellite instability accounts for 15% of all CRCs. A major cause of MSI development is an inactivation of DNA mismatch repair mechanisms (MRR), either by mutation or downregulation of repair gene expression by promoter hypermethylation (Söreide et al. 2006). The MMR pathway is a complex system that repairs accidental changes in DNA that arise by replication mistakes, thereby maintaining the integrity of the DNA (Jun et al. 2005, Kunkel and Erie 2005, Modrich 2006, Hsieh and Yamane 2008). The most important proteins of this pathway are MLH1, PMS2, MSH2 and MSH6, whose mutations play crucial roles in the development of the hereditary form of CRC known as Lynch syndrome. Sporadic CRCs with MSI have, in most cases, epigenetically silenced MLH1 promoters (Pino and Chung 2011). The phenotype of MMR inactivation involves a change in the length of microsatellite regions, which are mono-, di- or tri- nucleotide repetitions found in many genes. Inactivation of MMR leads to somatic mutations in the genes containing microsatellite regions (mostly by reading frame shifts and the production of shortened

or non-functional proteins). Some of these genes, such as PTEN, BAX and TGFβRII, are important for CRC development (Iacopetta et al. 2010).

Difficulties in the determination of MSI status lie in the identification of the best markers for assessing the microsatellite region length differences. The original marker panel, approved in 1997, contained two mononucleotide repetitions (BAT25 and BAT26) and three dinucleotide repetitions (D2S123, D17S250 and D5S345) (Boland et al. 1998). However, the presence of the dinucleotide repetitions in the panel led to a misclassification of some microsatellite-stable tumours as tumours with low-level MSI (MSI-L) (Murphy et al. 2006). In recent years, a new panel with five mononucleotide repetitions (BAT25, BAT26, NR21, NR22 and NR24) (Suraweera et al. 2002) has become preferred to the previous one.

From a clinical perspective, CRCs with MSI have a different phenotype than other subtypes, with a higher amount of tumour-infiltrating lymphocytes, a tendency to arise mainly in the proximal part of the large intestine and a lower differentiation status (Boland and Goel 2010). MSI status is not currently used for disease prognosis or prediction, but based on available experimental data, patients with MSI-positive tumours show better survival than patients with MSI-negative or CIN-positive tumours. This effect is further altered by the presence of other mutations in the genome (Popat and Houlston 2005). For example, a BRAF mutation on an MSI-negative background is prognostically very negative (Pai et al. 2012). MSI status is not an independent predictive marker because the results of studies are affected by the simultaneous occurrence of CIN or CIMP positivity. The effect of MSI status on treatment with 5-FU, irinotecan and oxaliplatin has been examined. In the case of 5-FU, a functional MMR system is necessary to achieve cell cycle arrest after the incorporation of 5-FU into the DNA and subsequent incorrect base pairing (Jo and Carethers 2006). In some cases, detrimental effects were reported when 5-FU treatment was used in MSI-positive patients in stage II and III of the disease (Sargent et al. 2010). In another study, patients with MSI-positive tumours in stage IV showed benefits and prolonged survival after 5-FU and leucovorin treatment (Liang et al. 2002). There are also sporadic reports indicating a higher sensitivity of MSI-high tumour cells to treatment by irinotecan (Fallik et al. 2003, Vilar et al. 2008). Recent meta-analysis did not show an association between MSI status and adjuvant chemotherapy (Des Guetz et al. 2009).

The presence of MSI can be used as an important marker for the screening of Lynch syndrome, particularly in younger patients with CRC or in families with a known genetic burden. In the case of early detection of MSI associated with germinal mutations in the MMR genes, special care and attention can be focused on these patients (Schofield et al. 2009).

CpG island methylator phenotype

A characteristic feature of the third CRC subtype is the presence of a CpG island methylator phenotype resulting from the aberrant methylation of DNA CpG islands (Toyota et al. 1999, Issa 2004). CpG islands are regions rich in cytosine and guanine that are situated in the promoter region or first exon of 70% of human genes (Saxonov et al. 2006). Normally, most of these islands are not methylated (in contrast to the CpG dinucleotides outside of promoter regions), except for those connected to imprinted genes or genes located on the inactivated X chromosome (Reik and Lewis 2005, Cotton et al. 2011). Approximately 5% of genes have aberrant methylation of CpG islands in colorectal carcinoma compared to normal tissue (Schuebel et al. 2007), which is a much greater amount than the number of genes affected by mutations (Wood et al. 2007).

Currently, there is no standard set of promoter regions for the assessment of CIMP status. Among the utilised panels for methylation level measurement, two are based on five studied regions (Chan et al. 2002, Weisenberger et al. 2006) and one is based on eight different chromosomal areas (Ogino et al. 2007). These panels have different sensitivities and specificities and results obtained by the different panels are not comparable between each other. Additionally, interpretation of the results is not unified; in addition to simple division of the tumours as CIMP-positive or CIMP-negative (Weisenberger et al. 2006), it is possible to divide them into three classes (CIMP-high, CIMP-low and CIMP-negative – Shen et al. 2007) or four classes (CIMP-high, CIMP-low and two CIMP-negative groups depending on the TP53 mutation status) (Hinoue et al. 2012), depending on the amount of methylated promoters.

CIMP is often connected to MSI because the majority of sporadic MSI-positive tumours arise via the epigenetic silencing of MLH1 gene expression, one of the most important proteins in the MMR pathway. Combining CIMP and MSI status, we can divide CRC into 4 to 6 groups with different clinical behaviours (Ogino and Goel 2008, Kang 2011). For example, the subgroup with the CIMP-high/MSI-positive phenotype usually has disease localised in the proximal part of colon and is more common in women and older patients (Kim et al. 2009, Bae et al. 2011, Hughes et al. 2012). Another important feature is the connection of CIMP and specific mutations.

CIMP-high CRCs correlate with mutations in BRAF (Weisenberg 2006), while the previously described connection between KRAS mutation and a CIMP-low phenotype is currently controversial (Ang et al. 2010). The most serious prognosis has been observed in patients with a CIMP-high/MSI-negative phenotype combined with the BRAF mutation (Lee et al. 2008).

The role of CIMP in treatment response prediction is unclear. Some studies have described a correlation between a CIMP-high status and a benefit from 5-FU adjuvant chemotherapy (Iacopetta et al. 2008, Min et al. 2011), but this result has not been confirmed by other groups (Jover et al. 2011) and requires more research.

MOLECULAR MARKERS WITH ROLES IN DISEASE PROGNOSIS AND TREATMENT PREDICTION

In addition to the described subtypes of CRC that alter the genotype and phenotype of cells globally, there is a large number of mutations in individual genes that can largely affect disease prognosis and prediction of treatment efficacy. Despite an enormous effort to identify new prognostic and predictive markers that can start a new era of personalised medicine for CRC patients, in current clinical practice, only two genes are monitored, which modify the usage of monoclonal antibodies blocking the function of EGFR. The studied markers indicate expression level status of the receptor itself and mutation status of its proximal effector KRAS. Other genes whose functional status should help with more precise disease prediction and more effective use of treatments are in various phases of experimental research. Some of them are described in the following text.

Epidermal growth factor signalling pathways

EGFR is a receptor-tyrosine kinase belonging to the HER-Erb2 protein family (Warren and Landgraf 2006). This single transmembrane glycoprotein can be specifically activated by the binding of its cognate ligand to the extracellular domain of the receptor. After ligand binding, the receptor dimerises (there can be homodimerisation as well as the formation of heterodimers with other members of its family). Dimerisation leads to activation of the intracellular kinase domain, which phosphorylates proximal members of signalling pathways emanating from the EGFR receptor. The most important proximal effector is the KRAS protein. Among the pathways activated by EGFR signalling are the MAPK, PI3K/AKT and Jak2/Stat3 pathways, all of which have crucial roles in maintaining cellular homeostasis (Yarden and Sliwkovski 2001, Lurje and Lenz 2009) (Fig. 2). Aberrant cell proliferation and its consequences are one possible outcome of the dysregulation of these pathways (Spano et al. 2005). The role of EGFR in CRC development seems to be essential, but the physiological function of EGFR signalling in the intestinal epithelium is still a matter of debate. According to current information, EGFR signalling plays an important role in the maintenance of the intestinal stem cell population (Sato et al. 2009, 2011, Xu et al. 2011, Nautiyal et al. 2012).

The EGFR pathway has a unique position in CRC treatment because it is one of the two pathways where the indication of the targeted treatment has been approved for patients with metastatic CRC (Cunningham et al. 2004). There are two types of EGFR inhibitors – small molecule tyrosine kinase inhibitors (TKI), such as erlotinib and gefitinib, and monoclonal antibodies, such as cetuximab and panitumumab (Ng and Zhu 2008). TKIs function to block the intracellular kinase domain (Takeuchi and Ito 2001), while antibodies target the extracellular domain and block receptor dimerisation (Okamoto 2010). When this type of treatment was introduced in clinical practice, only a small percentage of treated patients (approximately 10%) benefited from monoclonal antibody usage (Cunningham et al. 2004). Further research revealed that cellular EGFR expression on its own is not sufficient for the prediction of a treatment benefit. The first marker discovered to be responsible for a lack of treatment benefit in response to the anti-EGFR monoclonal antibodies was the mutated KRAS protein. Mutations resulted in its constitutive activity and consequent independence from EGFR stimulation (Lièvre et al. 2006).

KRAS mutations can be found in 40% of CRC patients (Amado et al. 2008). The KRAS mutations with the greatest impact are located in codons 12 and 13 in the second exon. These two changes impair the intrinsic hydrolytic activity of the KRAS protein and stop the degradation of GTP to GDP. The role of the KRAS mutation was first observed in small retrospective studies (De Roock et al. 2008, Lievre et al. 2008) and later confirmed in large randomised prospective studies including CRYSTAL (Cetuximab Combined with Irinotecan in First-line Therapy for Metastatic Colorectal Cancer; Van Cutsem et al. 2009), OPUS (Oxaliplatin and Cetuximab in First-line Treatment of Metastatic Colorectal Cancer; Bokemeyer et al. 2009) and PRIME (Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer

to Determine Efficacy; Douillard et al. 2010). Based on their conclusions, a benefit from moAb treatment was detected only in CRC patients with wild-type KRAS protein. This inference was essential because it allowed the stratification of patients based on the presence/absence of the mutation and protected a population of the patients (those with mutated KRAS) from the unnecessary side effects of an ineffective therapy. Several recent reports have disrupted part of this theory, as different authors have shown that not only the mutation of KRAS in general is important for treatment effect prediction, but the particular mutation type must also be assessed. For example, patients with a specific change in codon 13 (p.G13D) had a partial response to the cetuximab antibody, which was unlikely to occur in patients with mutations in other portions of the KRAS protein (De Roock et al. 2010b, Tejpar et al. 2012).

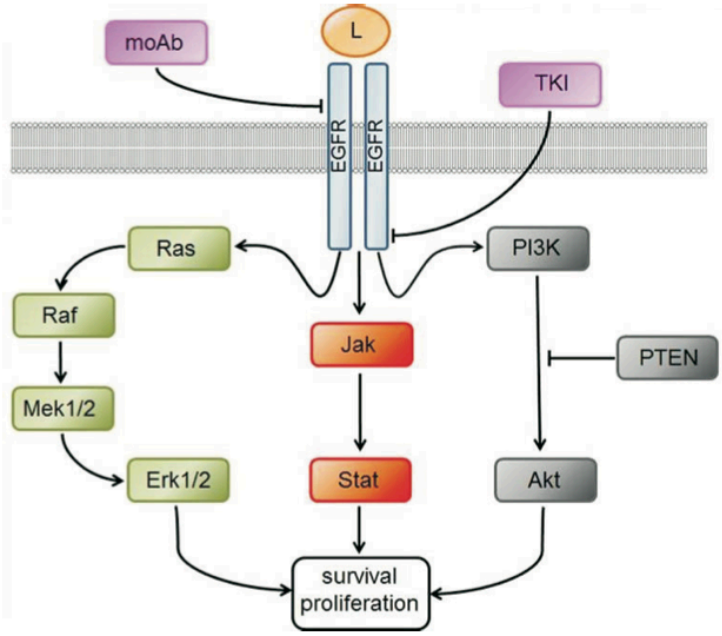

Fig. 2. **Overview of the EGFR signaling pathways.** The role of EGFR signaling in colorectal cancer is crucial mainly from the therapeutic point of view. EGFR function can be blocked by the monoclonal antibodies (moAb) affecting the extracellular domain by inhibiting the ligand binding (L), or by the small tyrosine kinase inhibitors (TKI), affecting the kinase domain. Three depicted pathways emanating from the EGFR are often modified in the tumour, mainly by the acquisition of different genetic/epigenetic abberations and resulting change in the signaling capacity.

Unfortunately, even KRAS wild-type patients fail to exhibit a homogeneous response to moAb therapy. With the KRAS-mutated patients excluded, a positive reaction was still only achieved in 20–40% of patients (Vecchione et al. 2011). One possible explanation might be the functioning of another RAS family protein, NRAS. Nevertheless, it can be responsible only for a minor part of the unresponsiveness because it is mutated in only 3% of patients (De Roock et al. 2010a). Examination of other members of the EGFR signalling cascade uncovered molecular changes in additional genes that cause tumour resistance to moAb therapy, particularly mutations in the genes coding for the BRAF and PI3K proteins and decreases in PTEN expression levels (PTEN is the major negative regulator of the PI3K pathway) (Grossmann and Samowitz 2011).

BRAF is a member of the RAF kinase family and the first kinase in the MAP kinase pathway (Chong et al. 2003). This protein is mutated in 10–15% of CRC patients. The point mutation V600E causes constitutive activity of BRAF's kinase domain (Davies et al. 2002, Samowitz et al. 2005b). Like the KRAS mutation, mutated BRAF has been repeatedly connected to tumour resistance to anti-EGFR moAb treatment (Di Nicolantonio et al. 2008, Loupakis et

al. 2009). The large randomised studies OPUS and CRYSTAL did not reach a clear conclusion regarding the predictive role of BRAF mutation in the treatment with cetuximab (Bokemeyer et al. 2012). However, they were able to demonstrate a prognostic effect of BRAF mutation, as the patients with the mutated variant had shorter lengths of overall survival than the patients with wild-type BRAF (Roth et al. 2010, Yokota et al. 2011, Bokemeyer et al. 2012). Because KRAS and BRAF are both members of one signalling pathway, their mutations are mutually exclusive. For tumour program activation in the cell, it is therefore likely that only one mutation in the given signalling pathway is necessary.

A second branch of EGFR signalling is the signalling cascade commenced by phosphatidil-inositol-3 kinase. This pathway, with the protein kinase AKT as the main node, performs a broad range of functions in the cell, including the regulation of glucose metabolism, gene expression, antiapoptotic actions and others (Vivanco and Sawyers 2002). Generally, this pathway regulates cell survival and metabolism. There are two changes in this pathway that have been connected to CRC development: mutation in the catalytic subunit of PI3K and change in the expression of the negative regulator protein PTEN. PIK3CA gene mutations (encoding the p100α catalytic subunit of PI3K) can be found in 15–25% of CRC patients (Samuels et al. 2005, De Roock et al. 2010a) and are located in exons 9 and 20 (approximately 70% of mutations are in exon 9 and 30% are in exon 20). The results of several studies focused on the predictive role of PIK3CA mutations in relation to the anti-EGFR treatment are ambiguous (Perrone et al. 2009, Prenen et al. 2009, Sartore-Bianchi et al. 2009), and it seems that a predictive role for these mutations is dependent upon the presence of other mutated genes (mainly KRAS and BRAF). Similar to the KRAS gene, each type of PIK3CA mutation has a different biological effect (De Roock et al. 2010a, Mao et al. 2012). In the case of the PTEN protein, the lack of standardised methods have caused diverse results in the individual studies, however, a connection between the loss of PTEN expression and resistance to the anti-EGFR-targeted drugs was observed (Colakoglu et al. 2008, Sawai et al. 2008, Sood et al. 2012). The roles of PTEN and PI3K expression and mutation status in treatment outcome prediction need to be validated in larger studies. The situation is very similar to the use of the PIK3CA or PTEN aberrations as prognostic markers, but recently published data have described potentially enhanced malignant behaviour in tumours with doubly mutated PIK3CA (Liao et al. 2012).

OTHER PATHWAYS IN CRC DEVELOPMENT

Despite the importance of the EGFR pathway in CRC development and treatment, other signalling cascades also have high potential for use in acquiring prognostic or predictive information or for targeting by CRC therapy. A currently existing method in CRC treatment is the blocking of VEGF signalling by the anti-VEGF monoclonal antibody bevacizumab. The VEGF pathway appears to be crucial for tumour angiogenesis, and its blocking proved to be efficient in a group of CRC patients (Hurwitz et al. 2004, Giantonio et al. 2007). This pathway can also be partially used for disease prognosis because particular single nucleotide polymorphisms in the VEGF gene were described to have prognostic roles (Vidauretta et al. 2010).

Other possible pathways crucial for CRC development have been identified in recent years thanks to technological advances such as whole-genome sequencing and exome scanning. These methods have described in higher detail the heterogenity of individual CRC cases, but have also revealed some common features. A recently published article from the authors contributing to The Cancer Genome Atlas Network focused on deep exon and whole-genome sequencing in 276 samples (Cancer Genome Atlas Network 2012). The authors found new types of alterations at all levels, starting from individual gene mutations, methylation or amplification and ending with chromosomal deletions or translocations. One of the most interesting results described the frequency of aberrations in entire signalling pathways. The most affected were the WNT, TGFβ, RAS-MAPK, PI3K and p53 pathways. Another interesting fact was that almost all genes had changes in the MYC target genes, demonstrating the great importance of this oncogene in CRC development. Currently, there are only two pathways of those described above that can be blocked by targeted treatment against the EGFR, RAS-MAPK and PI3K pathways. Therefore, there is a clear need to develop new types of treatments to target the other pathways essential for tumour development and growth.

NON-INVASIVE DETECTION OF CRC HETEROGENITY

In the previous paragraphs, we attempted to convey that CRC heterogenity causes major complications in the treatment of individual patients. In clinical practice, the only method for assessing the subtype or major mutations of the CRC without surgical removal

of the tumour is by biopsy, but this intervention brings additional stress to the patient. Therefore, in recent years, much effort has been dedicated to developing new, non-invasive methods that would enable us to determine the tumour subtype, its mutations and other characteristics, to better predict response to treatment. Two promising options are the detection of circulating or disseminated tumour cells, which are released from the tumour into the blood stream or bone marrow (Bidard et al. 2012), and the detection and analysis of cell-free DNA from stool samples (Miller and Steele 2012).

Circulating tumour cells

The fact that tumours release individual cells into bodily fluids was first observed by T. R. Ashworth almost 150 years ago (Sleijfer et al. 2007), and since this discovery, there have been attempts to use them for diagnostic as well as therapeutic purposes. However, it took until the last decades to transfer those ideas into practice, mainly because of the development of modern methods for cell isolation, detection and analysis.

The main complication is the low amount of circulating tumour cells (CTC) in contrast to normal blood or bone marrow cells. The first step in CTC detection is sample enrichment. The most common method is the use of positive immunoselection; other possibilities include negative immunoselection or centrifugation in a density gradient, which is based on the different physical properties of CTCs and blood cells. An overview of the individual types of CTC enrichment has been published elsewhere (Mikulová et al. 2011, Sun et al. 2011, Pantel and Alix-Panabières 2012). Because CRC is a tumour of epithelial origin, it is possible to use characteristic epithelial markers for CTC enrichment, for example, expression of the cell surface protein EpCAM, or markers typical for individual types of tumours, because these markers are usually not present on normal blood cells, which are mesodermal in origin (Sleijfer et al. 2007). In initial CTC studies, lack of standardisation in the enrichment step caused heterogeneous and incomparable results among the individual studies. Even the type of anti-EpCAM antibody used for positive immunoselection could yield different results (Antolovic et al. 2010), and individual methods had different sensitivities, specificities and reproducibilities. This complication was circumvented by the introduction of several standardised systems, such as the CellSearch™ system, which was introduced in 2004 (Allard et al. 2004). This process consists of three steps: CTC enrichment using the anti-EpCAM antibody, subsequent staining of the sample with an anti-cytokeratin antibody (characteristic for epithelial tissue) and immunostaining with an anti-CD45 antibody, which is expressed by haematopoietic cells and serves to control for nonspecifically selected cells. The result of these three steps is a cell suspension highly enriched for CTCs, which can be used for further analysis. This system is currently widely used and many studies employing CellSearch for CTC quantification in different types of carcinomas have been published (Cohen et al. 2008, Thorsteinsson et al. 2011, Munzone et al. 2012). It is also the only system approved by the U.S. Food and Drug Administration as a method for the ancillary diagnosis of patients with metastatic CRC. The number of CTCs itself was found to be a prognostic marker in patients with metastatic CRC (Rahbari et al. 2010).

In addition to counting the CTCs in peripheral blood, it is also possible to assess the mutational status of the selected genes in the isolated CTCs and to uncover information about the primary tumour. This method is at the beginning of its development but has already been tried in several other types of tumours (Kirby et al. 2012, Magbanau et al. 2012, Sakaizawa et al. 2012). In CRC, it will be possible to focus, for example, on the mutational status of genes in the EGFR signalling cascade or even to identify the main genetic and epigenetic tumour subtype.

Cell-free stool DNA

Rapidly dividing epithelial tissues release large amount of dying cells into their surroundings. In the case of the epithelial tissue covering the gastrointestinal tract, these cells are released into the lumen along with their nucleic acids. DNA, in the form of smaller fragments, passes through the digestive system and is ultimately excreted with the stool. This DNA can be isolated from the stool and used for molecular biological analysis (Ahlquist 2010). The biggest challenge to this technique is the very low ratio of epithelial DNA to other DNA found in the stool, a major part of which originates from gut microbiota (usually >99%) (Klaassen et al. 2003). Technological advances of recent years have fortunately provided new methods such as BEAMing (Beads, Emulsions, Amplification, Magnetics) (Diehl et al. 2008) or DMC (digital melt curve) (Zou et al. 2009), which have very high sensitivities allowing the detection of specific mutations, even if present in only 0.1% of gene copies. Using these methods, it is possible to assess mutations in the KRAS or BRAF genes (Deng et al. 2012, Li et al. 2012). A very promising alternative to mutational screening is the identification of specific methylation in the isolated stool DNA. Panels of markers for the best identification of the individual gastrointestinal malignancies are currently under development (Elliott

et al. 2013, Kisiel et al. 2012). Stool DNA analysis is beginning to be experimentally used as an alternative to the classical faecal blood test, mainly for preventive population screening and early tumour detection. In contrast to the common faecal blood test, stool DNA analysis has some disadvantages, including high cost and more complicated sample handling. However, it can also provide very useful information about the tumour, has very high sensitivity and can also identify tumours of the proximal regions of the gastrointestinal system (Ahlquist 2009).

CONCLUSION

As mentioned, many tumours are very heterogeneous and it is not an exception that morphologically identical tumours, which have the same tissue of origin, have developed by completely different pathways. For colorectal cancer, this heterogeneity is applicable with all its consequences. Rather than one homogeneous disease, colorectal cancer is many different types of tumours affecting one organ. However, these types are not strictly different from each other; they rather form a type of continuum of subtypes with many individual changes, which provides specific characteristics to each individual tumour. Recently, aspects of this tremendous heterogeneity have been revealed, and the most important ones were described in the individual chapters above, including novel diagnostic methods and the effects of molecular changes on patient survival.

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Přínos molekulární biologie v diagnostice a léčbě kolorektálního karcinomu – současnost a budoucnost

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Souhrn

Pitule P., Liška V., Třeška V., Novák P., Čedíková M., Králíčková M.: Přínos molekulární biologie v diagnostice a léčbě kolorektálního karcinomu – současnost a budoucnost

Kolorektální karcinom, jedno z nejčastějších maligních onemocnění, je i přes pokroky moderní chirurgické a onkologické léčby pro pacienta stále velmi závažnou diagnózou. Přežití pacienta po radikálním chirurgickém zákroku ovlivňuje zejména časnost záchytu onemocnění a výběr vhodné onkologické léčby, jejíž úspěšnost závisí především na vlastnostech samotné nádorové tkáně. V posledním desetiletí stoupá role molekulárně biologických metod v obou výše zmíněných kategoriích. Genetické a molekulárně biologické determinanty umožňují předpovědět prognózu onemocnění a rovněž napomoci ve výběru nejvhodnější léčby, která pacientovi přinese kýžený benefit v podobě prodloužení bezpříznakového a celkového přežití. Předkládaný přehledný článek popisuje nejdůležitější molekulárně-biologické znaky s prognostickým či prediktivním významem, které jsou využívané v klinické praxi nebo se nacházejí ve vysokém stupni klinické studie.

Klíčová slova: kolorektální karcinom – molekulárně biologické metody – prognostické faktory – prediktivní faktory

Summary

Pitule P., Liška V., Třeška V., Novák P., Čedíková M., Králíčková M.: Contribution of Mollecular Biology to the Diagnosis and Therapy of Colorectal Carcinoma – The Present and Future

Colorectal cancer is one of the most frequent malignant disease and despite of the development of modern surgical and oncological treatment, it is still a very severe diagnosis for the patient. The survival of the patient after the radical surgery is mostly affected by the time of detection of the disease and by the selection of the appropriate oncological treatment. The effectivity of the oncological treatment depends mainly on the features of the malignant tissue. During the last decade, the importance of the molecular biology and it´s methodology have been growing for both detection of the disease and the selection of the best treatment for the individual patient. Genetic and epigenetic characteristics of the tumours helps to predict the prognosis of the disease and also select the best treatment, which extends the disease-free and overall survival of the patient. The presented review describes the most important molecular-biological characteristics with the prognostic or predictive function, which are used in the clinical practice or are in the later phase of clinical study.

Key words: colorectal neoplasms – molecular biology methods – prognostic factors – predictive factors

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ÚVOD

Kolorektální karcinom (KRCa) je jedním z nejčastějších maligních onemocnění na světě a Česká republika v jeho výskytu zaujímá přední pozici [1]. Každý rok je v České republice nově diagnostikováno v průměru 8 000 nových případů a 5 000 pacientů na tuto chorobu zemře. Nebezpečí této choroby spočívá především ve vysokém počtu případů časné recidivy a v časté tvorbě metastáz, především v játrech, kam mohou migrující nádorové buňky s metastatickým potenciálem snadno vcestovat díky mezenterickému cévnímu systému.

Hlavní úlohu v léčbě primárního KRCa i jeho jaterních metastáz má radikální chirurgický zákrok, při kterém dojde k odstranění masy nádoru. V kombinaci s chemoterapií, která snižuje riziko recidivy a eliminuje nediagnostikovatelné mikrometastázy, dochází k poklesu počtu recidiv u radikálně operovaných pacientů. Nejstarším typem chemoterapie, využívané při léčbě pokročilého karcinomu tlustého střeva, je 5-fluorouracil a jeho deriváty v kombinaci s leucovorinem. Od konce devadesátých let se do klinické praxe postupně dostávaly nové typy chemoterapeutik, například inhibitor jaderného enzymu topoisomerázy I irinotecan (Camptosar®) či platinové deriváty (oxaliplatina, Eloxatin®), které výrazně zvýšily množství pacientů pozitivně reagujících na léčbu a prodloužily bezpříznakové i celkové přežití pacientů. Poslední novinkou v oblasti léčby pacientů s KRCa jsou protilátky blokující funkci proteinů, které jsou nezbytné pro růst nádoru. V praxi jsou již používané protilátky blokující receptor epidermálního růstového faktoru (EGFR) cetuximab (Erbitux®) a panitumumab (Vectibix®) a protilátka proti vaskulárnímu-endoteliálnímu růstovému faktoru (VEGF) bevacizumab (Avastin®).

V současné době je velkým problémem zvolit nejvhodnější kombinaci chirurgické a onkologické léčby pro jednotlivého pacienta postiženého KRCa. V této souvislosti se stále častěji využívají metodiky a poznatky molekulární biologie, které přinášejí nové důležité podklady pro diagnózu, prognózu i výběr terapie, čímž pomáhají šetřit nemalé náklady, které jsou na léčbu potřebné, a především zvyšují šanci pacientů na delší bezpříznakové i celkové přežití.

V následujícím přehledovém článku autoři prezentují nejvýznamnější molekulárně biologické mechanismy, které jsou v současné době považovány za nejdůležitější pro prognózu KRCa a jsou využívány v klinické praxi nebo probíhá jejich evaluace ve vysokém stupni klinické studie.

CIRKULUJÍCÍ NÁDOROVÉ BUŇKY

V posledních letech velmi diskutovaným směrem výzkumu v diagnóze a prognóze kolorektálního karcinomu, a karcinomů obecně, je možnost detekce a charakterizace cirkulujících nádorových buněk v periferní krvi, či tumorózních buněk diseminovaných do kostní dřeně. Přestože myšlenka samotná sahá již do poloviny minulého století, až vývoj vysoce citlivých detekčních metod v posledních letech umožnil výzkum a následně i aplikaci získaných poznatků do klinické praxe. Problémem byla především velice nízká koncentrace buněk uvolněných z tumoru do krve či kostní dřeně – řádově jednotky buněk v 10 ml periferní krve. Vzorek krve musí být proto nejprve specificky obohacen o potenciální nádorové buňky a až poté analyzován. K obohacení se nejčastěji používá centrifugace v hustotním gradientu nebo sofistikovanější imunomagnetické obohacení, kdy se využívají magnetické částice konjugované s protilátkami proti proteinům, které definují nádorovou buňku (v tomto případě především proteiny typické pro epiteliální buňky, například epiteliální adhezivní povrchový protein (EpCAM)). Po zahuštění následuje samotná detekce, použít lze imunocytochemii kombinovanou s vysokorychlostní automatickou mikroskopií, kvantitativní PCR (polymerase chain reaction), FISH (fluorescence *in-situ* hybridisation), průtokovou cytometrii (FC, flow cytometry), či nové metody typu mikrofluidních destiček, které jsou vysoce citlivé, ale stále ve fázi vývoje [2].

Z karcinomů je nejdále rozpracováno využití cirkulujících nádorových buněk při diagnóze a prognóze onemocnění u rakoviny prsu, kde je od roku 2007 jejich detekce jako tumorového markeru zahrnuta v doporučení Americké asociace klinické onkologie (ASCO, American Society of Clinical Oncology). U kolorektálního karcinomu je situace komplikovanější, dosud provedené studie zahrnovaly většinou malé množství pacientů a velmi se lišily metodicky a to nejen typem použité detekce, ale i dobou odběru krve či kostní dřeně (preoperační, postoperační) a charakteristikami pacientů zařazenými do studie. Výsledky jednotlivých studií jsou nekonzistentní, přesto většinou přiřazují cirkulujícím či rozesetým tumorózním buňkám určitý prognostický význam. Recentní meta-analýza všech těchto studií prokázala signifikantní korelaci mezi detekcí cirkulujících kmenových buněk, špatnou prognózou onemocnění a zkráceným bezpříznakovým i celkovým přežitím.

Další výzkum je nezbytný. Analýza cirkulujících kmenových buněk, jejich počtu, ale i mutačních a expresních profilů, může přinést užitečný, v případě periferní krve i neinvazivní, nástroj pro hodnocení rizika časné recidivy a vzniku vzdálených metastáz, ale i pro průběžné sledování účinku používané léčby s možností její okamžité změny.

DETEKCE VOLNÉ DNA VE STOLICI

Vysoká incidence kolorektálního karcinomu a nemalé finanční náklady na léčbu pacientů v pokročilém stadiu onemocnění vedou ke snaze prospektivně sledovat rizikovou část populace a diagnostikovat onemocnění v jeho počáteční fázi. Jednou z nejpoužívanějších metod je detekce okultního krvácení ve stolici. Metoda je neinvazivní, levná a pacient je schopen provést si ji sám bez nutnosti návštěvy lékaře a teprve až k jejímu vyhodnocení je zapotřebí vzorek dopravit do laboratoře. Nepřináší sice vysokou senzitivitu, přesto její využití vede díky odhalení nemoci před propuknutím potíží ke snížení mortality spojené s kolorektálním karcinomem [3]. S rozvojem molekulární biologie, a především metod pro detekci mutací pomocí PCR či přímou sekvenací, se objevila možnost detekovat počínající nádory kolorekta, ale i jiných částí gastrointestinálního traktu, z volné DNA ve stolici. Význam těchto testů stále narůstá díky jejich vysoké senzitivitě, nízké ceně a využití nejen pro detekci kolorektálního karcinomu.

Významnou výhodou diagnostiky DNA tumoru oproti klasickému testování přítomnosti krve (respektive hemoglobinu) ve stolici, je schopnost odhalit nejen karcinomy, ale i jejich prekurzory, jako například adenomy. V principu lze odhalit všechny typů nádorů a premalignit, ze kterých se uvolňují buňky do intraluminálního prostoru, jelikož po těchto buňkách zůstane ve stolici částečně degradovaná DNA. Problémem první generace testů DNA ve stolici byla nižší schopnost detekce a bylo zapotřebí, aby mutaci neslo více než jedno procento všech kopií studovaného genu. Množství relevantní DNA ve stolici je přitom relativně malé – zbytky lidské DNA tvoří zhruba 0,01 % veškeré DNA ve stolici, zbytek tvoří DNA bakteriální mikroflóry. V posledních letech se objevily nové metody zvyšující analytické schopnosti testů, jako BEAMing (beads, emulsions, amplification, magnetics; metoda pojmenována po základních principech a materiálech, které využívá) a digitální křivka tání struktury DNA (DMC, digital melt curve), u kterých je zapotřebí jen více než 0,1 % mutovaných kopií charakteristických pro malignitu.

Další výhodou testování DNA je vyšší hladina detekce nádorů proximálních částí tlustého střeva, protože z nich

uvolněná krev je ve stolici již téměř kompletně degradovaná a tedy nedetekovatelná klasickými testy. Nová metodika přináší navíc možnost diagnostiky dalších nádorů gastrointestinálního traktu, jejichž incidence je nižší než u kolorektálního karcinomu, a proto ve většině případů nejsou vyšetřovány při screeningových vyšetřeních a jsou diagnostikovány až jako symptomatické a tedy v pokročilém stadiu. Jen typem zvolených markerů lze časně detekovat celou řadu různých malignit a pacienty tak léčit ještě před rozvojem nemoci. To by přineslo významné snížení nákladů na péči o pacienty a zvýšení jejich celkového i bezpříznakového přežívání. Experimentálně bylo využití metod testování DNA ve stolici ověřeno například u nádorů jícnu, žaludku, slinivky a dalších [4].

Technický pokrok a stejné metodiky lze využít i pro detekci DNA neinvazivním způsobem v tělních tekutinách, například v krvi či v moči. Nové postupy již byly experimentálně ověřeny a v blízké době se očekává jejich zavedení do klinické praxe.

MOLEKULÁRNÍ PREDIKTIVNÍ A PROGNOSTICKÉ FAKTORY KOLOREKTÁLNÍHO KARCINOMU

Kolorektální karcinom není homogenním onemocněním. Molekulární klasifikace jednotlivých typů se stále vyvíjí a v současnosti lze rozeznat tři základní typy definované podle přítomnosti chromozomální nestability (CIN), mikrosatelitní nestability (MIN) či metylovaných CpG ostrůvků (CIMP). Každý z těchto typů má mírně odlišné klinické projevy a jinak reaguje na léčbu. Kromě rozdělení podle majoritních změn v genomu lze nalézt celou řadu pozměněných signalizačních drah a mutací v jednotlivých genech, které se vyskytují vždy jen u určitého procenta kolorektálních karcinomů [5]. Se snahou co nejvíce individualizovat léčbu je potřeba identifikovat další spolehlivé prediktivní a prognostické znaky, díky kterým by bylo možné pacientům indikovat nejvhodnější léčbu.

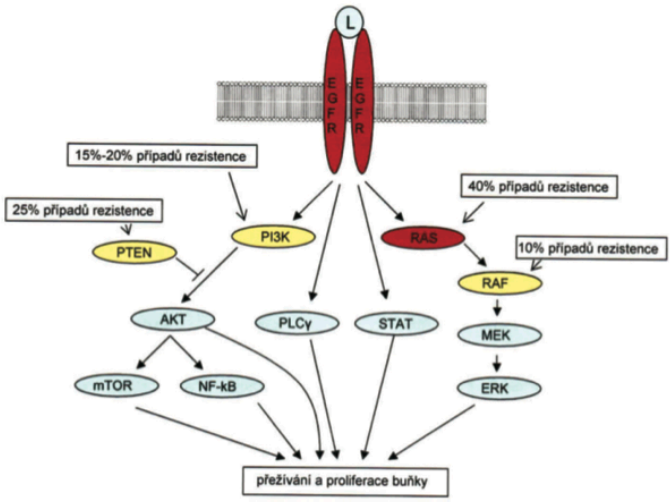
Prediktivní znaky jsou takové, které umožňují předpovědět efekt konkrétní léčby u individuálního pacienta. Znalost těchto znaků pomůže při výběru nejvhodnější léčby pro pacienta, který tak může být ušetřen případných nežádoucích efektů léčby, která by u něj nebyla účinná, a naopak může profitovat z časného nasazení efektivní chemoterapie či biologické léčby. Prognostické znaky naopak nejsou vázány na konkrétní typ léčby, ale poskytují informaci o prognóze onemocnění. Některé molekulární znaky mohou být jak prediktivní, tak prognostické, například přítomnost estrogenových receptorů u rakoviny prsu, ale u kolorektálního karcinomu zatím žádný znak s kombinovanou rolí identifikovaný nebyl.

Hledání a následná evaluace prognostických i prediktivních znaků je běh na dlouhou trať. V prvním kroku se kandidátní faktory většinou hledají pomocí metod molekulární biologie – měření proteinové exprese, genové exprese, hledání mutací v DNA apod. Poté většinou následují retrospektivní a prospektivní klinické zkoušky na větších skupinách pacientů, které ovšem často přinášejí nekonzistentní výsledky, například kvůli heterogenitě skupin pacientů zařazených do studií či odlišné metodice, která byla pro studium daného faktoru použita různými výzkumnými týmy. Popis v současnosti identifikovaných a nejvíce studovaných prognostických a prediktivních znaků u KRCa je uveden v následujících odstavcích.

Signalizační dráha vedoucí od receptoru epidermálního růstového faktoru

Rostoucí znalosti o biologickém chování různých typů tumorů v posledních deseti letech ukazovaly na podstatnou roli signalizačních drah vedoucích od receptorů pro růstové faktory ve vývoji a růstu různých typů tumorů. U KRCa se jedná především o receptor pro epidermální růstový faktor (EGFR), přes který buňka přijímá signály vedoucí k přežívání a k proliferaci. EGFR je transmembránový protein s intracelulární kinázovou doménou. Extracelulární oblast, ve které se nachází ligand-vazebná doména, je vhodným cílem pro vazbu specifických monoklonálních protilátek, které kompetují s ligandem o vazbu k receptoru, ale nespouštějí signalizaci a tedy přenos signálu přes daný receptor zablokují.

Popsaného mechanismu využívají dvě, v léčbě KRCa rutinně používané monoklonální protilátky proti EGFR, cetuximab (chimerická protilátka izotypu IgG1) a panitumumab (plně lidská protilátka izotypu IgG2). Nasazení těchto protilátek bylo v počátku indikováno pro pacienty s pozitivní expresí EGFR při imunohistochemickém vyšetření. Následné studie ovšem ukázaly, že v průměru pouze 10 % léčených EGFR-pozitivních pacientů reagovalo pozitivně na přidání monoklonální protilátky do léčebného režimu [6]. Předpokládaným důvodem snížené reakce některých pacientů byla mutace či jiná změna v proteinech signální dráhy EGFR, a jako hlavní kandidát se ukázal být známý onkogen KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog). Ve velkých randomizovaných studiích, které porovnávaly účinnost léčby zavedených chemoterapeutických postupů bez a s monoklonální protilátkou (například OPUS, CRYSTAL), byla opět u řady pacientů pozitivních na expresi EGFR zaznamenána snížená nebo žádná reakce na léčbu monoklonální protilátkou. V případě panitumumabu dokonce použití protilátky u některých pacientů vedlo ke sníženému celkovému přežívání. Testování pacientů na přítomnost KRAS mutace potvrdilo, že pozitivní efekt monoklonální protilátky proti EGFR je svázán s nemutovanými alelami genu KRAS. U jedinců s mutovaným proteinem KRAS je nejčastěji postižena autoinaktivační funkce, protein je konstitutivně aktivní a k signalizaci nevyžaduje aktivaci EGFR. U pacientů s konstitutivně aktivním proteinem KRAS je tedy použití protilátky blokující EGFR bezúčelné. V současnosti je kromě proteinu KRAS studováno několik dalších proteinů v signalizační kaskádě EGFR i receptor samotný. Stručný přehled signalizačních kaskád vedoucích od EGFR je zachycen na obrázku 1.



Obr. 1. Přehled základních drah vedoucích od receptoru pro epidermální růstový faktor (EGFR). U kolorektálního karcinomu hrají zásadní roli při rozvoji malignity především dráhy aktivované proteiny PI3K a RAS (všechny jeho typy). Kromě PI3K a RAS signalizační kaskády jsou aktivovány i další dráhy, například dráha fosfolipázy Cγ či rodiny transkripčních faktorů STAT. Červeně označené proteiny mají potvrzený prediktivní či prognostický význam a jsou zkoumány během výběru vhodné léčby pro pacienta či předpověď závažnosti onemocnění. Žlutě označené proteiny v současnosti využívány pro prognostické či prediktivní účely nejsou, ale po dalším ověření by jejich mutační či expresní změny mohly sloužit jako důležité faktory pro indikaci vhodné terapie. U proteinů RAS, PI3K, RAF a PTEN jsou uváděn odhadovaný procentuální podíl na rezistenci k léčbě monoklonálními protilátkami proti EGFR. Upraveno podle [20] a podle dat z internetové databáze signálních drah http://www.biocarta.com/genes/allPathways.asp; mTOR = mechanistic target of rapamycin (serine/threonine kinase); PLCγ = phospholipase C, gamma; STAT = signal transducer and activator of transcription.

Fig. 1. Overview of the signaling cascades activated by the epidermal growth factor receptor. The major role in the development of the colorectal carcinoma play pathways activated by PI3K and RAS proteins. In addition to PI3K and RAS signalling cascades, EGFR signalling activates other pathways like phospholipase Cγ or the family of STAT transcription factors. Red marked proteins are confirmed and used as a prognostic or predictive factors and are impotant for the selection of appropriate treatment or for the prediction of the disease outcome. Yellow marked proteins are not at present use for the prediction or prognosis, but after the future evaluation they can serve as a important factors for the selection of best treatment or for the disease prognosis. In the boxes next to the RAS, PI3K, RAF and PTEN proteins is the estimated percentage of colorectal carcinoma resistance to the anti-EGFR monoclonal antibody treatment caused by the mutation in the particular protein. Modified from [20] and from the signaling pathways database http://www.biocarta.com/genes/allPathways.asp; mTOR = mechanistic target of rapamycin (serine/threonine kinase); PLCγ = phospholipase C, gamma; STAT = signal transducer and activator of transcription.

KRAS jako prediktivní a prognostický faktor

Aktivační mutace v protoonkogenu KRAS jsou potvrzené jako negativní prediktivní znak léčby KRCa monoklonálními protilátkami (moAb) proti EGFR. Mutace se vyskytují u zhruba 40 % pacientů s KRCa a ti tak mohou být po vyšetření na KRAS mutační profil ušetřeni potenciálně toxické a bezvýsledné léčby, která by u nich nevedla k žádnému zlepšení stavu nemoci. Negativní efekt potvrdila řada nezávislých studií. Mutace v KRAS signifikantně korelovaly s odpovědí pacientů na léčbu. Například ve studii porovnávající efekt panitumumabu v porovnání s nejlepší podpůrnou léčbou (BSC, best supportive care) u pacientů, u nichž selhalo použití klasické chemoterapie, odpovídalo na léčbu 0 % pacientů s KRAS mutacemi a 17 % pacientů bez KRAS mutace. Rozdíl byl vidět i v efektu panitumumabu u jednotlivých skupin pacientů na délku období bez progrese onemocnění – 12,3 týdnů u pacientů s nemutovaným KRAS v porovnání se 7,4 týdny u pacientů s mutací (pro srovnání, u pacientů s podpůrnou léčbou bylo období bez progrese 7,3 týdne) [7]. Podobný efekt byl pozorován i u cetuximabu.

Zatímco prediktivní role KRAS mutací byla spolehlivě potvrzena, o jeho roli jako prognostického faktoru se stále diskutuje. Velká randomizovaná studie RASCALII popsala prognostický efekt mutace v KRAS, kdy je v kodónu 12 namísto glycinu valin – takto postižení pacienti měli snížené bezpříznakové i celkové přežití. Podobného výsledku dosáhla i studie prováděná na Chirurgické klinice KN v Pardubicích, ve které autoři na skupině 76 pacientů potvrdili negativní vliv KRAS mutace na prognózu [8]. Nicméně již zmiňovaná studie vlivu panitumumabu na léčbu KRCa neobjevila žádný efekt mutace KRAS na bezpříznakové přežití u pacientů s podpůrnou léčbou [7].

BRAF jako prediktivní a prognostický faktor

I přes vyřazení pacientů s KRAS mutacemi je odpověď na léčbu moAb poměrně nízká, což svědčí o faktu, že musí existovat ještě další geny podílející se na rezistenci k léčbě moAb proti EGFR. Serin/threoninová kináza BRAF (v-raf murine sarcoma viral oncogene homolog B1) je dalším proteinem v kaskádě EGFR signalizace a aktivační mutace (V600E) v BRAF genu se vyskytuje u 10–15 % nádorů kolorekta. Vliv této mutace na rezistenci k léčbě moAb potvrdila retrospektivní studie na 113 pacientech s metastatickým KRCa léčených jednou z používaných moAb. Autoři určili stav KRAS a BRAF mutací a korelovali je s odpovědí na léčbu, bezpříznakovým a celkovým přežitím. U KRAS mutace došlo k potvrzení předchozích závěrů, u 11 pacientů s nemutovaným KRAS byla detekována mutace BRAF. Ani jeden z těchto pacientů neodpovídal na léčbu a všichni měli kratší období bez progrese onemocnění i celkové přežití. Zároveň ani jeden z pacientů odpovídajících na léčbu nenesl mutovanou BRAF alelu [9].

BRAF mutaci V600E lze tedy považovat za prediktivní vzhledem k odpovědi pacientů na léčbu moAb. Kromě prediktivní role byla k této mutaci přiřazena i role prognostického faktoru – porovnání bezpříznakového a celkového přežití mezi pacienty s mutovanou a nemutovanou alelou BRAF signifikantně prokázalo zkrácení obou parametrů u pacientů s BRAF mutací a překvapivě byly oba typy přežití ještě nižší, než u pacientů s mutací v KRAS genu. I další práce napovídají, že BRAF mutace by mohla v některých případech (například v subtypu KRCa definovaného žádnou či nízkou mikrosatelitní nestabilitou) opravdu mít prognostický význam. Přes zajímavé experimentální výsledky je potřeba prediktivní i prognostickou roli BRAF mutace ověřit při randomizovaných klinických studiích.

PI3KCA mutace a ztráta exprese PTEN jako prediktivní a prognostické faktory

Phosphatidyl-inositol-3-kináza (PI3K) tvoří druhou hlavní efektorovou větev dráhy EGFR signalizace. Jedná se o kinázu se specifitou pro lipidy a může být aktivována nejen přímo EGFR, ale také interakcí s aktivní formou proteinu KRAS. Hlavním uzlem v PI3K aktivované dráze je další kináza AKT (v-akt murine thymoma viral oncogene homolog 1), která má v buňce řadu rolí, od regulace metabolismu glukózy přes blokování funkce pro-apoptotických proteinů až k regulaci genové exprese. Aktivace dráhy obecně vede k vyššímu přežívání buňky a její větší odolnosti k nepříznivému vlivu okolí. Protein PTEN (phosphatase and tensin homolog) je hlavní negativní regulátor funkce PI3K, jedná se o fosfatázu, která je schopná defosforylovat lipidy a tím blokovat spuštění PI3K signalizační kaskády.

PI3K, konkrétně její katalytická podjednotka označovaná p100α (PIK3CA), je velmi často mutovaná v celé řadě různých typů nádorů. U kolorektálního karcinomu se mutace v PIK3CA vyskytuje zhruba v 25 % případů sporadických KRCa, častěji u žen a u nádorů proximálních oblastí tlustého střeva. Mutace vedou ke katalytické aktivitě i bez předchozí stimulace, a dochází tak k zapnutí drah pro přežívání a proliferaci buňky. Stejný výsledný efekt má i ztráta exprese proteinu PTEN, která se vyskytuje zhruba u 30 % pacientů s KRCa.

U kolorektálního karcinomu proběhlo několik studií, které se pokoušely jednotlivým mutacím PIK3CA přiřadit prognostický či prediktivní efekt, výsledky jsou ale velmi nekonzistentní. Sartore-Bianchi s kolegy ukázala, že mutace v PIK3CA vede ke zkrácení bezpříznakového přežití a pacienti s PIK3CA mutací neodpovídají na léčbu monoklonálními protilátkami cetuximabem a panitumumabem. Efekt mutace byl však pozorovatelný jen u pacientů s nemutovaným proteinem KRAS. U osob s KRAS mutací byl vliv PIK3CA mutace neznatelný [10]. Naproti tomu Prenen s kolegy nepozorovali vliv PIK3CA mutací na léčebný efekt cetuximabu (samostatně či v kombinaci s irinotecanem) u pacientů s metastatickým KRCa neodpovídajícím na léčbu irinotecanem samotným [11]. Pro PI3K je ještě zapotřebí zevrubné studium jejího vztahu ke klinickým parametrům a její použití jako prediktivního či prognostického znaku v současnosti není možné, i přes existující náznaky, že PIK3CA mutace by mohly být z prediktivního hlediska považovány za negativní faktor indikace léčby monoklonálními protilátkami proti EGFR, stejně jako v případě proteinu KRAS, a mutace PIK3CA by mohly ukazovat na snížené přežívání postižených pacientů. Ztráta exprese PTEN má poté podobné dopady, jako mutace v PIK3CA, navíc byla pozorována korelace mezi PTEN expresí a lokální recidivou KRCa. Zajímavé je, že stejně jako v případě mutací v BRAF a KRAS, jsou i mutace v PIK3CA a ztráta PTEN vzájemně výlučné. Ke spuštění onkogenní malformace buňky tedy pravděpodobně stačí jen jedna změna v dané signalizační dráze.

Prediktivní a prognostické mutace a změny v dalších genech EGFR dráhy

Kromě výše zmíněných hlavních mutací a expresních změn byly zkoumány další proteiny a jejich molekulární změny v jednotlivých úsecích EGFR signální kaskády. Výsledky různých studií stejného proteinu jsou často velmi odlišné, například z důvodu odlišných použitých experimentálních technik či malého počtu studovaných pacientů. Žádný další prediktivní či prognostický znak nebyl dodnes přesvědčivě univerzálně potvrzen. Mezi nejzajímavější studované změny patří expresní hladina EGFR, jeho ligandů (především amphiregulin a epiregulin) a efektorového transkripčního faktoru NF-κB (nuclear factor-κB), počet genomových kopií EGFR, mutace a polymorfismy v EGFR, NRAS (neuroblastoma RAS viral (v-ras) oncogene homolog) a typ heteroduplexních receptorů, které EGFR tvoří po aktivaci s dalšími členy své receptorové rodiny.

V následujícím textu jsou stručně shrnuty informace o dalších zajímavých prognostických a prediktivních znacích v EGFR signalizační kaskádě. Samotná hladina exprese EGFR nepřináší prognostickou ani prediktivní informaci. Vyšší počet genomových kopií EGFR je spojován s delším celkovým přežitím pacientů, a byla popsána i prediktivní hodnota počtu EGFR kopií pro léčbu panitumumabem v konkrétních případech KRCa [12]. Vyšší exprese NF-κB v nádoru vede ke snížené odpovědi na léčbu nejen protilátkami proti EGFR, ale také snižuje léčebný efekt radioterapie [13] a klasické chemoterapie. Na malém vzorku pacientů bylo prokázáno, že pacienti s vyšší expresí NF-κB mají kratší období do progrese tumoru a nižší celkové přežití. Mezi pacienty se zdravým typem KRAS proteinu se stále nachází celá řada těch, kteří nemají žádný benefit z léčby EGFR blokujícími protilátkami. Dodatečnou informaci u těchto pacientů kromě mutačního statutu genů PIK3CA a BRAF nese i gen pro protein NRAS, který, pokud je mutovaný, snižuje výrazně odpověď na léčbu anti-EGFR protilátkami [14].

Genetické subtypy KRCa (CIN, MSI, CIMP) a jejich prognostická hodnota

Jak již bylo zmíněno výše, kolorektální karcinom lze z genetického a epigenetického hlediska rozdělit podle tří

hlavních kritérií: podle přítomnosti/nepřítomnosti chromozomální nestability (chromosomal instability, CIN), podle přítomnosti/nepřítomnosti mikrosatelitní nestability (microsatellite instability, MSI) a podle stupně metylace DNA (CpG insland methylator phenotype, CIMP). CIN se vyskytuje u 65–70 % případů KRCa a je rozpoznána jako mnohočetná změna v ploidii či struktuře chromozomů. MSI je definována jako změna v délce repetitivních sekvencí (mikrosatelitů) a vyskytuje se zhruba v 15 % případů KRCa. CIMP je často asociovaný s přítomností MSI u pacientů bez vrozené mutace v genech pro opravu DNA a je definovaný jako metylace určitého počtu analyzovaných oblastí v genomu. Zmíněné kategorie navíc vzájemnou kombinací vytvářejí další heterogenitu. Poměrné zastoupení jednotlivých typů se nachází na obrázku 2. Molekulární mechanismy jednotlivých genetických odlišností přesahují rámec tohoto článku, zájemci naleznou řadu informací v recentních souhrnných publikacích na toto téma [5].

U některých ze subtypů KRCa byla popsána korelace s prognózou onemocnění či s odpovědí na určitou léčbu. Pozitivita pro CIN a MSI u nádorů byla podrobena rozsáhlým meta-analýzám a z jejich závěrů vyplývá, že pacienti s CIN pozitivním nádorem mají horší prognózu onemocnění a pacienti s MSI pozitivním KRCa naopak lepší prognózu než CIN či MSI negativní. Původní představa o vzájemné neslučitelnosti CIN a MSI byla v posledních letech zpochybněna a jelikož CIN a MSI mohou nést odlišnou prognostickou informaci, je důležité ověřit jejich vzájemnou nezávislost, aby mohly být jako jednoznačné prognostické faktory používány.

CIMP lze rozdělit na dvě skupiny jako CIMP – high a CIMP – low, podle počtu metylovaných analyzovaných oblastí. U nádorů, které jsou MSI pozitivní a mají vysoký stupeň CIMP, se lze často setkat s mutací v genu BRAF a pacienti s tímto typem nádoru mají lepší prognózu. Naproti tomu nádory s MSI negativitou a vysokým stupněm metylace v kombinaci s BRAF mutací mají prognózu horší a se zvyšujícím se stupněm metylace CpG oblastí se prognóza zhoršuje. Druhou epigenetickou změnou u KRCa bývá celková DNA hypometylace, která je často asociována s CIN pozitivitou, a bylo ukázáno, že hypometylace vede ke zhoršení prognózy.

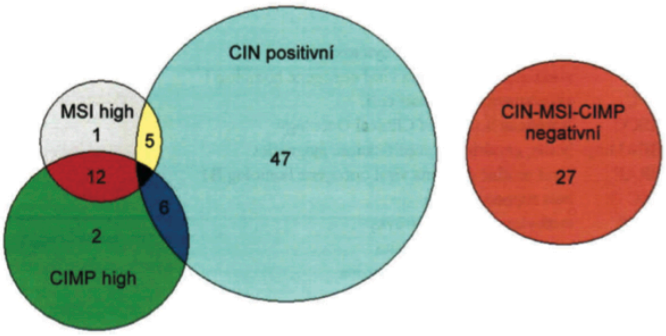
I přes řadu jednotlivých náznaků o prognostické významnosti subtypů KRCa je potřeba prokázat podíl jednotlivých genetických a epigenetických změn na celkový dopad prognózy v rámci rozsáhlých klinických studií, které v současnosti probíhají. I proto nebyl doposud žádný z těchto znaků schválen k využití v klinické praxi.

Prediktivní význam subtypů KRCa

Každý genetický a epigenetický subtyp KRCa je asociován s celou řadou expresních či mutačních změn v jednotlivých genech. Často jsou mutací, která alteruje či ruší jejich funkci, zasaženy geny, které hrají roli při působení či odbourávání různých chemoterapeutik. Tím dochází ke změně intenzity jejich účinku, v krajních případech mohou být silně toxické či na ně rakovinné buňky mohou být rezistentní. Určení subtypu KRCa tedy může přinést důležité informace pro výběr léčby pro konkrétního pacienta.

Systém genů pro opravu nesprávného párování (mismatch repair system, MMR) bází v DNA je nezbytný pro správnou funkci chemoterapeutik typu 5-fluorouracilu (5-FU), které se inkorporují do DNA a díky nesprávnému párování dojde ke spuštění signální kaskády, která končí zastavením buněčného cyklu. MMR systém je nefunkční v případě MSI pozitivních nádorů a byl dokumentován nepříznivý efekt při podávání 5-FU pacientům s tímto subtypem KRCa ve stadiu II a III [15], u léčby pacientů po paliativní resekci KRCa ve stadiu IV byla naopak odpověď na léčbu 5-FU v kombinaci s leukovorinem lepší. Existují i data o vyšší senzitivitě MSI pozitivních pacientů k léčbě irinotecanem.

Kolorektální karcinom s chromozomální instabilitou je prediktivní pro léčbu taxany, které ovšem obecně pro léčbu KRCa nejsou vhodné, jelikož výrazně neovlivňují celkové ani bezpříznakové přežití. Pacienti s CIN pozitivním nádorem nevykazují žádné benefity z léčby těmito chemoterapeutikami. Důvodem je nepřítomnost intaktního dělícího vřeténka v aneuploidních buňkách, které je nezbytné pro senzitivitu na léčbu využívající taxany, například paclitaxel (Taxol®).



Obr. 2. Poměrné zastoupení jednotlivých genetických a epigenetických subtypů KRCa. Chromozomální nestabilitu (CIN positivní) vykazuje 58 % KRCa, mikrosatelitní nestabilitu (MIN high) 18 % případů KRCa, metylaci CpG (CIMP high) ostrůvků 20 % KRCa a negativitu ve všech třech znacích 27% KRCa. Volně praveno podle [5].

Fig. 2. Proportional representation of the genetic and epigenetic subtypes of colorectal carcinomas (CRC). Chromosomal instability (CIN positivity) is present in 58% of CRC, microsatellite instability (MIN high) in 18% of CRC, CpG island methylation (CIMP high) in 20% of CRC and 27% of CRC is negative in all three criterions. Modified from [5].

Další molekulárně-biologické změny s prognostickým či prediktivním potenciálem u KRCa

Polymorfismy v Fc gamma receptorech

Fc-γ receptory (FcγR) se podílejí na efektivitě léčby monoklonálními protilátkami

izotypu IgG1 mechanismem protilátkově závislé buněčné toxicity (ADCC – antibody dependent cell cytotoxicity). Změny v FcγR, které ovlivní afinitu receptoru k Fc oblasti protilátky, tak mohou změnit účinnost imunitní reakce proti buňkám opsonizovaným protilátkami. U KRCa byly studovány dva polymorfismy s prognostickým využitím. Delší období bez progrese onemocnění (PFS) bylo více asociováno s FcγRIIa genotypy 131H/H nebo 131R/H než u varianty 131R/R, u polymorfismu 158V/V a 158 F/V nejsou výsledky jednotné [16]. Pro jednoznačné určení role jednotlivých alelických variant FcγR při prognóze odpovědi na léčbu anti-EGFR protilátkami je nutné uskutečnit rozsáhlejší prospektivní studii.

Mutace v genech pro reparaci DNA

Vrozené mutace v genech pro opravu nepřesného párování v DNA jsou příčinou Lynchova syndromu a vyskytují se rovněž u 15–20 % případů sporadického KRCa. Z prognostického hlediska jsou mutace v těchto genech často asociovány s mikrosatelitní nestabilitou a pro pacienty tyto změny znamenají výhodu delšího celkového přežití.

U jednoho z genů pracujících v dráze nukleotidové excizní opravy DNA – genu ERCC1 (excision repair cross-complementing rodent repair deficiency, complementation group 1) – byl popsán prediktivní charakter jeho polymorfismu v kodónu 118 pro odpověď na léčbu oxaliplatinou [5].

Expresní hladina topoizomerázy I

V randomizované studii FOCUS byla jako prediktivní biomarker studována expresní hladina genu TopoI. Proteinový produkt toho genu se účastní procesu stříhání dvouvláknové DNA při opravě jejích chyb a je inhibován irinotecanem. U pacientů s nízkou expresí TopoI nebylo PFS prodlouženo přidáním irinotecanu do léčebného procesu, u pacientů se střední či vysokou expresní hladinou došlo k prodloužení PFS [17].

Thymidylát syntetáza

Thymidylát syntetáza (TYMS) je hlavním cílem působení aktivního derivátu 5-FU. Bylo prokázáno, že vyšší expresní hladina TYMS koreluje s kratším celkovým přežitím pacientů. Změna exprese je zajišťována 3 mechanismy: tandemovou repeticí v promotorové oblasti TYMS genu, jednonukleotidovým polymorfismem v této repetici a inserčním/delečním polymorfismem v 3´ne-

Tab. 1. Přehled nejdůležitějších prediktivních a prognostických faktorů u kolorektálního karcinomu. Následující přehled obsahuje až na několik výjimek (například KRAS mutace v predikci odpovědi na moAb proti EGFR) znaky, které nejsou v současné době zavedené v klinické praxi, ale řada studií ukazuje na jejich možný význam. U většiny z nich je v současné době potřeba další evaluace velkými randomizovanými studiemi. DFI = disease-free interval; OS = overall survival
Tab. 1. Overview of the most important predictive and prognostic factors in the colorectal carcinoma. The following overview contains, with few exceptions like KRAS mutation in the prediction of the response on the treatment by moAb again EGFR, mainly factors which are not at present use in the clinical practice, but exist several studies which indicates their importance for the prognosis or prediction of the disease outcome. Majority of the factors need further evaluation in randomised studies. DFI = disease-free interval; OS = overall survival

Prediktivní faktory	
Faktor	Vliv na výběr léčby
EGFR pozitivita	Možnost použití moAb proti EGFR
KRAS aktivační mutace	Negativní znak pro použití moAb proti EGFR
BRAF aktivační mutace	Negativní znak pro použití moAb proti EGFR
PI3KCA mutace	Hypotetický negativní znak pro použití moAb proti EGFR
PTEN ztráta exprese	Negativní znak pro použití moAb proti EGFR
Vyšší exprese NF-κB	Snížení účinku moAb proti EGFR, radioterapie i klasické chemoterapie
NRAS aktivační mutace	Negativní znak pro použití moAb proti EGFR
MSI pozitivita	Negativní faktor pro chemoterapeutika na bázi 5-FU
ERCC1 polymorfismus	Prediktivní znak pro použití oxaliplatiny
Nízká exprese TopoI	Negativní prediktivní znak pro použití irinotecanu
Prognostické faktory	
Faktor	Vliv na prognózu
Cirkulující a diseminované nádorové buňky	Horší prognóza, zkrácení DFI i OS
KRAS mutace v kodonu 12	Zkrácení DFI i OS
BRAF aktivační mutace V600E	Zkrácení DFI i OS
PTEN ztráta exprese	Vyšší riziko lokální recidivy
Zvýšený počet genomových kopií EGFR	Delší OS
Vyšší exprese NF-κB	Zkrácení PFS a OS
CIN pozitivita	Negativní prognostický faktor
MSI pozitivita	Pozitivní prognostický faktor
Vysoká exprese TYMS	Zkrácení OS

překládané oblasti. První dvě změny vedou k vyšší expresi TYMS, poslední změna vede k nižší stabilizaci TYMS mRNA a ve výsledku k nižší expresi. Identifikace všech těchto změn může být využita k prognóze zvýšeného nebezpečí recidivy u pacientů s KRCa ve stadiu II a III [18].

TP53 a APC

Gen pro TP53 (tumor protein p53) se nachází na chromozómu 17p a u KRCa dochází velice často ke ztrátě heterozygozity této oblasti. Mutace v TP53 jsou u KRCa rovněž velmi časté, ale výsledky studií hledající význam v prognóze či predikci byly nejednotné, často zřejmě díky odlišným metodickým přístupům.

Mutace jednotlivých genů ve WNT (wingless-type MMTV integration site family) signalizační dráze jsou velice častým jevem u pacientů s KRCa (až v 90 % případů [19]) a nejčastěji mutovaným zástupcem je gen APC (adenomatous polyposis coli). APC je součást komplexu degradující ß-catenin a jeho mutace vede k deregulaci buněčného cyklu. Oba tyto proteiny jsou tak často změněny po mutační či expresní stránce, že je nelze spolehlivě použít pro prognózu či predikci. Určité typy mutací v APC mohou vést k horší prognóze (především ty, které ovlivní vazebnou doménu k ß-cateninu), ale v klinické praxi není jednoduché vzhledem k velkému počtu popsaných APC mutací jednotlivé mutace přesně identifikovat. U ß-cateninu je spíše než vyšší exprese důležitá jeho lokalizace, která nese určitou prognostickou hodnotu.

Ztráta 18q

Na dlouhém raménku 18. chromozomu se nacházejí geny DCC (deleted in colorectal carcinoma), SMAD4 (SMAD family member 4) a SMAD2 (SMAD family member 2), a ztráta heterozygosity v této oblasti je velice častou cytogenetickou abnormalitou u KRCa. Existují údaje, že tato ztráta vede k horší prognóze [5]. Ani jeden z genů nebyl potvrzen jako nezávislý prognostický faktor, protože ztráta 18q je asociována s CIN pozitivitou a vliv těchto genů na prognózu tedy může být jen jedním z důsledků chromozomální nestability.

ZÁVĚR

Molekulární biologie nabízí klinickým oborům podílejícím se na komplexní léčbě KRCa nové možnosti, které mohou ovlivnit diagnostiku a léčbu tohoto onemocnění. V současné době tento obor spíše zkoumá, které z nepřeberných molekulárně biologických metod mohou skutečně a významně přispět svou prognostickou nebo prediktivní hodnotou v klinické praxi. Z uvedeného přehledového článku je zřejmé, že lze očekávat v následujícím období zvýraznění významu molekulární biologie a tím začlenění tohoto oboru mezi obory zabývající se v součinnosti diagnostikou a léčbou tohoto onemocnění.

Seznam zkratek

5-FU	5-fluorouracil
ADCC	antibody dependent cell cytotoxicity
AKT	v-akt murine thymoma viral oncogene homolog 1
APC	adenomatous polyposis coli
ASCO	American Society of Clinical Oncology
BEAMing	beads, emulsions, amplification, magnetics
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BSC	best supportive care
CIMP	metylované CpG ostrůvky
CIN	chromozomální nestabilita
DCC	deleted in colorectal carcinoma
DFI	disease-free interval
DMC	digital melt curve
EGFR	receptor epidermálního růstového faktoru
EpCAM	epiteliální adhezivní povrchový protein
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1
FC	průtoková cytometrie
FcγR	Fc-γ receptory
FISH	fluorescence *in-situ* hybridisation
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
KRCa	kolorektální karcinom
MIN	mikrosatelitní nestabilita
MMR	mis-match repair system
moAb	monoklonální protilátka
mTOR	mechanistic target of rapamycin
NF-κB	nuclear factor-κB
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
OS	overall survival
PCR	polymerase chain reaction
PFS	období bez progrese onemocnění
PI3K	phosphatidyl-inositol-3-kináza
PLCγ	phospholipase C, gamma
PTEN	phosphatase and tensin homolog
SMAD2	SMAD family member 2
SMAD4	SMAD family member 4
STAT	signal transducer and activator of transcription
TP53	tumor protein p53
TYMS	tymidylát syntetáza
VEGF	vaskulární-endoteliální růstový faktor
WNT	wingless-type MMTV integration site family

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Příloha VIII:

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Research Article

Isocitrate Dehydrogenase-1 Mutations as Prognostic Biomarker in Glioblastoma Multiforme Patients in West Bohemia

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Introduction. Glioblastoma multiforme (GBM) is the most malignant primary brain tumor in adults. Recent whole-genome studies revealed novel GBM prognostic biomarkers such as mutations in metabolic enzyme IDH—isocitrate dehydrogenases (IDH1 and IDH2). The distinctive mutation IDH1 R132H was uncovered to be a strong prognostic biomarker for glioma patients. We investigated the prognostic role of IDH1 R132H mutation in GBM patients in West Bohemia. *Methods*. The IDH1 R132H mutation was assessed by the RT-PCR in the tumor samples from 45 GBM patients treated in the Faculty Hospital in Pilsen and was correlated with the progression free and overall survival. *Results*. The IDH1 R132H mutation was identified in 20 from 44 GBM tumor samples (45.4%). The majority of mutated tumors were secondary GBMs (16 in 18, 89.9%). Low frequency of IDH1 mutations was observed in primary GBMs (4 in 26, 15.3%). Patients with IDH R132H mutation had longer PFS, 136 versus 51 days ($P < 0.021$, Wilcoxon), and OS, 270 versus 130 days ($P < 0.024$, Wilcoxon test). *Summary*. The prognostic value of IDH1 R132H mutation in GBM patients was verified. Patients with mutation had significantly longer PFS and OS than patients with wild-type IDH1 and suffered more likely from secondary GBMs.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and most malignant primary brain tumor in adults with an incidence of 3-4/100,000/year. The median survival of patients with GBM is 12.1–14.6 months [1] and only 3–5% of patients survive longer than 3 years [2, 3]. The progress in genomics of GBM has revealed several abnormalities in signaling pathways and a diversity of mutated genes. One of great importance among them is isocitrate dehydrogenase (IDH) [4, 5]. Isocitrate dehydrogenases (three isoforms IDH1, IDH2, and IDH3) catalyze the oxidative carboxylation of isocitrate to alpha-ketoglutarate and reduce nicotinamide adenine dinucleotide phosphate (NADP) to NADPH, which is necessary for the regeneration of reduced glutathione that serves as the main antioxidant [6]. The genes for IDH1 and IDH2 carry specific mutations in 70%–80% of low-grade gliomas, in approximately 50% of anaplastic gliomas, and in more than 5% of glioblastomas [7, 8]. The mutations are involved in 90% single amino acid substitution—R132H in the IDH1 active site that leads to the loss of regular enzyme function—and are predominantly heterozygous. Mutations in IDH2 occurred rarely in brain tumors [7, 9]. The aberrant function of mutated IDH1 is the conversion of alpha-ketoglutarate to the novel oncometabolite 2-hydroxyglutarate (2-HG), which leads to genome-wide epigenetic changes in human gliomas [10]. Tumors with mutated IDH1 and corresponding epigenetic changes demonstrated better prognosis than gliomas with wild-type IDH1. This association was observed also for GBM [4, 6, 7, 11, 12]. The aim of this study was to assess the prognostic role of IDH1 R132H mutation in the relation

Table 1: Glioblastoma patient demographics and clinical characteristics.

Patient characteristics	
Sex	
Male to female	1
Male	22
Female	22
Age, years	
Median	64.3
Range	35–87
KPS	
Median	77.5
Range	30–100
Postoperative treatment	
RT (±CT)	29
CT alone	1
None	15

KPS: Karnofsky performance score; RT: radiotherapy; CT: chemotherapy.

to progression-free survival (PFS) as well as overall survival (OS) of our GBM patients in West Bohemia.

2. Patients and Methods

2.1. Patients. We performed a retrospective study of 44 patients with a diagnosis of WHO grade IV astrocytoma—GBM ($n = 44$; 22 males and 22 females; mean age 64.3 years) who were treated (total or subtotal tumor resection or tumor biopsy, radiotherapy, and chemotherapy with temozolomide) in the Faculty Hospital in Pilsen between the years 2009 and 2011. The study protocol was approved by the ethics committee (Table 1).

2.2. DNA Isolation. DNA was extracted from 10 μm FFPE sections following macrodissection of tumor tissue and normal brain tissue using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany). The 10 μm sections corresponded to HES-representative with tumor tissue verified by pathologist.

2.3. Mutation Detection. For detection of mutant allele IDH1 c.395G>A (p.R132H, COSMIC ID 28746), we use TaqMan Mutation Detection Assays (assay name: IDH1 28746 mu and IDH1 rf) with the TaqMan Mutation Detection IPC Reagent Kit (Life Technologies, Carlsbad, California, USA). Mutant allele detection we performed according to recommended procedure and reaction conditions is found in the manual. For the amplification, we used the Stratagene Mx3000P real-time PCR system instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). Detection of mutant alleles was performed in duplicate in a reaction volume of 20 μL. Detection of reference gene was also performed in duplicate. The analysis of the positive samples was repeated. Before analysis of our collection of tumor samples, we analyzed samples of normal brain tissue for detection of cut-off amplification curve. No amplification of mutant allele was present in normal brain tissue. On the basis of these results and the shape of amplification curve of positive tumor samples, we determined the ΔCt cut-off 25 value.

Table 2: The representation of IDH1 R132H mutation in primary versus secondary glioblastomas.

Glioblastoma type	Primary GBM ($n = 26$)	Secondary GBM ($n = 18$)
Mutation status [n]		
IDH1 R132H	4 (15.3%)	16 (89.9%)
IDH1 wild-type	22 (84.7%)	2 (11.1%)

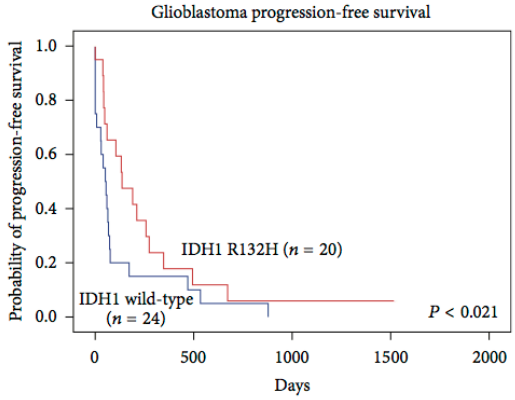

Figure 1: Progression-free survival of patients with glioblastoma with (red line) or without (blue line) IDH1 R132H gene mutation.

2.4. Statistical Analysis. Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Kaplan-Meier survival curves were plotted and the survival distributions were compared with the use of the Wilcoxon test. Reported P values are two-sided. P values of less than 0.05 were considered to indicate statistical significance.

3. Results

The examined mutation IDH1 R132H was observed in 20 of 44 GBM-patient tumor samples. Therefore we identified the IDH1 mutation in more than 45.4% of glioblastomas. The separation of primary and secondary glioblastomas (GBM that progressed from the low-grade glioma) was done on the basis of clinically relevant information, where possible. The IDH1 R132H mutation occurred in 4 of 26 primary GBMs (15.3%), whereas the majority, 16 of 18 (89.9%) were of secondary glioblastomas mutated (Table 2). The significant relation between IDH1 mutation status and clinical parameters such as PFS and OS was also observed (Table 3). Patients with IDH1 R132H mutation had longer PFS than patients with wild-type IDH1-136 versus 51 days ($P < 0.021$, Wilcoxon test) (Figure 1). Significantly longer OS was observed as well for

Table 3: Results for progression-free survival and overall survival differences in patients with GBM in relation to IDH1 mutation status.

Glioblastoma results	*N*	Median [days] (95% Cl)	*P* (Wilcoxon)
Overall survival (OS)			
IDH1 R132H	20	270 (139–400)	0.024
IDH1 wild-type	24	130 (87–172)	
Progression-free survival (PFS)			
IDH1 R132H	20	136 (22–249)	0.021
IDH1 wild-type	24	51 (19–82)	

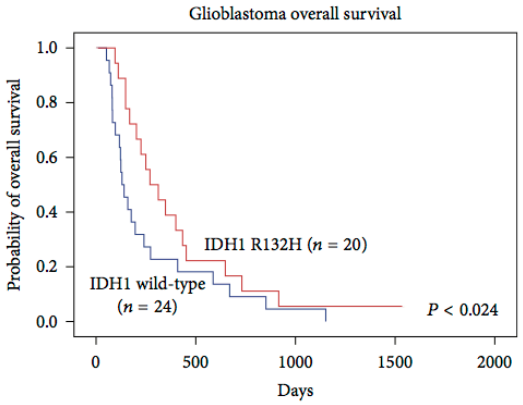

Figure 2: Overall survival of patients with glioblastoma with (red line) or without (blue line) IDH1 R132H gene mutation.

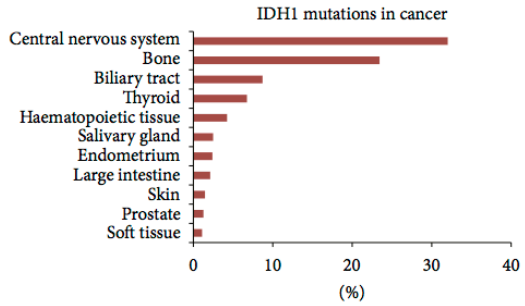

Figure 3: The representation of IDH1 mutations in various cancers [14].

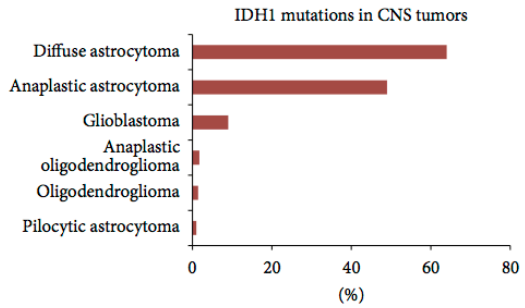

Figure 4: The representation of IDH1 mutations in various types of central nervous system tumors [14].

patients with IDH1 R132H mutation than for patients without the mutation-270 versus 130 days ($P < 0.024$, Wilcoxon test) (Figure 2).

4. Discussion

Recurrent IDH mutations and their role in oncogenesis and tumor progression were described for the first time in GBM [4]. This observation has led to new insights into GBM and cancer biology. Alterations in cancer cell metabolism are now well accepted as one of the principal hallmarks of the cancerogenesis and tumor progression [13]. Mutations in IDH1 were also identified in substantial portion of other tumor types. The data from the Sanger Institute Cancer Genome Project-Catalogue of Somatic Mutations in Cancer revealed the presence of IDH1 mutations in more than 32% of central nervous system tumors, 23% of bone tumors, 8% of biliary tract tumors, 6% of thyroid cancer, and many other tumor types [14] (Figure 3). In the primary brain tumors group, IDH1 mutations are presented mostly in diffuse astrocytomas (64%), anaplastic astrocytomas (49%), glioblastomas (9%), or oligodendrogliomas (2%) [14] (Figure 4). The R132H amino acid substitution is the most common form of IDH1 mutations with the prevalence of 90% among IDH1-mutant tumors. Less common mutants such as R132C, R132G, R132S, and R132L are also known [7, 9].

The fundamental shift in the understanding of mutated IDH and its role in cancer progression came with the observation of the neomorphic function of the mutated enzyme. Instead of the production of alpha-ketoglutarate, mutated IDH1 produced novel oncometabolite 2-hydroxyglutarate (2-HG) that was highly accumulated in the cancer cells [15]. It was subsequently discovered that 2-HG inhibits the functions of the alpha-ketoglutarate dependent superfamily of dioxygenases. These enzymes have diverse cellular functions including, but not limited to, histone demethylation and demethylation of hypermethylated DNA [16, 17]. Moreover, IDH1 mutations and 2-HG production were identified to be sufficient steps in the process leading to glioma hypermethylator phenotype. That observation was important for understanding of glioma oncogenesis and highlighted the interplay between genomic and epigenomic changes in human cancers [10, 18].

Mutations in IDH1 are important also for their clinical consequences. Recent studies revealed the important role of mutated IDH1 in the assessment of astrocytoma patient prognosis. Therefore IDH1 mutations could serve in the near future as the standard prognostic biomarkers for patients with grade II, III, and IV astrocytomas. The differences in OS between IDH1-mutant and IDH1 wildtype GBM were 3.8 versus 1.1 years [4], 2.6 versus 1.3 years [7], 2.3 versus 1.2 years [6], and 3 years versus 1 year in several studies [11]. Similar OS differences in IDH1-mutant versus IDH1-WT tumors were observed for anaplastic astrocytomas, such as 5.4 versus 1.7 years [7], 6.8 versus 1.6 years [6], and 7 versus 2 years [11] as well as for low-grade gliomas [19]. Recent meta-analysis also confirmed the prognostic role of IDH1/2 mutations in gliomas [20]. These data highlight the major impact of IDH1 mutation status on glioma patient survival and support the incorporation of this biomarker into the clinical assessments. Mutations in IDH1/IDH2 and production of oncometabolite 2-HG could be used as well for therapeutic intervention in the near future [21].

The results from our study also support the IDH1 mutation R132H to be the strong prognostic factor for patients with GBM. Although the differences in median PFS and OS between patients with IDH1 mutated and IDH1 wild-type tumors are not as big as in other studies, they are statistically significant. One reason for the relatively small differences in median PFS and OS between both groups could be the heterogeneity of the treatment protocols. The standard treatment with neurosurgery and concomitant chemo-radiotherapy with temozolomide was implemented in 29 patients and 1 patient had only radiotherapy and 15 patients were treated neither with radiotherapy nor with chemotherapy (Table 1). The proportion of IDH1 mutated tumors is also higher in our study than in other similar studies. The IDH1 mutations in glioblastomas were formerly identified predominantly in secondary GBM that progressed from the low grade tumors [22]. In our study, we tried to distinguish between the primary and secondary glioblastomas on the basis of clinically relevant information from the patient history. However, the distinction between primary and secondary GBM was not possible exactly. Only 5 patients had previously assessed low grade glioma (surgery in 2 cases, tumor biopsy in 3 cases). Patients with tumor corresponding neurological symptomatology (epileptic seizures, focal neurological deficit) present at least 6 months before the tumor diagnosis was considered as likely secondary GBM. Moreover the primary-like glioblastomas could be in fact secondary without the symptoms of low grade tumors.

The recent study of mutations in telomerase reverse transcriptase (TERT) gene promoter has revealed the high incidence of these aberrations in a large portion of primary GBMs (about 80%) [23]. In the perspectives of our further research, we will use TERT promoter mutations in addition to clinically relevant information for the separation of primary and secondary glioblastomas. The assessment of other IDH1 mutations than R132H as well as the analysis of mutations in IDH2 is also planned.

Despite the drawbacks of our study mentioned above, IDH1 R132H mutation still serves as a strong prognostic biomarker for our patients with GBM.

5. Summary

The IDH1 R132H mutation was observed in the interestingly higher number of patients with GBM that was previously published by other groups. On the other hand, the majority of mutated GBMs in our cohort are probably secondary glioblastomas. The prognostic value of the IDH1 R132H mutation was also observed. Patients with this mutation had significantly longer PFS as well as OS than patients with wild-type IDH1. The IDH1 mutation status could be used as a strong prognostic factor for patients with GBM, but further validation of this biomarker in large prospective clinical trials is urgently needed.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgments

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Příloha IX:

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in vivo 26: 683-692 (2012)

Immediately Preoperative use of Biological Therapy Does not Influence Liver Regeneration after Large Resection - Porcine Experimental Model with Monoclonal Antibody against Epidermal Growth Factor

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Abstract. *Background: The aim of this work was to study the influence of isolated biological therapy administered immediately before extended liver resection on liver function and regenerative capacity of future liver remnant (FLR) in a large-animal experiment. Materials and Methods: Nineteen piglets were included in this study (10 in the control group and 9 in the experimental group). A port-a-cath was introduced into the superior caval vein. On days 11 and 4 before liver resection, cetuximab was administered via this port at 400 mg/m2 of piglet body surface. Physiological solution was applied to the control group. Resection of the left lateral, left medial and right medial hepatic lobes was followingly performed (reduction of 50-60% of liver parenchyma). Blood samples were collected at different times before the operation and after liver resection. Serum levels of bilirubin, urea, creatinine, alkaline phosphatase, gamma glutamyltransferase, cholinesterase, aspartate aminotransferase, alanine aminotransferase, albumin, C-reactive protein and transforming growth factor-β1 were assessed. The ultrasonographic examinations at different time points were performed pre-operatively and after liver resection in order to assess the liver volume. The biopsies from the liver parenchyma were examined for proliferative activity, binocluated hepatocytes, size of hepatocytes, and the length of the lobuli. The comparison of distribution of the studied parameters between the groups was carried out using the Wilcoxon test. The Spearman rank correlation co-efficient was used because of the non-Gaussian distribution of the parameter values. The whole development of the studied parameters over time was compared between the groups using ANOVA. Results: There were no important complications of administration of biologic therapy during the operation or throughout the peri-operative period. There was no statistically significant difference in the regeneration of FLR nor were any differences in biochemical, immunoanalytical and histological parameters detected. Conclusion: The achieved results of comparable liver regeneration in both the experimental and control groups confirms the use of biological treatment with cetuximab in the pre-operative period for minimizing the recovery period.*

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Key Words: Epidermal growth factor, epidermal growth factor receptor, liver surgery, biological therapy, liver regeneration, porcine model, cetuximab.

Possibilities for liver surgery have been extended in recent years by new surgical techniques, and more highly developed procedures and skills. At present, surgery is the only curative option for the treatment of liver metastases from primary colorectal carcinoma. Today we are able to perform large radical resections of malignant liver lesions. Nevertheless, many patients with primary or secondary liver malignancies that undergo oncologic treatment before liver surgery are not directed to the radical surgical therapy that could extend their chance of complete remission for the malignancy. We fear the increased risk of acute liver failure after extended liver resection, where the liver parenchyma could be affected by previous chemotherapy and the liver functions could be reduced (1-3). Many patients are indicated for neoadjuvant oncologic treatment with the aim of down-staging and down-sizing the liver malignancy (4-7).

The use of standard combination of chemotherapy [oxaliplatin/5-fluorouracil (5-FU) or irinotecan/5-FU) has increased the resection rate up to 20-40% in selected patients (8). The addition of anti-EGFR monoclonal antibody, and

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anti VEGF mononclonal antibody therapy to doublet chemotherapy has demonstrated consistent improvements in the response rate in a number of randomized trials. However the intensification of treatment can lead to an increased rate of severe toxicity. Therefore careful selection of the patients that will undergo intensive chemotherapy and surgical intervention is very important (9, 10).

Oxaliplatin, irinotecan, cetuximab, and bevacuzimab are valuable drugs. The usage of these drugs can lead to a maximal response rate before the liver surgery; on the other hand, the prolonged exposure to these drugs may results in hepatotoxicity and the potential for further complications (11). The protracted use of cytotoxic drugs can therefore lead to progressive liver damage and surgical intervention is often not possible afterwards (12). The usage of oxaliplatin in neoadjuvant regimens of chemotherapy can lead to morphological lesions involving hepatic microvasculature. Sinusoidal obstruction, complicated by perisinusoidal fibrosis and veno-occlusive lesion of the non-tumoral liver are the most often adverse side-effect of to the use of oxaliplatin (13). The hepatotoxicity of a combination of 5-FU and levamisole in an adjuvant or palliative setting is mild and only rarely symptomatic (14). Irinotecan regimens are associated with a higher frequency of steatosis and steatohepatitis. Such damage can affect post-operative mortality of patients (15).

The impact of anti-angiogenesis agents on liver regeneration and wound healing is not yet fully understood. The potential side-effect of bevacizumab is delayed wound healing and possible delayed hepatic regeneration. But no specific data are available to guide the optimal timing before the elective surgery. The optimal time for termination of the treatment before elective surgery is at least 4-6 weeks. A similar delay is also seen in the timing of the continuation of bevacizumab treatment after liver surgery (16).

A crucial end point of neoadjuvant treatment is the achievment of a high R0 resection rate (17). The efficacy of cetuximab in patients with unresectable hepatic metastases and wild-type *KRAS* was confirmed in the multicentric randomised CELIM study.

The study enrolled a total of 111 patients with unresectable hepatic metastases. Unresectability criteria in this study were as follows: five and more metastatic lesions; technical unresectability; infiltration of the hepatic vessels; infiltration of both hepatic arteries or infiltration of both branches of the portal vein. In these patients, a combination therapy with (FOLFIRI) or (FOLFOX 6) was evaluated. A significant reduction of 79% in the size and spread of metastases, observed in patients with wild-type *KRAS* gene, enabled resection of metastases in 43% of patients (radical R0 resection was achievable in 34% of patients) (18, 21). Chemotherapy-associated hepatotoxicity has often been described using many classical oncologic regimens (19, 20). The question remains whether the chemotherapy can be administered immediately before liver surgery, or if a recovery period is required between the end of the neoadjuvant chemotherapy and proper surgical procedure. A particular question is also the use of biological treatment that interferes with the key growth factors that are involved in liver parenchyma regeneration and so play key roles in the restoration of future liver remnant volume and also liver function after extended liver resection. The most frequently used type of biological treatment in relation to neoadjuvant chemotherapy of secondary liver malignancies is cetuximab (2, 22-23). Cetuximab inhibits the effects of EGF *via* EGFR. The role of EGF in the promotion of hepatocyte proliferation has also been demonstrated (24-26).

The aim of the present animal experiment was to study the influence of cetuximab administered immediately before extended liver resection. The results could be used in human liver surgery to increase the number of patients who can undergo radical extended liver resection for malignancy because the recovery period between the chemotherapy and liver resection could be shortened.

Materials and Methods

The performed experimental surgical and anaesthesiological procedures and the use of piglets were certified by the Commission for Work with Experimental Animals at the Pilsen Medical Faculty of the Charles University, Prague, and were under the control of the Ministry of Education of the Czech Republic. All the performed procedures were prepared and performed under the law of the Czech Republic, which is compatible with legislation of the European Union.

Choise of biological therapy. The most frequently used type of biological treatment in neoadjuvant chemotherapy for secondary liver malignancies is cetuximab (Erbitux; Merck KGaA, Germany) that inhibits the effect of EGF *via* EGFR (2, 22, 23). Cetuximab recognizes the structural epitope in the extracellular region of EGFR. Based on the known cetuximab binding site in human EGFR, we have compared sequences of human and porcine EGFR using BLAST software. From 14 amino acids important for proper binding of cetuximab to human EGFR, 10 are identical, 2 similar and 2 different, which can reduce the affinity of antibody to porcine EGFR, but the binding is still possible (37, 38). We have concluded that the correspondency of domains is sufficient for inactivation of this receptor and the use of cetuximab for this porcine experimental model is efficient.

Surgical procedure. Nineteen piglets were included in this study (10 in the control group and 9 in the experimental group). No piglet was excluded because of untimely death or any type of surgical complication. The piglets were premedicated intramuscularly with 1.0 mg atropine, 200 mg ketamine (approximately 5-10 mg/kg) and 160 mg azaperon (2-8 mg/kg). Anesthesia was administered continually through a peripheral or central venous catheter in the following total average doses: propofol (1% mixture, 5-10 mg/kg/h) and fentanyl (1-2 μg/kg/h). Muscle relaxation was ensured by bolus administration of 0.1-0.2 mg/kg pancuronium at the beginning of surgery. The piglets were intubated and mechanically ventilated during the surgical procedure and received infusion and volume substitution when needed

[Plasmalyte (Baxter Healthcare Ltd., UK) and Gelofusine (B.Braun Melsungen AG, Germany) respectively]. Aminopenicilline and clavulic acid (1.2 g) was used as antibiotic prophylaxis throughout the procedure. Monitoring of electrocardiogram, oxygen saturation and central venous pressure was implemented. The surgical procedure was performed under aseptic and antiseptic conditions.

Firstly a port-a-cath was introduced into the superior caval vein. On days 11 and 4 before liver resection, cetuximab at 400 mg/m^2 of piglet body surface was administered (23). Physiological solution was applied to the control group.

Liver resection followed. Firstly, a central laparotomy was performed. The resection of the left lateral, left medial and right medial hepatic lobes was performed (reduction of 50-60% of liver parenchyma). The laparotomy was closed in anatomical layers.

The animals were extubated and monitored each day for the next 14 days, with particular emphasis on the clinical examination (attention to wound healing, infection of the port-a-cath and function of the gastrointestinal system) to diagnose possible surgical complications. Postoperative analgesia was provided by intramuscular application of small dosis of Azaperon (10 mg).

Biochemistry. Blood samples were collected from the central vein catheter: on the 14th, 11th and 4th preoperative days; immediately before the operation, immediately after liver resection; 2 hours after liver resection; and on the 1st, 3rd, 7th, 10th and 14th post-operative days. Biochemical serum parameters were assessed with focus on liver functions to detect the influence of the applied monoclonal antibody on the animal and to recognise possible differences between experimental and the control groups. Serum levels of bilirubin, urea, creatinine, alkaline phosphatase (ALP), gammaglutamyltransferase (GGT), cholinesterase (CHE), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin were assessed by an Olympus 2700 biochemical analyzer. Serum levels of C-reactive protein (CRP) and transforming growth factor- β1(TGF-β1) were also assessed to provide information about the termination of liver proliferation.

Ultrasonography. Ultrasonographic examinations were performed on the 14th and 4th preoperative days; immediately before the operation; immediately after liver resection; on the 3rd, 7th, 10th and 14th postoperative days (ultrasound apparatus, Medison Sonoace 9900; convex probe with a frequency of 3.5 MHz). The diameters of the hypertrophic lobes were measured in B-mode in all three basic planes (axial, sagittal and coronal). The volume of the lobes was assessed by using the standard ultrasonographic formula that is also used in human medicine: axial × sagittal × coronal 2. The volumes are presented as a percentage of baseline values (immediately postoperative liver lobe volumes) to provide better information about the changes in volumes that were achieved.

Termination of experiment. The piglets were sacrificed on the 14th post-operative day under deep general anesthesia with a concentrated solution of potassium chloride administered into the central venous catheter. The piglets were dissected and measurement of the hypertrophic liver lobes was performed. These data were compared with the proportions estimated by ultrasonography. Bioptic samples from the atrophic and hypertrophic liver lobes were collected and stored in 10% formaldehyde (methanal) and also frozen below –70°C.

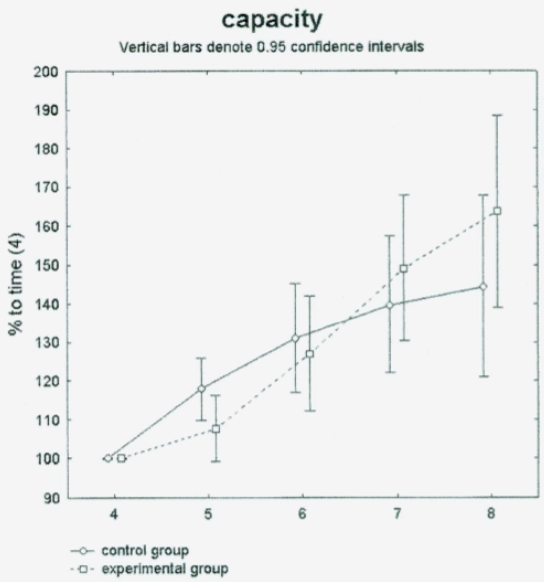

Figure 1. *Comparison of hypertrophic liver lobe growth relative to these point 4 between experimental and control groups. Time points: 4, after liver resection; 5-8, 3rd, 7th, 10th and 14th postoperative days, respectively.*

Histology. Biopsies from the liver parenchyma were examined after staining with hematoxylin-eosine and periodic acid-Schiff (PAS) staining after digestion of preparations with diastase. The proliferative activity was examined using the Ki 67 antibody (MIB1, 1:1000; DakoCytomation, Denmark). The primary antibodies were visualised using a streptavidin- biotin-peroxidase complex (DakoCytomation). The number of binucleated hepatocytes was measured in 20 microscopic fields with the aid of an eyepiece micrometer (Olympus). The size of the hepatocytes and the length of the lobuli were examined twice using the eyepiece micrometer.

Statistical analysis. Statistical analysis was performed with the CRAN 2.4.0 and the STATISTICA (98 Edition) software. The assessed parameters (biochemistry, ultrasonography, histology) were analyzed by the following statistical tests: the comparison of distribution of the studied parameters over the groups was counted using a Wilcoxon distribution-free test. The Spearman rank correlation co-efficient was used because of the non-Gaussian distribution of parameter values. The development of the studied parameters over time was compared between the groups using ANOVA.

Results

The volumes of hypertrophic liver lobes measured by ultrasonography and physical examination were comparable on the 14th postoperative day. The differences in absolute volume of remnant hypertrophic lobes (right lateral and caudate lobes) between experimental and control groups were not statistically significant (Figure 1).

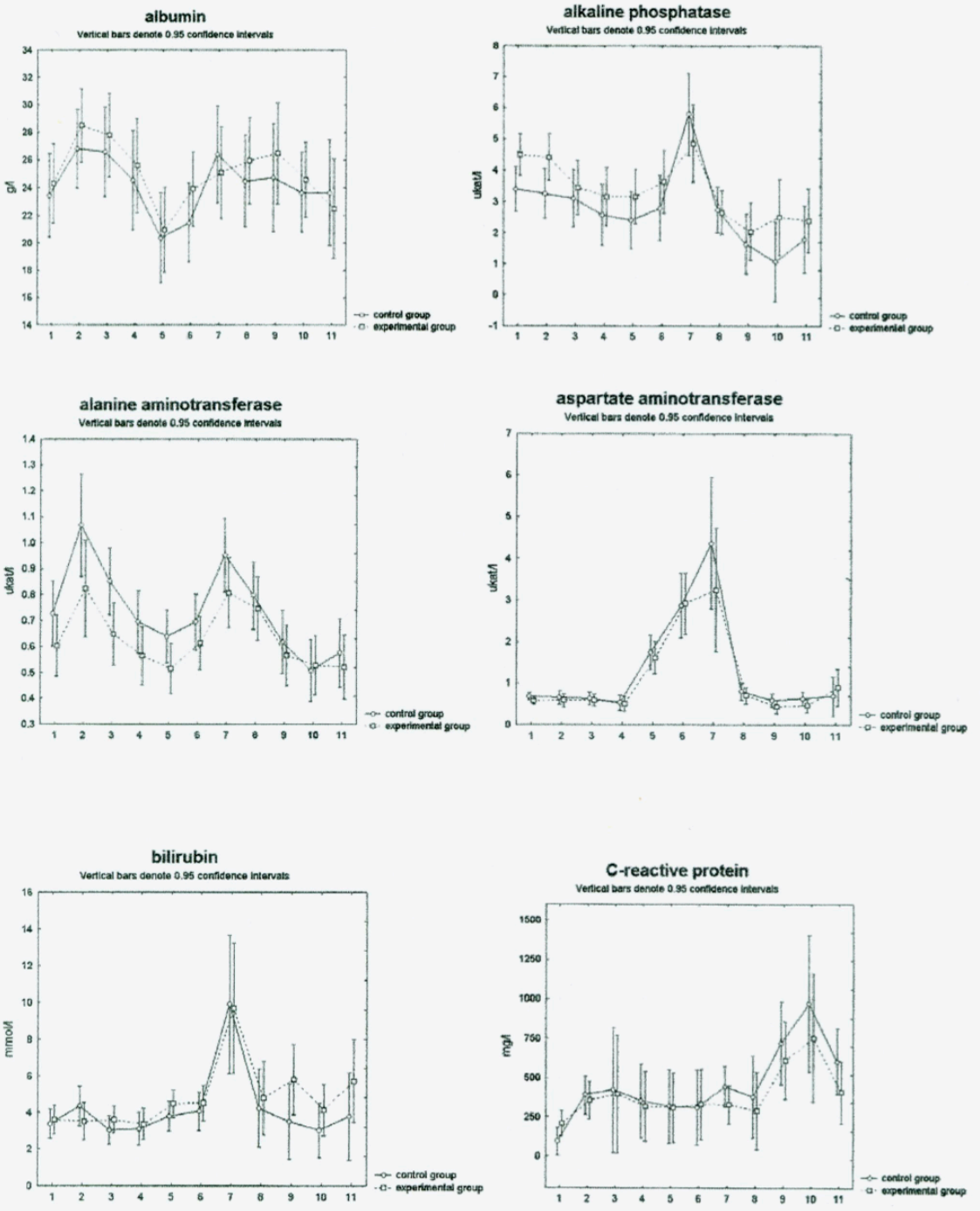

Figure 2. *Continued*

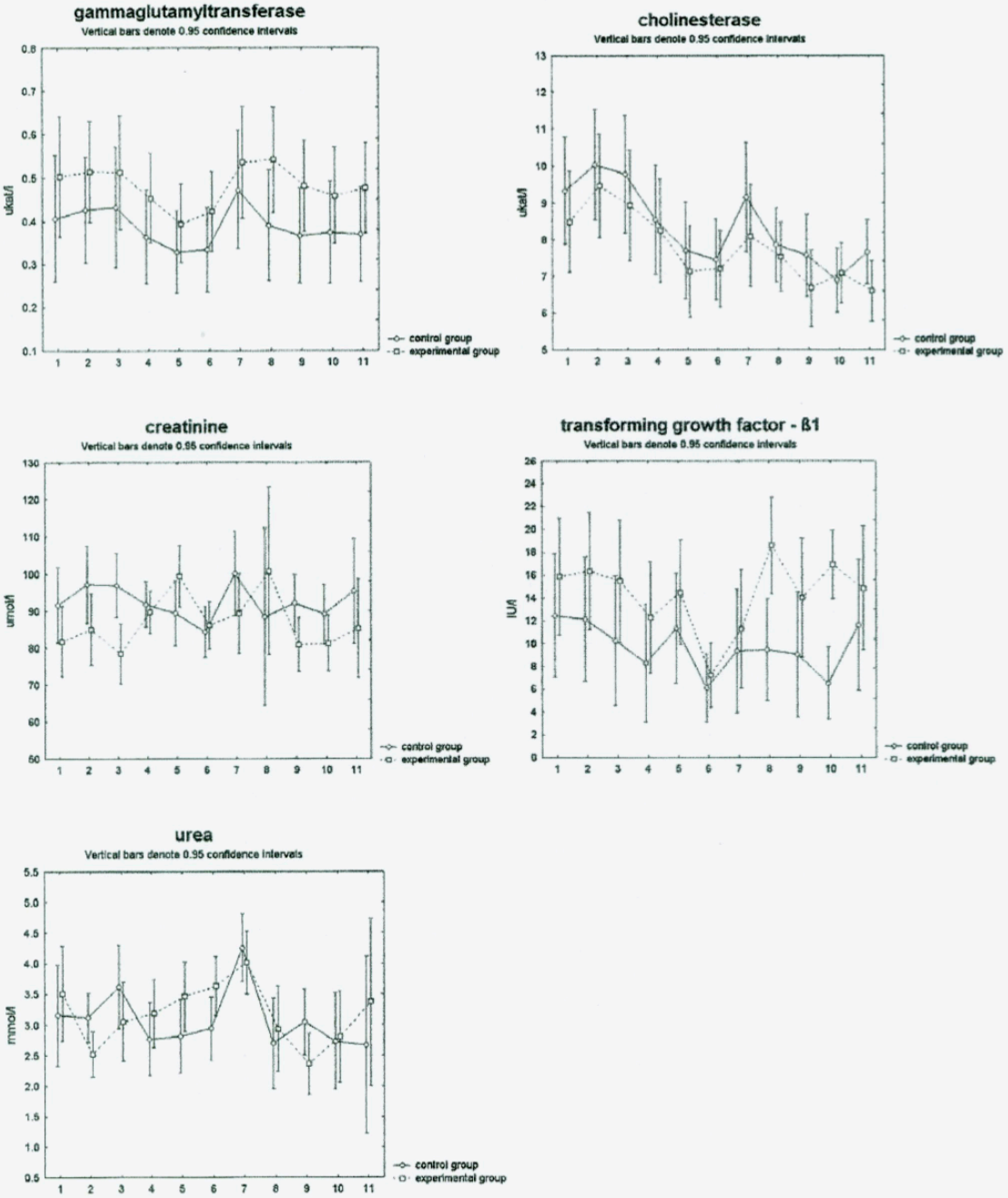

Figure 2. *Comparison of serum levels of studied biochemical parameters between experimental and control groups. Time points: 1-3, 14th, 11th and 4th days before surgery; 4, immediately before the operation; 5,immediately after liver resection; 6, 2 hours after liver resection; 7-11, 1st, 3rd, 7th, 10th, 14th postoperative days, respectively.*

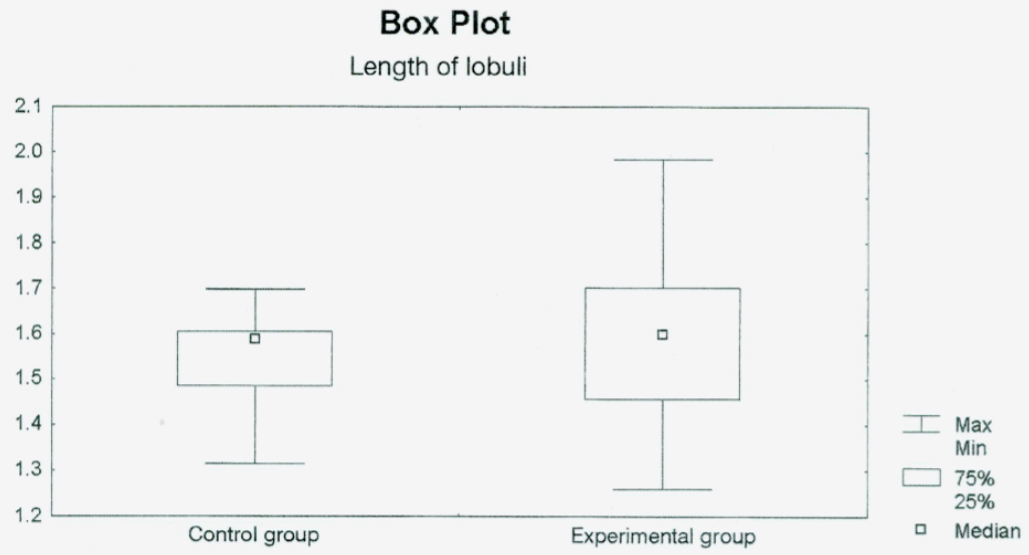

Figure 3. *Comparison of the length of lobuli (cm) in hypertrophic lobes between experimental and control groups.*

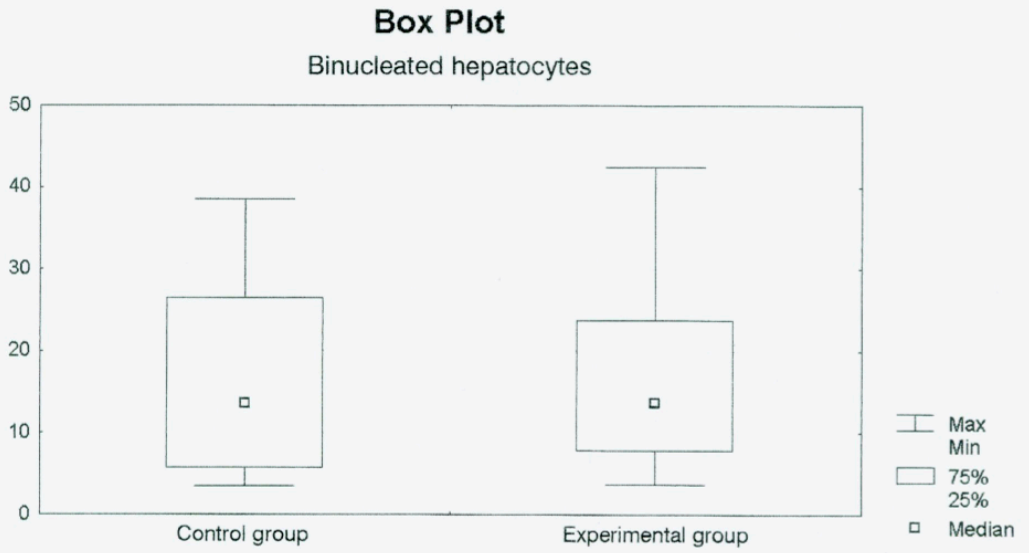

Figure 4. *Comparison of the frequency of binucleated hepatocytes in hypertrophic lobes between experimental and control groups. The number of binucleated hepatocytes were counted in 20 microscopic fields.*

The serum levels of all the studied biochemical parameters at the different time points are presented in Figure 2. All the studied serum biochemical parameters were comparable in both experimental groups and the differences were not statistically significant between the experimental and the control group at each time point (Figure 2). No adverse reaction to cetuximab application was observed.

The histological examination was performed on biopsies that were conducted at the end of the experiment after the animals had been sacrificed, when the proliferative phase of

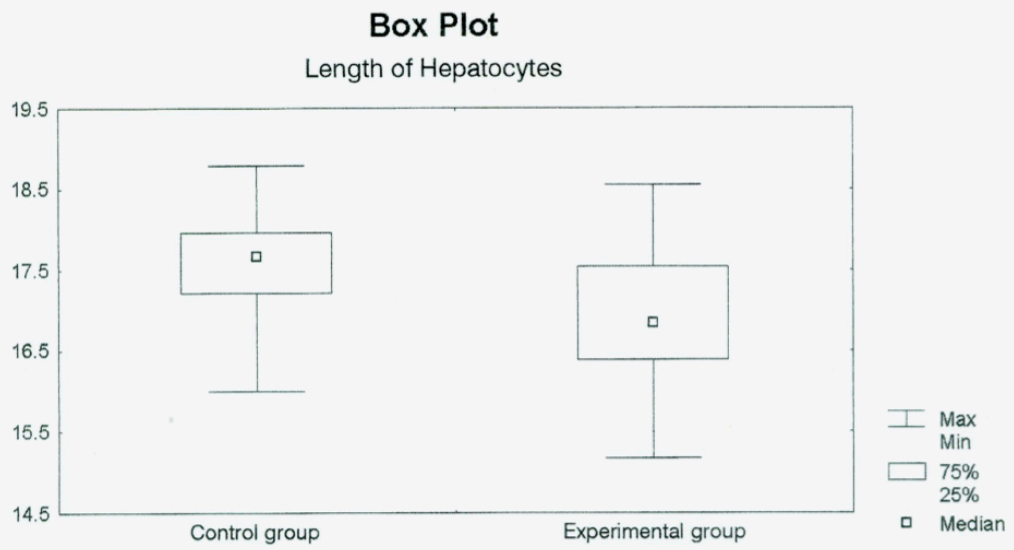

Figure 5. *Comparison of the size of hepatocytes (cm) in hypertrophic lobes between experimental and control groups.*

liver regeneration had in fact finished. The differences in the length of lobuli between studied groups were not statistically significant (Figure 3). Statistical analysis also showed no significant increase in the number of binucleated hepatocytes in the hypertrophic liver lobes in the experimental group (Figure 4). The size of hepatocytes was also not a statistically significant parameter for differences between the experimental group and the control group (Figure 5). Proliferative activity in both experimental and control groups was greatly reduced.

Discussion

This experimental study presents the possibility of the application of extrinsic monoclonal antibody against EGF immediately before surgery. The regenerative capacity of liver parenchyma was not reduced by cetuximab administration. We demonstrated that liver functions were also not affected. The use of large animals, especially piglets, in this experimental surgical study is appropriate in relation to human medicine and clinical surgery because of the similar physiology of piglets and humans. The results of the present study show that the recovery period could be shortened for patients indicated for liver resection who undergo oncologic treatment. The effect of a monoclonal antibody against EGFR is lost rather quickly and the recovery period differs between patients that undergo classical chemotherapy and those patients that were treated by biological therapy (39). The concentration (recommended dose) and timing of the application of the monoclonal antibody selected for our study did not reduce liver parenchyma regeneration in comparison to the one in the control group. The secondary effects that could be hypothesized after application of the monoclonal antibody against the key pleiotropic growth factor (changes in immune reactions and homeostasis) were also not observed, neither during application nor throughout the postoperative period. In previous experiments, we have demonstrated that administration of cytokines of the first phase of liver regeneration, interleukin-6 and tumor necrosis factor–α, increase regeneration of future liver remnant (27, 39). These cytokines have pleiotropic functions, which could be altered or changed by application of these cytokines from an extrinsic source (28). It was our aim in this experiment to select a monoclonal antibody against the growth factor that plays a role during the first phase of liver regeneration. By blocking of its receptor, we wanted to inhibit the proliferation of hepatocytes. Nevertheless, the regenerative capacity of the liver parenchyma was not negatively influenced by cetuximab administrations. No statistically significant differences were observed between serum levels of the studied biochemical parameters at particular points in time. This also demonstrates that there was no unsuitable effect of the applied monoclonal antibody on liver function. These results support our choise of a monoclonal antibody against EGFR for future clinical studies in human liver surgery.

The broad distribution of the of binucleated hepatocytes in the hypertrophic parenchyma of individual animals from

both groups could be explained by incomplete liver regeneration at the end of experiment. Because there were practically no mitotic figures, or the number was the same as in the normal liver parenchyma without any surgical procedures or toxic insult, it was possible to hypothesize that the first phase of liver regeneration was finished and the next phase of regeneration was proceeding, namely the remodelling phase and the phase when the liver microstructure is restored (29).

The next objective for future studies would be the detection of changes on the extracellular matrix during the process of liver regeneration. The size of hepatocytes and the length of lobuli were not statistically different between study and control groups. The same size of hepatocytes and length of lobuli in the bioptical samples from the hypertrophic parenchyma could be also explained in the same way as for the binucleated hepatocytes. This hypothesis is supported by the restitution of all liver functions monitored by biochemical parameters and completion of the proliferative phase of liver regeneration at the moment of sacrificing of the experimental animals.

The most important point for discussion among all clinicians is the problematic recovery period which is necessary for restoration of liver functions after administration of classical chemotherapeutics (30). On the other hand, we have observed that a proportion of our patients experience progression of malignancy during this period. This could also bring more experimental oriented experience into the discussion of whether the administration of biological therapy, for example bevacizumab, is associated with increased risk of postoperative complications (31-36). We conclude that immediate pre-operative administration of cetuximab in the experimental model used here did not increase this risk. The results of this experiment could be useful to support the administration of biological therapy until surgery in order to reduce the risk of the preoperative progression of malignancy.

The present study describes a new usage of a monoclonal antibody against EGFR in large-animal experimental model of extended liver resection, which simulates the situation in human medicine. The achieved results of comparable liver regeneration in both experimental and control groups confirms the use of biological treatment with cetuximab in the preoperative period with minimal recovery period. The experimental results could lead to a clinical study in patients with neoadjuvant treatment with biological therapy.

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Original Paper

Inhibition of Transforming Growth Factor Beta-1 Augments Liver Regeneration after Partial Portal Vein Ligation in a Porcine Experimental Model

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ABSTRACT

Background/Aims: TGF-β1 is a pleiotropic cytokine that is over expressed in terminal phase of liver regeneration. **Methodology:** Twenty-four hours after partial portal vein ligation monoclonal antibody against TGF-β1 (TGF-β1 group, 7 piglets) or physiological solution (control group, 9 piglets) were applied into the central venous catheter. The biochemical parameters (bilirubin, urea, creatinine, alkaline phosphatase, gamma-glutamyl transferase, cholinesterase, aspartate aminotransferase, alanine aminotransferase and albumin) were assessed. The compensatory hypertrophy of the non-occluded liver lobes was evaluated by periodic ultrasonography during the next fourteen days and by histological examination. **Results:** The acceleration of growth of the hypertrophic liver lobes was maximal between 3rd and 7th postoperative days in comparison with the control group ($p<0.05$). No important differences in the biochemical or studied histological parameters were proved. **Conclusions:** The present study describes a new usage of monoclonal antibody against TGF-β1 in large animal experimental model of partial portal vein ligation.

Key Words: Transforming growth factor beta-1; Monoclonal antibody; Liver surgery; Portal vein embolization; Portal vein ligation; Liver regeneration; Experimental study; Porcine model.

Abbreviations: Alkaline Phosphatase (ALP); Alanine Aminotransferase (ALT); Aspartate Aminotransferase (AST); Cholinesterase (CHE); Future Liver Remnant Volume (FLRV); Gamma-Glutamyl Transferase (GGT); Monoclonal Antibody (MAB); Postoperative Day (POD); Portal Vein Embolization (PVE); Portal Vein Ligation (PVL); Transforming Growth Factor-Beta1 (TGF-β1).

INTRODUCTION

New surgical techniques and more highly developed procedures and skills have extended the possibilities of liver surgery and have made it possible to perform large radical resections of malignant liver lesions. Nevertheless, many patients with primary or secondary liver malignancies are not directed to the radical surgical therapy that could extend their chance of complete remission of the malignancy because of the increased risk of acute liver failure after extended liver resection, where the liver remnant volume is too small to sustain the liver functions (1). Portal vein embolization could increase the future liver remnant volume (FLRV) before the scheduled extended liver resection (2). The essential condition for indication of this procedure is that only one of the liver lobes is affected by malignant diseases or that the lesions in the remnant lobe have to be resected or destructed before the performance of PVE (3-5). Compensatory hyperplasia of the contralateral liver lobe is initiated by PVE of the portal branch of the liver lobe afflicted by the malignancy that underlies atrophy (6). Liver surgery is performed in only 63-96% of patients after successfully performed PVE (7,8). The main reason for resorting to liver resection after PVE is unsuccessful hypertrophy of the FLRV or progression of the malignancy.

In practice, portal vein ligation (PVL) could be used instead of PVE. The differences between PVE and PVL were not shown to be statistically significant for the achieved FLRV (9). However, contemporary literature has considered portal vein ligation only on a murine experimental model (10). The key roles of interleukins (tumor necrosis factor-α, Interleukin-6) during first phase (priming of hepatocytes) of liver regeneration after surgical or toxic insult are known (11,12). The meaning of growth factors (hepatocyte growth factor, transforming growth factor-α, epidermal growth factor) for progression of hepatocyte proliferation has also been shown (13). The proliferative phase of liver regeneration is terminated by an increased serum level of the transforming growth factor-beta1 (TGF-β1) (14).

TGF-β1 plays the most important role in starting the remodelling of the extracellular matrix and restoration of the original structure of the liver parenchyma. TGF-β1 inhibits DNA synthesis and plays a pivotal role in the down-regulation of liver regeneration as has been demonstrated in toxic models of liver regeneration (15,16). TGF-β1 also down-regulates the production of the hepatocyte growth factor that sustains hepatocyte proliferation (17).

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Increased expression of TGF-β1 prevents uncontrolled growth during liver regeneration by the regulation of hepatocyte transition from the G1 to the S phase of the cell cycle (18,19). TGF-β1 helps maintain the differentiation of hepatocytes and non-parenchymal liver cells. The proposed HGF/TGF-β1 ratio could reflect the proliferation/differentiation status of hepatocytes (20). Increased expression of TGF-β1 was also shown to be a crucial factor for the progression of hepatic fibrosis (21). This could be explained by increased or prolonged production of the liver extracellular matrix (22).

The aim of the present study was to accelerate and amplify the regeneration of liver parenchyma after portal vein ligation (PVL) by application of monoclonal antibody against TGF-β1 and so extend first phase of liver regeneration in a large animal experimental model that could simulate the situation in human body. These results could be used in human liver surgery to increase the number of patients undergoing radical extended liver resection for malignancy.

METHODOLOGY

The performed experimental surgical and anaesthesiological procedures and the use of piglets were certified by the Commission for Work with Experimental Animals at the Pilsen Medical Faculty of the Charles University, Prague and were under the control of the Ministry of Education of the Czech Republic. All the performed procedures were prepared and performed under the law of the Czech Republic, which is compatible with legislation of the European Union.

Surgical procedure

Fifteen piglets were included in this study (9 in the control group and 7 in the TGF-β1 group). No piglet was excluded because of untimely death or any type of surgical complication. The piglets were pre-medicated intramuscularly with atropine 1.5mg and azaperon 1.0mg/kg. Anaesthesia was administered continually through a central venous catheter in the following total average doses: azaperon 1.0mg/kg/hour, thiopental 10mg/kg/hour, ketamin 5-10mg/kg/hour and fentanyl 1-2μg/kg/hour. Muscle relaxation was ensured by bolus administration of pancuronium 0.1-0.2mg/kg at the beginning of surgery. The piglets were intubated and mechanically ventilated during the surgical procedure. Monitoring of electrocardiogram, oxygen saturation and central venous pressure was implemented. The surgical procedure was performed under aseptic and antiseptic conditions. An antibiotic prophylaxis was administered in a total dose of 1.2g amoxicillin with clavulanic acid divided into two doses (before surgery and two hours later).

The surgical procedure was performed as described in detail by Liska, as a new established model of partial portal vein ligation (23-25). The following description will summarise the key details of the surgical procedure. First, a central laparotomy was performed. The portal vein branches for the caudate, right lateral and right medial lobes (50-60 per cent of the supposed liver parenchyma) were prepared and ligated without injury or ligation of the hepatic artery branches. The blood flow in the hepatic artery branches and occlusion of the portal vein branches were controlled by Doppler ultrasonography (Medison Sonoace 9900, linear probe with frequency 7.5MHz). The borders between atrophic and hypertrophic liver lobes were marked by titanium staples to simplify the postoperative ultrasonography. The laparotomy was closed in anatomical layers. At the end of operation the portacath was introduced into the superior caval vein. The animals were extubated and monitored each day for the next fourteen days with a particular emphasis on the clinical examination (attention to wound healing, infection of the portacath and function of the gastrointestinal system) to diagnose possible surgical complications. The monoclonal antibody against TGF-β1 (MAB TGF- β1, MAB1032, Chemicon International, Inc., USA) in amount 40μg/kg of body weight (TGF-β1 group) or a physiological solution (control group) were applied into the central venous catheter 24 hours after partial portal vein ligation (the start of production of endogenous TGF-β1 according to works of Armendariz Borunda (15,26). The postoperative analgesia was provided by intramuscular application of small doses of Azaperon (10mg).

Biochemistry

Blood samples were collected from the central vein catheter before the operation, after ligation of the last portal branch, 30 minutes after partial portal vein ligation, 2 hours after partial portal vein ligation, on the 1st (before application of MAB TGF-β1 or the physiological solution), 3rd, 7th, 10th and 14th postoperative days (POD). Biochemical serum parameters focused on liver functions to detect the influence of the applied monoclonal antibody on the animal organism and to recognise possible differences between TGF-β1 and the control group. Serum levels of bilirubin, urea, creatinine, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), cholinesterase (CHE), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin were assessed by the biochemical analyzer Olympus 2700.

Ultrasonography

The ultrasonographic controls were performed immediately after surgical procedure and on the 3rd, 7th, 10th and 14th PODs (ultrasound machine Medison Sonoace 9900, convex probe with a frequency of 3.5 MHz). The diameters of the atrophic and hypertrophic lobes were measured in B-modus in all three basic planes (axial, sagittal and coronary). The volume of the lobes was assessed by using the standard ultrasonographic formula that is also used in human medicine: axial x sagittal x coronary/2. The volumes are presented as a percentage of base line (immediately postoperative liver lobe volumes) to provide better information about the changes in volumes that were achieved.

Termination of experiment

The piglets were sacrificed on the 14th day under deep general anaesthesia with a concentrated solution of potassium chloride administered into a central venous catheter. The piglets were dissected and measurement of the atrophic and hypertrophic liver lobes was performed. These data were compared with the proportions estimated by ultrasonography. Bioptic samples from the atrophic and hypertrophic liver lobes were taken and stored in 10% formaldehyde (methanal) and also frozen below -70°C.

Histology

The biopsies from the atrophic and hypertrophic parenchyma were examined after staining with hematoxylin-eosine and periodic acid-Schiff (PAS) staining after digestion of preparations with diastase. The proliferation activity was examined using antibody Ki67 (MIB 1MW,

1:1000 DakoCytomation, Denmark). The primary antibodies were visualised using a streptavidin-biotin-peroxidase complex (DakoCytomation). The amount of binucleated hepatocytes were measured in 20 microscopic fields by an eyepiece micrometer (Olympus). The size of the hepatocytes and the length of the lobuli were examined twice using the eyepiece micrometer (Olympus).

Statistical analysis

Statistical analysis was performed with the CRAN 2.4.0 and STATISTICA (98 Edition) software. The assessed parameters (biochemistry, ultrasonography, histology) were analysed by the following statistical tests: the comparison of distribution of the studied parameters over the groups was counted using a distribution-free test (Wilcoxon). The Spearman Rank Correlation Coefficient was

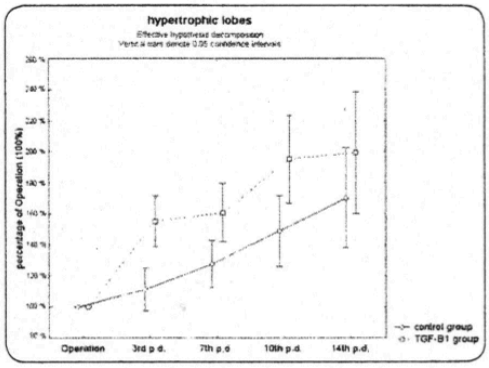
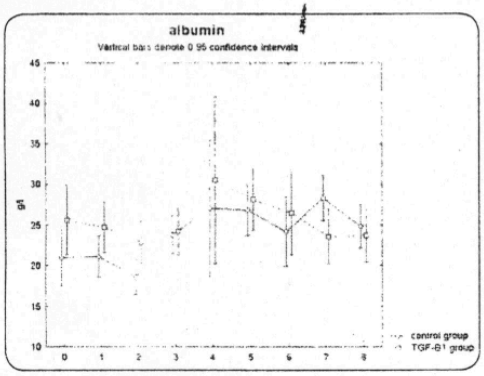
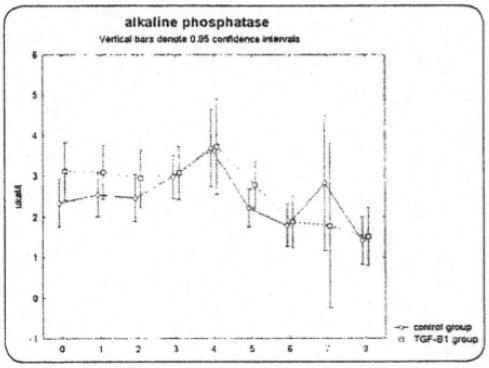
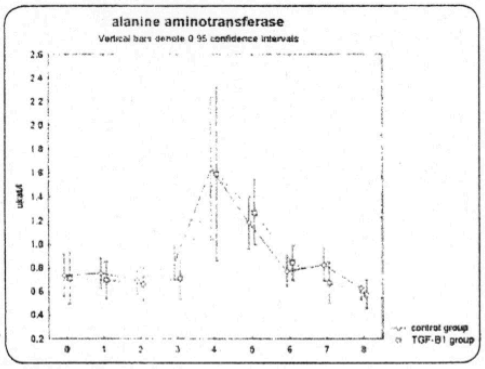
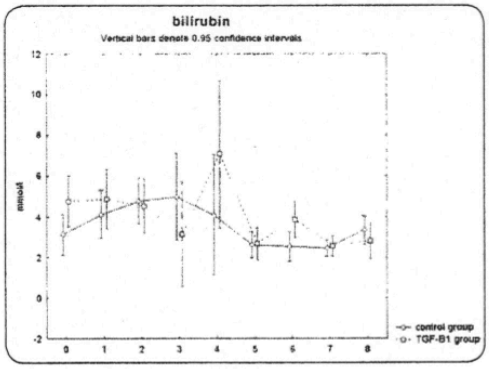


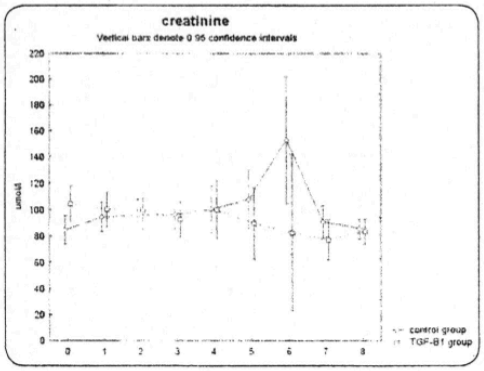
FIGURE 1.
Comparison of the growth of hypertrophic liver lobes between TGF-β1 and control groups. (p.d. = postoperative day).











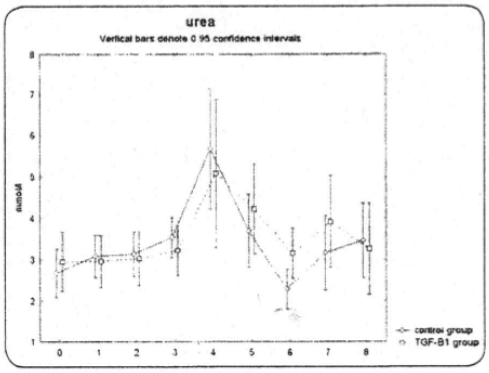


FIGURE 2.
Comparison of serum levels of some of the studied biochemical parameters between TGF-β1 and control groups. (0) before operation, (1) after ligation of the last portal branch, (2) 30 minutes after partial portal vein ligation, (3) 2 hours after partial portal vein ligation, (4) 1st POD (before application of MAB TGF-β1 or physiological solution), (5) 3rd POD, (6) 7th POD, (7) 10th POD, (8) 14th POD.

used because of the non-Gaussian distribution of parameter values. The whole development of the studied parameters over time was compared between the groups using the ANOVA test.

RESULTS

The volumes of liver lobes measured by ultrasonography and physical examination were compatible on the 14th POD. The absolute volume of hypertrophic lobes (left lateral and medial lobes) grew more rapidly after application of MAB TGF-β1, whereas the control group had a slow but continual growth in the hypertrophic liver lobes during the whole follow-up period. The augmentation of growth of the hypertrophic lobes was maximal between the 3rd and 7th PODs in comparison with the control group ($p<0.05$). Nevertheless this growth accelerating effect was lost during the next ultrasonographic controls and on the 14th POD there were no statistically significant differences (**Figure 1**). The serum levels of all studied biochemical parameters and the progression of curves are presented in **Figure 2**. All the studied serum biochemical parameters were comparable in both experimental groups and the differences show no statistical significance between the TGF-β1 and the control group at each time point (**Figure 2**). No adverse reaction on MAB application was observed.

The histological examination was performed on biopsies that were conducted at the end of the experiment after the animals had been sacrificed when the proliferative phase of liver regeneration had in fact finished. The differences in length of lobuli were not statistically significant (**Figure 3**). Statistical analysis also showed no significant increase in the quantity of binucleated hepatocytes in the hypertrophic liver lobes in the TGF-β1 group (**Figure 4**). We observed a larger distribution of the number of binucleated hepatocytes per field in the hypertrophic lobe from TGF-β1 group (1-4 binucleated hepatocytes per field) in contrast to the control group (2-3, 5 binucleated hepatocytes per field). The test of normality demonstrated a normal distribution of this parameter in each animal in both experimental groups. The size of hepatocytes was also not proved to be a statistically significant parameter for differences between the TGF-β1 group and the control group (**Figure 5**). Proliferative activity in both groups was greatly reduced.

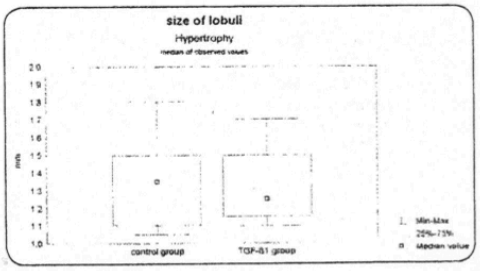


FIGURE 3. Comparison of length of lobuli in hypertrophic lobes between TGF-β1 and control groups.

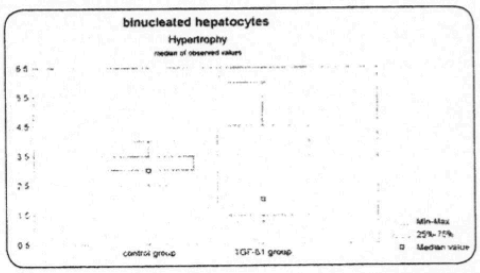


FIGURE 4. Comparison of the concentration of binucleated hepatocytes in hypertrophic lobes between TGF-β1 and control groups. The binucleated hepatocytes were detected in 20 microscopic fields.

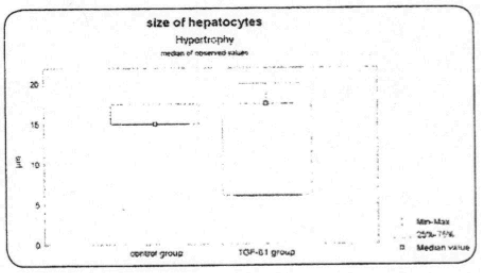


FIGURE 5. Comparison of size of hepatocytes in hypertrophic lobes between TGF-β1 and control groups.

DISCUSSION

This experimental study presents possibilities of the application of extrinsic MAB TGF-β1 to increase the required future remnant liver volume after partial portal vein ligation. The absolute volume of hypertrophic lobes (left lateral and medial lobes) grew more rapidly after application of MAB TGF-β1, whereas the control group had a slow but continual growth in the hypertrophic liver lobes during the whole follow-up period. The augmentation of growth of the hypertrophic lobes was maximal between the 3rd and 7th PODs in comparison with the control group ($p<0.05$). Nevertheless this growth accelerating effect was lost during the next ultrasonographic controls and on the 14th POD there were no statistically significant differences. The use of large animals especially piglets in this experimental surgical study is very appropriate in relation to the human medicine and clinical surgery because of the similar physiology of piglets and the human body. The results of the present study confirm previous findings gained using *in vitro* models and in experiments with small laboratory animals (15,16,26,27). The selected concentration and timing of the application of monoclonal antibody prolonged acceleration of liver parenchyma regeneration in non-occluded liver lobes (15,26). The secondary effects that could be hypothesized after application of the monoclonal antibody against key pleiotropic growth factor (changes in immune reactions and homeostasis) were not observed during application or in the whole postoperative period. In previous experiments we have shown the importance and usefulness of cytokines of the first phase of liver regeneration that increases priming of the hepatocytes interleukine-6 and tumor necrosis factor α (23,24). These cytokines have pleiotropic functions which could be altered or changed by application of these cytokines from an extrinsic source. The choice of the monoclonal antibody against growth factor that terminates this first phase of liver regeneration and stimulates hepatocytes to differentiation, production of extracellular matrix and remodelation of liver tissue structure, imitates the same effect of these named cytokines (18).

No statistically significant differences were shown between serum levels of the studied biochemical parameters at particular points in time. This also demonstrates that there was no unsuitable influence of the applied cytokine on the liver function. These results support our ideas to choose monoclonal antibody against TGF-β1 for future clinical studies in human liver surgery.

The larger distribution of the number of binucleated hepatocytes in the hypertrophic parenchyma of TGF-β1 group in comparison with the control group could be explained by incomplete liver regeneration at the end of experiment. Because there are practically no mitotic figures or the amount is the same as in the normal liver parenchyma without any surgical procedures or toxic insult, it was possible to hypothesize that the first phase of liver regeneration was finished and the next phase of regeneration was proceeding, namely the remodelling phase and the phase when the liver microstructure is restored (28). The next objective for future study would be the detection of changes to the intracellular or extracellular matrix during the process of liver regeneration. The size of hepatocytes and the length of lobuli were not proved to be statistically different between study and control group. The same size of hepatocytes and length of lobuli in the bioptical samples from the hypertrophic parenchyma could be also explained in the same way. This hypothesis is supported by the restitution of all liver functions monitored by biochemical parameters and completion of the proliferative phase of liver regeneration at the moment of sacrificing of the experimental animals.

This presented experimental model of liver regeneration is considered to be more compatible with the human organism and with the situation in clinical surgery than other published models in small animals (15,16,26,27). The performed portal vein ligature is also very similar to PVE (9). However, contemporary literature has considered the application of MAB TGF-β1 after partial portal vein ligation only on a murine experimental model and we did not find any references in the literature about the experimental verification of MAB TGF-β1 in the process of liver regeneration on large animals and it has not been transferred to clinical studies (10). The results of this experiment could be useful for the support of liver regeneration during complicated liver procedures with a high risk of liver failure and a small future liver remnant volume.

TGF-β1 stimulates the growth of tumor remnants after non-complete liver tumor resections (29,30). We could hypothesize that systemic application of MAB TGF-β1 could silence these effect and reduce the supposed increase of tumor volume between portal vein ligation and planned resection after achievement of a satisfactory FLRV. These results have encouraged the preparation of further studies in human medicine and the initiation of hypertrophy after PVE in high-risk patients where a weak hypertrophic reaction after PVE can be expected. The application of MAB TGF-β1 could hypothesize an increase in future liver remnant volume after performance of PVE.

The present study describes a new usage of a monoclonal antibody against TGF-β1 in a large animal experimental model of partial portal vein ligation which simulates the situation in human medicine. The achieved acceleration of growth of the hypertrophic liver lobes after application of monoclonal antibody against TGF-β1between 3rd and 7th PODs confirmed the key role of the studied cytokine in terminating the regeneration of liver parenchyma after portal vein ligation. The experimental results could be settings for a clinical study in patients with a low response regeneration of future liver remnant volume after portal vein embolization that does not allow surgical treatment.

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