# UNIVERZITA PALACKÉHO V OLOMOUCI

Lékařská fakulta


# Cytogenetické změny u nemocných se solidními nádory

**DOKTORSKÁ DIZERTAČNÍ PRÁCE**


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Olomouc 2015

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|---|---|
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V Olomouci, 12.6.2015 Mgr. Vladimíra Koudeláková

# Obsah

# Seznam použitých zkratek

| | |
|---|---|
| AKT | rodina signálních Ser/Thr protein-kináz |
| ALK | gen anaplastic lymphoma kinase |
| ASCO | Americká společnost pro klinickou onkologii (American Society for Clinical Oncology) |
| BCL2 | gen B-cell lymphoma 2 |
| BCSS | breast cancer-specific survival |
| BL1/2 | basal-like 1/2 |
| BRAF | gen v-raf murine sarcoma viral oncogene homolog B1 |
| BRCA1 | gen breast cancer 1 |
| CAP | společnost Amerických patologů (College of American Pathologists) |
| CCNE1 | gen pro cyklin E1 |
| CEP17 | centromerická oblast chromozomu 17 |
| CNS | centrální nervová soustava |
| EGFR | gen pro epidermální růstový faktor |
| EML4 | gen echinoderm microtubule associated protein like-4 |
| EMT | epiteliální-mezenchymální tranzice |
| ER | estrogenový receptor |
| ERK | gen pro extracelulárně regulovanou MAP kinázu |
| ESMO | Evropská společnost pro lékařskou onkologii (European Society for Medical Oncology) |
| EU | Evropská unie |
| FDA | úřad pro kontrolu potravin a léčiv (Food and Drug Administration) |
| FGFR1/2 | gen pro receptor pro fibroblastový růstový faktor 1/2 |
| FFPE | formalinem fixované v parafinu zalité tkáně |
| FISH | fluorescenční *in situ* hybridizace |
| GIST | gastrointestinální stromální tumory |
| IM | immunomodulatory |
| INPP4B | inozitol-polyfosfát-4-fosfatáza |
| ΔHER2 | varianta alternativního sestřihu HER2, delece exonu 16 |
| HER2 | gen pro epidermální růstový faktor 2 |
| HER3 | gen pro epidermální růstový faktor 3 |
| HIP1 | gen huntingtin interacting protein 1 |
| HNSCC | karcinom hlavy a krku |
| IHC | imunohistochemie |
| Ki-67 | protein kódovaný genem MKI67 |

| | |
|---|---|
| KIF5B | gen kinesin family member 5B |
| KIT | gen pro tyrozin-kinázu s ligandem SCF; CD117 |
| KRAS | gen kirsten rat sarcoma viral oncogene homolog |
| LAR | luminal androgen receptor |
| M | mesenchymal |
| MAPK | gen mitogen activated protein kinase |
| MDM2 | gen mouse double minute 2 |
| MEK | mitogeny aktivovaná protein kináza kináza |
| MSL | mesenchymal stem-like |
| MYC | gen v-myc myelocytomatosis viral oncogene homolog |
| NCCN | National Comprehensive Cancer Network |
| NF1 | gen pro neurofibromin |
| NOS | blíže nespecifikovaný (not otherwise specified) |
| NPI | Nottinghamský prognostický index (Nottingham Prognostic Index) |
| NRAS | gen neuroblastoma RAS viral (v-ras) oncogene homolog |
| NSCLC | nemalobuněčný plicní karcinom |
| OS | celkové přežití (overal survival) |
| PAM50 | Prediction Analysis for Microarrays |
| PCR | polymerázová řetězová reakce |
| PDFGRA | gen pro receptor pro destičkový růstový faktor |
| PI3K | fosfatidyl-inositol-3-kináza |
| PIK3CA | fosphatidyl-inositol-4,5-bifosfát-3-kináza, katalytická podjednotka alfa |
| PLC-γ | fosfolipáza C gamma |
| PR | progesteronový receptor |
| PTB | phosphotyrosine-binding region |
| PTEN | gen phosphatase and tensin homolog deleted on chromosome ten |
| RARA | gen pro alfa receptor kyseliny retinové |
| RB1 | gen pro retinoblastom 1 |
| RET | gen pro tyrozin-kinázový receptor |
| RFS | doba přežití bez relapsu (relapse-free survival) |
| ROS1 | gen kódující protein ROS |
| RS | skóre rekurence |
| RT-PCR | reverzně-transkriptázová PCR |
| SCF | gen pro faktor kmenových buněk (stem cell factor) |
| SH2 | Src Homology 2 region |
| STAT3 | gen signal transducer and activator of transcription 3 |

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|---|---|
| T-DM1 | trastuzumab emtansine |
| TFG | gen TRK-fused gene |
| TKI | tyrozin-kinázový inhibitor |
| TNBC | triple-negativní karcinom prsu |
| TNM | klasifikace nádorů (T-tumor, N-node, M-metastasis) |
| TOP2A | gen pro topoizomerázu 2 alfa |
| WNT3 | gen wingless signaling pathway |
| wt | wild type |
| + | pozitivní |
| - | negativní |
| ↓ | nízký |
| ↑ | vysoký |

# 1. Teoretická část

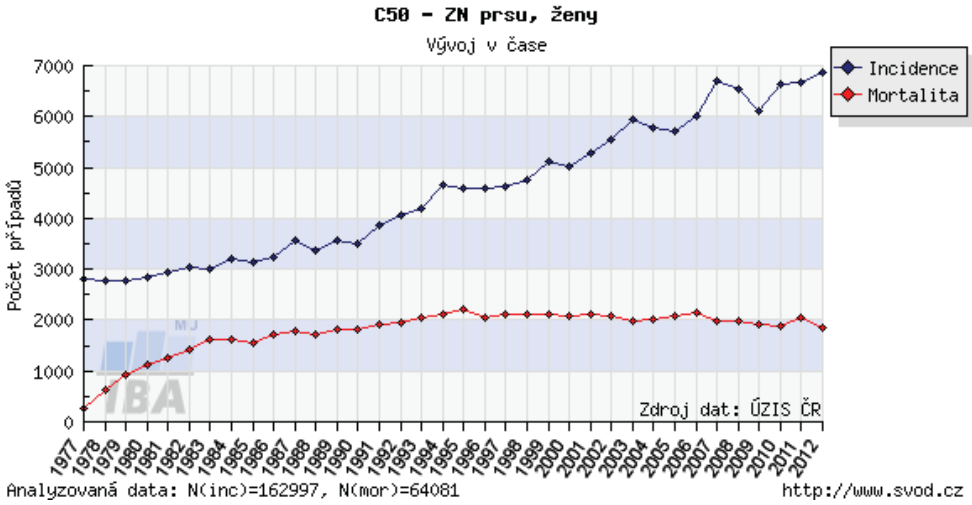
## 1.1 Úvod

Nádorová onemocnění jsou, po onemocněních kardiovaskulárního systému, druhou hlavní příčinou úmrtí v Evropě i České republice. Rostoucí incidence je především důsledkem stárnutí populace, životního stylu a prostředí a v neposlední řadě zlepšující se diagnostiky. I přes rostoucí incidenci však mortalita stagnuje či klesá, což signalizuje jednak úspěšnost screeningových programů a včasnější diagnostiku, ale také zvyšující se kvalitu léčby [1].

Chemoterapie patří společně s chirurgií a radioterapií mezi nejdůležitější léčebné postupy v onkologii. Již řadu let dochází k vývoji nových typů chemoterapeutik, hledání možností snížení jejich toxicity, zvýšení efektivity a cílení terapeutika do místa nádoru. Současná onkologie se zaměřuje především na studium diagnostických, prognostických a prediktivních biomarkerů. Intenzivní výzkum v této oblasti přinesl za poslední desetiletí celou řadu nových léčiv a prediktorů léčebné odpovědi, což umožnilo personalizovat terapii na základě genetických/expresních změn nádoru. Do popředí zájmu se dostávají především protinádorová léčiva, specifická pro konkrétní typ nádoru a biomarkery, které s dostatečnou pravděpodobností definují skupinu pacientů, kteří z této léčby budou profitovat. Dochází tak k personalizaci onkologické léčby na základě molekulárně-genetických a/nebo proteomických charakteristik samotného nádoru. Nádorové biomarkery jsou v současné době používány v oblasti diagnostiky, prognózování nemoci, predikci léčebné odpovědi a monitorování průběhu onemocnění.

## 1.2 Karcinom prsu

Karcinom prsu je celosvětově druhé nejčastější nádorové onemocnění u mužů i žen a nejčastější maligní onemocnění u žen, u nichž zaujímá přibližně čtvrtinu ze všech diagnostikovaných nádorových onemocnění. V roce 2012 bylo celosvětově nově diagnostikováno 1,67 milionů případů, v Evropské Unii (EU) 362.000 případů a v České republice 6852 nových případů. Věkově specifická incidence postupně narůstá od 30 do 70 let s maximem mezi 60 a 70 lety. I přesto, že je ve většině vyspělých zemí dostupný organizovaný screening a kvalitní terapie, je karcinom prsu celosvětově pátou nejčastější příčinou úmrtí na nádorová onemocnění. Mortalita na tento typ nádorového onemocnění byla v roce 2012 522.000 případů (celosvětově), 92.000 případů (EU) a 1840 případů (ČR). Karcinom prsu je poměrně dobře léčitelné onemocnění, což dokazuje časový vývoj incidence a mortality, kdy i přes vzrůstající incidenci, mortalita stagnuje (Obrázek 1) [2, 3].




**Obrázek 1** Incidence a mortalita karcinomu prsu v České republice v letech 1977-2012 [2].

Prognóza onemocnění je závislá na stádiu onemocnění, gradu, věku a v neposlední řadě na podtypu nádoru a zvolení vhodného léčebného režimu. Pro volbu správné léčebné strategie je třeba zhodnotit prognostické a prediktivní biomarkery. K prognostickým faktorům patří hodnocení velikosti nádoru, zasažení uzlin, klinické stádium, míra invazivity, histologický typ nádoru a míra exprese estrogenových (ER), progesteronových (PR) a HER2 receptorů. K prvním prediktivním biomarkerům, které byly u karcinomu prsu rutinně zavedeny pro individualizaci léčby, patří vyšetření exprese ER a PR receptorů. Nadměrná exprese těchto receptorů se vyskytuje až u 70% pacientů s karcinomem prsu a je prediktorem dobré léčebné odpovědi na hormonální terapii [4]. Dalším z velmi významných prediktivních biomarkerů je u karcinomu prsu pozitivita HER2 receptoru, kterou se zabývá část předložené dizertační práce.

Dizertační práce je zaměřena na 3 prognosticky nepříznivé skupiny karcinomu prsu, a to na karcinom prsu s *HER2* amplifikací (kapitola 1.2.2), polyzomií chromozomu 17 (kapitola 1.2.3) a triple-negativní karcinom prsu (kapitola 1.2.4).

### 1.2.1 Klasifikace nádorů prsu

1.2.1.1 TNM a histologická klasifikace nádorů prsu

Základní diagnostikou a klasifikací nádorů prsu je klasifikace TNM, kde se na základě klinického vyšetření a zobrazovacích vyšetřovacích metod hodnotí sublokalizace primárního nádoru a jeho velikost (T), zasažení mízních uzlin (N) či přítomnost vzdálených metastáz (M). Na základě těchto kritérií je nádor rozdělen do jednotlivých stádií [5].

Klinicky nejpřínosnější je rozdělení karcinomů prsu na základě histologického vyšetření, a to gradu a typu nádoru. Histologický grade odráží agresivitu nádoru, míru proliferace a diferenciace (hodnotí se mitotický index, tubulární formace, jaderný polymorfismus) [6]. Z histologických typů nádorů prsu jsou nejčastější karcinomy duktální (přibližně 70-80%) a lobulární (přibližně 10%). Méně často se vyskytují karcinomy tubulární, medulární, kribriformní, mucinózní, metaplastické či méně časté podtypy [7]. Histologická klasifikace má však, na rozdíl od určení TNM a gradu onemocnění, pro klinickou praxi pouze minimální význam.

1.2.1.2 Molekulární klasifikace nádorů prsu (tzv. „intrinsic subtypes“)

Na základě výsledků high-throughput technologií byla v roce 2000 popsána molekulární klasifikace nádorů prsu (tzv. „intrinsic subtypes“). Následným hierarchickým klastrováním expresního profilu byly nádory prsu rozděleny do 4 základních skupin: luminal A, luminal B, basal-like a HER2-enriched. Pátou nalezenou skupinou byl normal breast-like podtyp, který se klastruje s fyziologickými vzorky prsu [8,9]. Luminal A nádory jsou hormonálně pozitivní (pozitivita ER a/nebo PR), HER2-negativní s nízkou proliferační aktivitou (Ki-67). Luminal B nádory jsou rovněž ER/PR-pozitivní, často HER2-negativní, jejich proliferační aktivita je však vysoká. Basal-like podskupina má triple-negativní (TN) stav receptorů (ER, PR, HER2-negativní), pozitivitu EGFR a cytokeratinu 5/6. Karcinomy z HER2-enriched skupiny jsou ER/PR-negativní a HER2-pozitivní. Normal breast-like karcinomy jsou TN a exprimují geny adipózní tkáně. Šestý, claudin-low podtyp byl identifikován v roce 2007 a má podobné charakteristiky jako basal-like karcinomy (ER, PR, HER2-negativní). Hlavní charakteristikou je nízká exprese claudinů 3, 4 a 7 a vysoká exprese genů imunitní odpovědi. Charakteristiky jednotlivých podskupin jsou shrnuty v Tabulce 1 [8–11].

Jednotlivé skupiny se liší v incidenci, věku pacientů při diagnóze, prognóze i odpovědi na terapii. Mezi skupinu s nejlepší prognózou patří karcinomy ze skupiny luminal A, které dobře odpovídají na hormonální terapii. Oproti luminal A skupině, jsou luminal B nádory více agresivní, mají vyšší histologický grade, vysokou proliferační aktivitu a horší prognózu. I přes hormonální pozitivitu luminal B nádorů je jejich citlivost na hormonální terapii nízká a je proto doporučena kombinace s chemoterapií (antracykliny, taxany). K nádorům se špatnou prognózou patří rovněž HER2-enriched, basal-like a claudin-low nádory. Basal-like nádory se vyskytují většinou u mladších žen, častěji Afro-Amerického původu. Jejich prognóza je velmi nepříznivá, jedná se většinou o velké nádory, s vysokým gradem a častým zasažením lymfatických uzlin. Claudin-low nádory mají podobně jako basal-like karcinomy vysoký grade, špatnou prognózu a odpověď na terapii, často se jedná o karcinomy metaplastické. U HER2-enriched skupiny se jedná často o karcinomy duktální s vysokou proliferační aktivitou, vysokým gradem a zasažením uzlin. HER2 pozitivita je negativním prognostickým faktorem, zároveň však prediktorem dobré odpovědi na anti-HER2 terapii (trastuzumab, pertuzumab, lapatinib, T-DM1). HER2-cílená terapie zásadně vylepšila prognózu

HER2-enriched pacientů. Normal breast-like skupina je pouze málo charakterizovaná, existence nádorů z této skupiny je často zpochybňována. Diskutuje se, zda se nejedná pouze o artefakt, resp. kontaminaci vzorků normální prsní tkání [12–17].

1.2.1.3 Molekulární klasifikace v klinické praxi

V klinické praxi je použití high-throughput technologií velmi obtížné a nákladné, proto bylo vyvinuto několik komerčně dostupných diagnostických testů, které jsou schopny zařadit pacienta do jedné z podskupin, stanovit míru rizika či vhodnost terapie. Na základě experimentů z čipových technologií byl vyvinut prediktor PAM50 (prediction of microarray), který je schopen pomocí reverzně-transkriptázové polymerázové řetězové reakce v reálném čase (real-time RT-PCR) stanovit expresi 50 genů, zahrnujících proliferaci, ER, HER2, myoepiteliální a bazální charakteristiky. Na základě PAM50 prediktoru lze pacienty zařadit do luminal A, luminal B, basal-like a HER2-enriched podskupin. V roce 2013 byl PAM50 prediktor certifikován a schválen Food and Drug Administration (FDA) pro klinické použití jako Prosigna test (NanoString Technologies). Tato assay je použitelná v klinické praxi především proto, že je možné analyzovat RNA izolovanou z formalinem fixovaných v parafinu zalitých vzorků (FFPE). Assay je navíc validovaná několika rozsáhlými studiemi [13, 18–20].

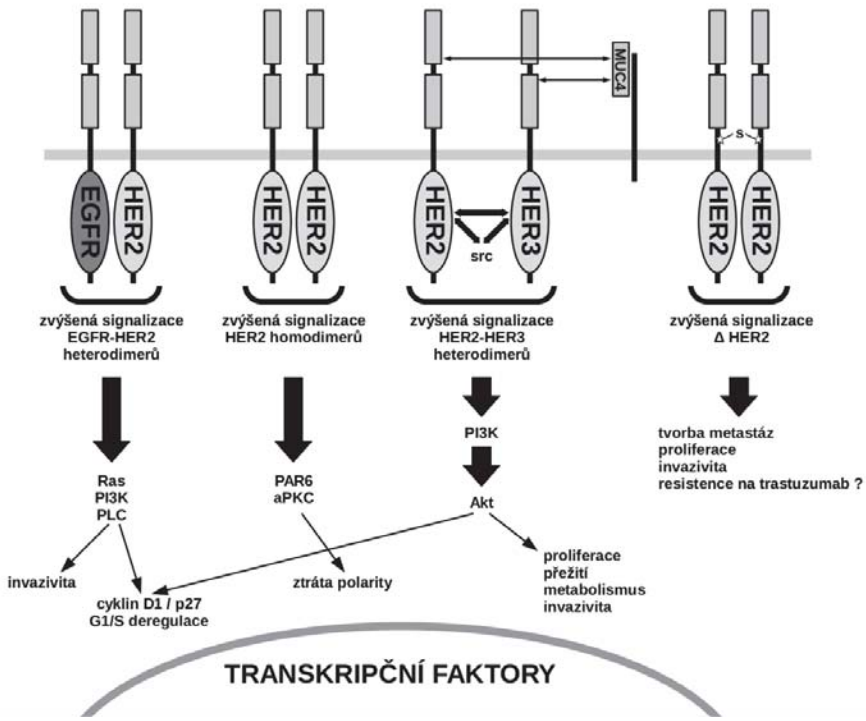
Klinicky nejrozšířenějším a nejvíce validovaným testem je Oncotype DX (Genomic Health, Inc.), který předpovídá riziko rekurence časných stádií karcinomu prsu na základě expresního profilu 21 genů (16 genů spojených s nádorovým onemocněním, 5 kontrolních genů). Jedná se o geny spojené s proliferací, invazivitou, ER a HER2 dráhou. Assay, založená na real-time RT-PCR, je proveditelná z RNA izolované z FFPE vzorků. Oncotype DX byl primárně validován na vzorcích pacientek ze tří nezávislých studií, u kterých bylo hodnoceno riziko recidivy s více než 10-letým sledováním. U Oncotype DX je hodnoceno tzv. skóre rekurence (RS), na základě kterého lze nádor zařadit do jedné ze tří rizikových skupin. Bylo prokázáno, že hodnota RS je jak prognostický faktorem, tak i prediktorem odpovědi na chemoterapii [21–27]. Na základě výsledků řady prospektivních i retrospektivních studií byl Oncotype DX assay zařazen do mezinárodních doporučení pro léčbu karcinomu prsu (ESMO, ASCO, St.Gallen, NCCN). Od roku 2014 je vyšetření zařazeno i do doporučení České onkologické společnosti pro adjuvantní léčbu časného ER+, HER- karcinomu prsu gradu 2. Toto vyšetření je u pacientek s nejednoznačným benefitem z chemoterapie hrazeno ze zdrojů zdravotního pojištění [16, 28–30].

V rutinní klinické praxi se pro klasifikaci nádorů prsu používá imunohistochemické, respektive molekulárně-biologické stanovení hormonálních receptorů (ER, PR), HER2 a proliferačního markeru Ki-67. Na základě exprese, resp. počtu kopií genu HER2 lze nádory prsu rozdělit do 5 základních skupin: luminal A-like (ER+ a/nebo PR+, HER-, Ki-67↓), luminal B-like, HER2- (ER+, HER-, PR-, Ki-67↓/ ER+, HER-, PR+, Ki-67↑ ), luminal B-like, HER2+ (ER+, HER+, PR+/-, Ki-67↓/↑ ), HER2+

(ER-, PR-, HER2+) a basal-like (ER-, PR-, HER-). Na základě tohoto rozdělení lze predikovat prognózu, stejně jako účinnost chemoterapie či cílené léčby [16,31,32].

### 1.2.2 HER2-pozitivní karcinom prsu

*HER2*, lokalizovaný na 17q12, kóduje transmembránový protein p185, patřící do rodiny receptorů pro epidermální růstový faktor (HER/EGFR/ErbB) s tyrozin-kinázovou aktivitou. Za fyziologických podmínek reguluje buněčný růst a diferenciaci. Protein HER2 se, stejně jako ostatní zástupci erbB rodiny, skládá ze tří funkčních domén - extracelulární, transmembránové a intracelulární (cytoplazmatické). N-koncová extracelulární doména je u proteinů erbB rodiny místem vazby ligandu [33,34]. Vazba ligandu na extracelulární doménu proteinů erbB rodiny způsobuje dimerizaci až oligomerizaci s HER2, což umožňuje transfosforylaci cytoplazmatických domén a tím aktivaci celé řady proteinů. Při nadměrné expresi HER2 dochází k aktivaci receptoru i bez přítomnosti ligandu [34,35]. Pro erbB receptory bylo nalezeno více než 12 ligandů, fyziologický ligand pro HER2 však dosud nebyl nalezen. Předpokládá se, že protein HER2 působí jako preferenční protein pro heterodimerizaci s ostatními ligandem aktivovanými členy erbB rodiny (EGFR a HER3) a jeho fyziologický ligand neexistuje [36]. C-koncová intracelulární doména je místem autofosforylace a aktivace řady buněčných proteinů. Po aktivaci receptoru a jeho dimerizaci dochází k fosforylaci specifických tyrozinů (1028, 1144, 1201, 1226/1227, 1258) [37]. Na ty se váží signalizační proteiny obsahující na fosfotyrozin specifické vazebné domény (SH2, PTB domény), které přenáší mitogenní signál přes Ras/MAPK, PI3K/Akt, Janus kinázovou a PLC-γ signální dráhu [38–40]. HER2 signalizace je znázorněna na Obrázku 2.



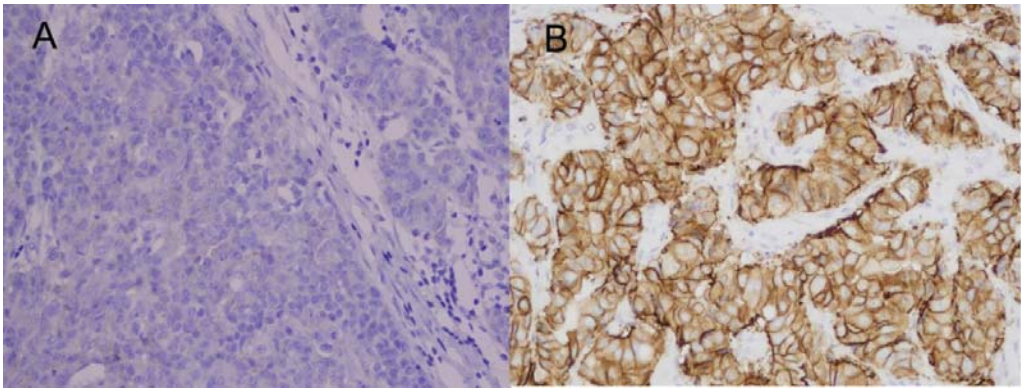

**Obrázek 2** Buněčná signalizace onkogenu HER2. Upraveno dle [41].

Amplifikace způsobující overexpresi proteinu HER2 bývá nalezena přibližně u 15-20% pacientů s karcinomem prsu, nejčastěji u duktálního typu s negativitou hormonálních receptorů. Overexprese proteinu HER2 vede ke zvýšené tvorbě dimerů všech typů. Tvorba EGFR-HER2 heterodimerů zvyšuje proliferaci a invazivitu tkáně. Homodimery HER2 narušují buněčnou polaritu a HER3-HER2 heterodimery ovlivňují proliferativní, invazivní, metabolické procesy a procesy přežití. Transaktivace HER3 a následná aktivace PI3K/AKT signální dráhy se zdá být jako klíčová pro tumorigenezi u HER2 overexprimovaných buněk. Overexprese HER2 rovněž umožňuje vznik méně časté ΔHER2 varianty (in-frame delece exonu 16, ztráta 16 aminokyselin v juxtamembránové doméně) s rozmanitějším signalizačním a transformačním potenciálem.

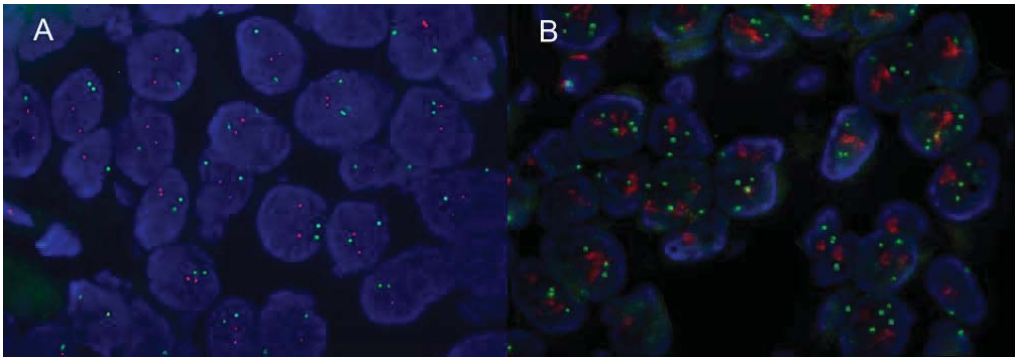
HER2 pozitivní nádory prsu jsou agresivnější, většinou málo diferenciované, s častým postižením lymfatických uzlin, výskytem distálních metastáz (často do CNS) a nepřítomností hormonálních receptorů, jsou méně citlivé na standardní chemoterapii. HER2 amplifikace/overexprese je nezávislým negativním prognostickým faktorem [41,42]. Amplifikace/overexprese HER2 byla nalezena i u dalších typů solidních nádorů, například u nemalobuněčného plicního karcinomu (NSCLC) [43], karcinomu žaludku, prostaty, kolorekta, ovariálního karcinomu a karcinomu hlavy a krku (HNSCC) [44].

Řada multicentrických, randomizovaných studií prokázala, že je amplifikace/overexprese genu HER2 prediktorem účinnosti anti-HER2 terapie. Humanizovaná monoklonální protilátka trastuzumab (Herceptin) je schválena pro léčbu časného i metastatického karcinomu prsu (v monoterapii či v kombinaci s chemoterapií) s prokázanou pozitivitou HER2. K léčbě HER2 pozitivního, lokálně pokročilého či metastatického karcinom prsu lze indikovat i další HER2 protilátky/nízkomolekulární inhibitory pertuzumab, T-DM1 či lapatinib [30, 45].

V současné době jsou odbornými společnostmi doporučeny dvě metody vyšetření HER2 a to immunohistochemie (IHC), která často slouží jako screeningová metoda a *in situ* hybridizace (ISH), která je potřebná především pro potvrzení IHC 2+ (středně pozitivních, nejednoznačných) výsledků. Příklady výsledků obou vyšetření jsou ilustrovány Obrázky 3 a 4. Dle aktuálních doporučení American Society for Clinical Oncology/ College of American Pathologists (ASCO/CAP) je anti-HER2 terapie podávána pacientům na základě ISH pozitivity (respektive pozitivního HER2/CEP17 poměru ≥ 2 či pozitivity průměrného počtu kopií HER2 ≥ 6 kopií/jádro) a/nebo imunohistochemické pozitivity 3+ [46].




**Obrázek 3** Vyšetření exprese proteinu HER2 u karcinomu prsu pomocí imunohistochemie (HercepTest) s negativním nálezem IHC 0 (A) a vysoce pozitivním nálezem IHC 3+ (B)

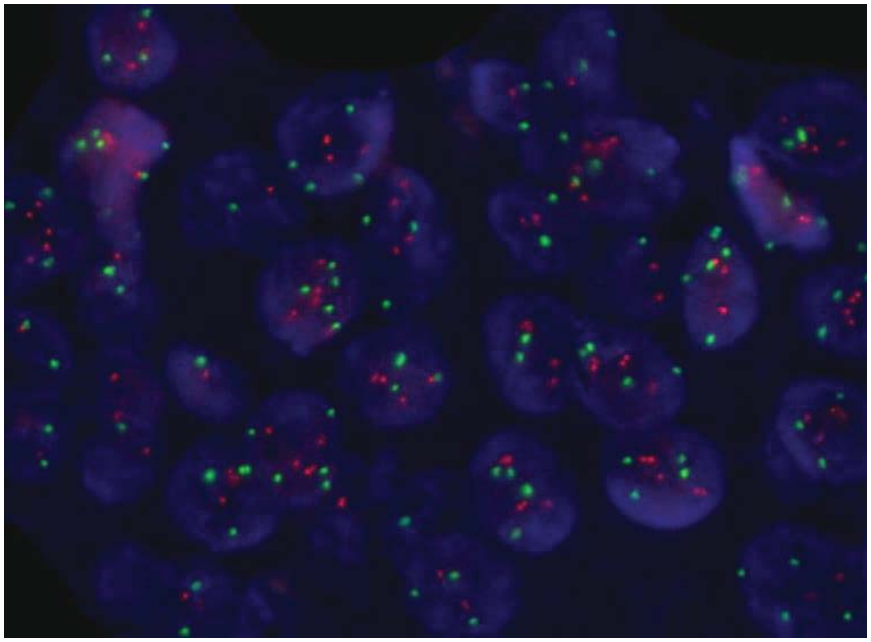



**Obrázek 4** Vyšetření počtu kopií genu *HER2* u karcinomu prsu pomocí fluorescenční *in situ* hybridizace (A) s negativním nálezem (2 kopie genu *HER2* a chromozomu 17) a (B) pozitivním nálezem (klastry více než 20 kopií genu *HER2* a 2-4 kopií chromozomu 17)

### 1.2.3 Karcinom prsu s polyzomií chromozomu 17

Počet kopií genu *HER2* je u karcinomu prsu vyšetřován pomocí ISH, nejčastěji duálně značenou sondou, která vyjma počtu kopií *HER2* detekuje rovněž počet kopií chromozomu 17, respektive centromerické oblasti chromozomu 17 (CEP17). Chromozom 17 je u karcinomu prsu velmi často alterován, a to jak důsledkem strukturálních přestaveb či bodových mutací, tak změnami počtu kopií genů, resp. různě dlouhých chromozomálních oblastí, až polyzomie. Hlavní příčinou je lokalizace celé řady významných protoonkogenů a tumor supresorových genů jako *p53*, *NF1*, *HER2*, *TOP2A*, *STAT3*, *BRCA1*, *WNT3* a *RARA* [47].

Vzhledem k lokalizaci významných genů byl intenzivně studován dopad polyzomie chromozomu 17, respektive zmnožení CEP17, kdy je počet kopií ≥ 3, na prognózu či predikci účinnosti terapie. Špatná prognóza u pacientů s karcinomem prsu se zmnožením CEP17, resp. zvýšená proliferační aktivita, vyšší Nottingham Prognostic Index (NPI) skóre či zasažení uzlin, byla popsána řadou studií. Vliv zmnožení CEP17 na přežití pacientů však nebyl pozorován [48–51]. Ve studii Krishnamurti et al. bylo zmnožení CEP17 asociováno s vyšším histologickým gradem, větší mitotickou aktivitou, vyšším NPI skóre a ER-negativitou i u pacientů s negativním *HER2*/CEP17 poměrem [52]. Naopak, v ostatních studiích nebyly špatné prognostické markery u pacientů se zmnožením CEP17 pozorovány [53,54]. Na klinickou praxi má však největší dopad prediktivní hodnota zmnožení CEP17. Retrospektivní analýzy prokázaly účinnost trastuzumabu u pacientů se zvýšeným počtem kopií CEP17 a negativním *HER2*/CEP17 poměrem, a to i u IHC negativních (0/1+) a středně pozitivních (2+) případů. Účinnost lapatinibu u HER2 negativních pacientů se zvýšením CEP17 však prokázána nebyla [55].



**Obrázek 5** Karcinom prsu se současně zvýšeným počtem kopií genu *HER2* (3-5 kopií; oranžové signály, SpectrumOrange) a CEP17, resp. chromozomu 17 (3-5 kopií; zelené signály, SpectrumGreen).

Polyzomie chromozomu 17, kdy je počet kopií CEP17≥ 3 (viz Obrázek 5), je řadou studií popisována jako poměrně častý jev s výskytem od 3 do 46% [55]. Recentní studie za pomocí high-throughput technologií odhalily, že se nejedná o polyzomii chromozomu 17 (tj. zmnožení celého chromozomu), nýbrž o zmnožení, resp. amplifikaci centromerické oblasti chromozomu 17 nebo o zmnožení dlouhého ramene chromozomu 17 s přesahem do centromerické oblasti [56–60]. Tito autoři ukázali, že polyzomie chromozomu 17 jako taková je u karcinomu prsu velmi ojedinělá událost, což bylo potvrzeno řadou studií založených na FISH [61–63]. Vzhledem k tomu, že se „pravá“ polyzomie chromozomu 17 vyskytuje u méně než 1% případů s karcinomem prsu, doporučuje Moelans et al. užívání termínu zvýšení počtu kopií CEP17 u případů, kdy byla „polyzomie“ detekována pouze pomocí centromerické sondy [64]. Dle ASCO/CAP kritérií by se ojedinělé případy, u nichž je průměrný počet kopií *HER2* mezi 4 a 6, poměr *HER2*/CEP17 < 2.0 a CEP17 > 2.0, měly vyšetřit pomocí alternativního markeru na chromozomu 17 [46], v případě pozitivního poměru *HER2* a vyšetřovaného markeru lze podat anti-HER2 terapii.

### 1.2.4 Triple-negativní karcinom prsu

Triple-negativní karcinom prsu (TNBC) se vyskytuje u 15-20% případů karcinomu prsu a je charakterizován negativitou ER, PR a HER2 receptorů. TNBC se z více než 70% překrývá s basal-like podtypem karcinomu prsu (viz výše) a tyto dvě skupiny jsou také velmi často zaměňovány. Podobně jako u basal-like karcinomů jsou tedy TNBC nádory nalézány častěji u mladších žen, Afro-Američanek a žen s familiárním karcinomem prsu, respektive *BRCA1/2* mutací [65,66]. TNBC karcinomy jsou v porovnání s non-TNBC karcinomy prsu agresivnější, rychle rostoucí nádory s vysokým gradem a častým zasažením uzlin. Na rozdíl od ostatních podtypů karcinomu prsu, recidivují TNBC nádory nejčastěji mezi 1.-3. rokem od operace, většina pacientek s TNBC umírá do 5 let od terapie [66,67].

Vzhledem k agresivitě onemocnění a tomu, že pacienti nemohou profitovat z cílené anti-HER2 nebo hormonální terapie, je velká snaha o identifikaci makeru nebo skupiny markerů, které by mohly predikovat účinnost chemoterapie. Stěžejní prací je studie Lehmann et al. z roku 2011, který analyzoval expresní profil 587 TNBC případů a na základě hierarchického klastrování identifikoval 6 různých TNBC podtypů. Podle expresního profilu byly podtypy nazvány jako basal-like 1 a 2 (BL1/2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) a luminal androgen receptor (LAR). Charakteristiky jednotlivých podskupin jsou shrnuty v Tabulce 2. Na základě této klasifikace byly identifikovány TNBC buněčné linie reprezentující jednotlivé podskupiny, u nichž byla testována účinnost terapie, zaměřená na alterované signální dráhy [68]. Molekulární podtypy byly navíc identifikovány jako nezávislý prediktor patologické kompletní odpovědi, přičemž nejlepší odpověď byla pozorována u BL1 skupiny, naopak nejhorší u BL2 a LAR skupin (52% vs. 0%/10%) [69].

Následně byly v roce 2012 publikovány dvě významné studie, zaměřené na genetické změny u TNBC. V souladu s velkou heterogenitou TNBC nádorů byla nalezena celá řada aberací, které se vyskytovaly pouze u malého procenta studovaných nádorů. U velké části nalezených mutací (64%) nebyl navíc potvrzen biologický význam, respektive přepis těchto mutací do mRNA. Nejčastěji nalezené mutace byly mutace v genu *p53*. Dalšími geny, u nichž byly nalezeny významné alterace, jsou geny *RB1*, *PTEN*, *MYC*, *PIK3CA*, *EGFR*, *MDM2*, *CCNE1*, *INPP4B* a *FGFR1/2* [70,71].

Hlavním problémem TNBC nádorů je obrovská heterogenita, která brání identifikaci klinicky relevantního biomarkeru. Pomocí high-throughput technologií byla identifikována celá řada slibných biomarkerů a cílených terapeutik, které je třeba validovat pomocí klinických studií. Problematika TNBC je shrnuta v přehledové publikaci (viz příloha 1).

**Tabulka 1 Charakteristika podtypů karcinomu prsu**

| Podtyp | ER/PR | HER2 | EGFR | CK 5/6 | Proliferační markery | Další významné markery | Frekvence | Prognóza | Cílená léčba |
|---|---|---|---|---|---|---|---|---|---|
| Luminal A | + | - | - | - | nízké | BCL2, CK8/18, GATA3 (+) | 40% | dobrá | hormonální |
| Luminal B | + | +/- | +/- | - | vysoké | MKI67, CCNB1, MYBL2 (+) | 20% | střední/špatná | hormonální |
| HER2-enriched | - | + | + | - | vysoké | GRB7 (+) | 15-20% | špatná | inhibitory HER2 |
| Basal-like | - | - | + | + | vysoké | CK5, CK17, caveolin 1/2, P-cadherin, nestin (+) | 15-20% | špatná | - |
| Claudin-low | - | - | + | - | vysoké | claudin 3,4,7, E-cadherin (-), geny imunitní odpovědi, EMT (+) | 12-14% | špatná | - |

**Tabulka 2 Charakteristika podtypů triple-negativního karcinomu prsu**

| Podtyp | Ovlivněné dráhy | Významné biomarkery | Chemosenzitivita | Potenciální léčba |
|---|---|---|---|---|
| BL1 | dráhy buněčného cyklu, proliferace, poškození DNA | ATR, BRCA, MYC, NRAS | velmi dobrá | cis-platina, PARP inhibitory |
| BL2 | dráhy buněčného cyklu, proliferace, signalizace růstových faktorů | EGFR, MET, EPHA2, TP63 | velmi špatná | cis-platina; inhibitory PARP, inhibitory růstových faktorů |
| IM | procesy signalizace imunitních buněk | JAK1/2, STAT1/4, IRF1/7/8, TNF | střední | - |
| M | EMT, buněčná pohyblivost, diferenciace, proliferace | Wnt, ALK, TGF-β | střední | PI3K/mTOR, Src inhibitory |
| MSL | EMT, buněčná pohyblivost, diferenciace, signalizace růstových faktorů, angiogeneze | EGFR, PDGFR, ERK1/2, VEGFR2 | střední | PI3K/mTOR, Src inhibitory |
| LAR | androgenový/estrogenový metabolismus, syntéza steroidů, metabolismus porfyrinu | AR, FOXA1, KRT18, XBP1 | špatná | AR antagonisté; inhibitory PI3K, Hsp90 inhibitory |

### 1.3 Ostatní nádorová onemocnění – prediktivní biomarkery v klinické praxi

Mezi nejdéle používané prediktivní biomarkery solidních nádorů patří, jak již bylo zmíněno, vyšetření exprese ER, PR a HER2 receptorů u pacientů s karcinomem prsu. Zavedení hormonální a anti-HER2 terapie do klinické praxe významně ovlivnilo prognózu velké části pacientů s tímto onemocněním. V poslední době byla identifikována celá řada potenciálních prediktivních biomarkerů, stejně jako cílených terapeutik. Tématu nádorových biomarkerů se věnují dvě přehledové prvoautorské publikace (viz příloha 2 a 3). Prediktivní biomarkery, které jsou v současné době součástí klinické praxe, jsou popsány v následujícím textu.

#### 1.3.1 Karcinom žaludku

Karcinom žaludku je zhoubné nádorové onemocnění žaludeční sliznice, z 95% se jedná o adenokarcinom. Přestože incidence i mortalita tohoto onemocnění významně klesá, jedná se o páté nejčastější nádorové onemocnění a třetí nejčastější příčinu úmrtí na nádorové onemocnění u mužů i žen [3]. U přibližně 20% případů karcinomu žaludku je nalézána amplifikace/overexprese genu HER2 (kapitola 1.2.2), která se častěji vyskytuje u intestinálního typu nádoru v oblasti gastroesofageální junkce [72–74]. Podobně jako u karcinomu prsu je amplifikace/overexprese genu HER2 prediktorem účinnosti anti-HER2 terapie. Humanizovaná monoklonální protilátka trastuzumab (Herceptin) je schválena pro léčbu metastatického adenokarcinomu žaludku či gastroesofageální junkce (v kombinaci s cisplatinou a kapecitabinem nebo fluorouracilem) s prokázanou pozitivitou HER2 [30].

#### 1.3.2 Nemalobuněčný karcinom plic

Karcinom plic je nejčastějším nádorovým onemocněním u mužů a nejčastější příčinou úmrtí na nádorové onemocnění u mužů i žen. U 85% případů se jedná o nemalobuněčný plicní karcinom, který lze dělit na adenokarcinom (~ 40%), spinocelulární karcinom (25-30%) a karcinom velkobuněčný (10-15%). V posledních letech bylo u plicního adenokarcinomu nalezeno několik významných prediktivních biomarkerů (*EGFR*, *ALK*, *ROS1*, *RET*), v klinické praxi jsou prozatím vyšetřovány geny *EGFR* a *ALK*.

*EGFR* gen je lokalizovaný na 7p11 a kóduje transmembránový tyrozin-kinázový receptor s obdobnou funkcí jako HER2. U NSCLC byly popsány aktivační mutace *EGFR* genu, jejichž výskyt je přibližně 30% u Asiatů a 15% v kavkazské populaci. Aktivační mutace, způsobující na ligandu nezávislou aktivaci receptoru, jsou preferenčně nalézány u nekuřáků/slabých kuřáků, asiatů a žen. Nejčastější mutace, delece v exonu 19 (báze 746-753) a substituce argininu za leucin (L858R) v exonu 21, bývají nalezeny až u 90% případů. Méně častými mutacemi jsou mutace v exonu 18 (G719X) a v exonu 21 (L861G). Tyto mutace jsou aktivační a predikují sensitivitu na tyrozin-kinázové inhibitory (TKI), zatímco pacienti s mutací v exonu 20 (inzerce, T790M) jsou na TKI rezistentní [75–78]. Pro léčbu

metastatického adenokarcinomu plic s prokázanou aktivační mutací *EGFR* lze v monoterapii indikovat reverzibilní TKI erlotinib, gefitinib či irreverzibilní TKI afatinib [30,79].

Dalším významným biomarkerem je gen *ALK*, lokalizovaný na 2p23. *ALK* kóduje transmembránový tyrozin-kinázový receptor, který hraje významnou roli ve vývoji nervového systému a je za fyziologických podmínek exprimován pouze v tenkém střevě, mozku a varlatech. U 2-7% pacientů s NSCLC bývá nalezena patologická exprese, způsobená nejčastěji inverzí malé části krátkého ramene chromozomu 2, inv(2)(p21p23), za vzniku fúzního proteinu EML4-ALK. Nejčastěji se jedná o fúzi exonu 13 (varianta 1) nebo exonu 6a/b (variant 3a/b) genu *EML4* s exonem 20 genu *ALK* [76,80]. Méně často se u NSCLC vyskytuje translokace *ALK* s geny *KIF5B* (kinesin family member 5B), *TFG* (TRK-fused gene) a *HIP1* (huntingtin interacting protein 1) [80].

*ALK* přestavba se preferenčně vyskytuje u mladších pacientů s adenokarcinomem a nekuřáků bez současných mutací *KRAS* či *EGFR*. Pro testování *ALK* přestaveb je doporučena FISH u pacientů s adenokarcinomem či NSCLC NOS (not otherwise specified). Pro ALK+ (ALK pozitivní) pacienty s již dříve léčeným pokročilým NSCLC lze indikovat léčbu crizotinibem [30,81].

### 1.3.3 Kolorektální karcinom

Kolorektální karcinom je třetí nejčastější nádorové onemocnění (po zhoubných nádorech kůže, karcinomu prsu a plic). U kolorektálního karcinomu je v současnosti významné vyšetření mutací genů skupiny *RAS*. *KRAS* gen, lokalizovaný na 12p12, kóduje na membránu vázaný protein s GTPázovou aktivitou, který zprostředkovává signální přenos mezi EGFR a dalšími proteiny proliferační kaskády Ras/Raf/MEK/ERK, což vede k buněčné proliferaci a přežití [76]. Přibližně u 35-40% pacientů s kolorektálním karcinomem bývá nelezena mutace genu *KRAS*, nejčastěji se jedná o bodovou mutaci exonu 2 kodonu 12 (G12V, G12D) a kodonu 13 (G13D) a méně často exonu 13 kodonu 61 [82]. U *KRAS* mutovaných případů je onkogenní signál, vzhledem k jeho umístění v signální kaskádě, nezávislý na *EGFR* aktivaci a u karcinomu kolorekta je proto mutovaný *KRAS* prediktorem špatné odpovědi na extracelulární blokátory EGFR (cetuximab/panitumumab) [83]. U metastatického kolorektálního karcinomu s wt-*KRAS* lze v monoterapii či kombinované terapii indikovat monoklonální protilátky cetuximab či panitumumab [30,84].

Mutace genu *KRAS*, způsobující rovněž rezistenci na EGFR TKI, se vyskytují přibližně u 20% případů NSCLC a jsou asociovány s adenokarcinomy, kavkazskou populací a kouřením [76,85]. Vyšetření *KRAS* mutací u pacientů s NSCLC však nemá klinický význam vzhledem k mutační exkluzivitě *EGFR* a *KRAS* mutací.

*NRAS* gen, lokalizovaný na 1p13, je úzce příbuzný genu *KRAS* a hraje rovněž významnou roli v regulaci proliferace a buněčného přežití. *NRAS* je mutovaný u 3-5% případů kolorektálního karcinomu. Mutace se podobně jako u *KRAS* genu vyskytují v kodonech 12,13 a 61, přičemž mezi nejčastější patří mutace kodonu 61 (Q61K, Q61R), následované mutacemi kodonu 12 [86–89]. Podobně

jako mutace *KRAS* jsou mutace *NRAS* prediktorem špatné odpovědi na extracelulární blokátory EGFR (cetuximab/panitumumab) [88], které lze indikovat pouze pacientům s wt-*NRAS* [30].

1.3.4 Maligní melanom

Maligní melanom je nejzávažnějším typem rakoviny kůže, vznikající proliferací melanocytů (neuroektodermový původ). Přestože maligní melanom reprezentuje pouze asi 4% nádorů kůže, je příčinou úmrtí v důsledku nádorového onemocnění kůže přibližně v 80%. Velmi špatná prognóza tohoto onemocnění byla v nedávné době zásadně zlepšena schválením TKI cílených na mutace v genu *BRAF* [90].

*BRAF* gen, lokalizovaný na 7q34, kóduje serin/threonin kinázu přenášející signál od proteinu RAS. Mutace *BRAF* bývá nalezena přibližně u 60% melanomů. Z více než 90% se jedná o substituci valinu za glutamát v kodónu 600 (V600E), která vede k aktivaci kinázové aktivity a přenosu mitogenního signálu [91]. Pacientům s neresekovatelným nebo metastazujícím melanomem s prokázanou mutací V600E lze indikovat v monoterapii BRAF inhibitory vemurafenib, dabrafenib a trametinib [30]. Vemurafenib inhibuje ERK kinázu a tím způsobuje programovanou buněčnou smrt pouze u buněk melanomu s V600E mutací. U melanomů bez V600E mutace se zdá, že vemurafenib paradoxně podporuje buněčný růst aktivací RAF1 [92].

U karcinomu kolorekta je mutace *BRAF* genu nalézána u 5-10% případů a je asociována s rezistencí na cetuximab/panitumumab. Vzhledem k mutační exkluzivitě s *KRAS* je testování *BRAF* genu doporučeno pacientům s wt-*KRAS* [30, 93].

1.3.5 Gastrointestinální stromální tumory

Gastrointestinální stromální tumory (GIST) jsou poměrně vzácné mezenchymální nádory trávicího traktu s nejčastější lokalizací v žaludku (50-60%) a tenkém střevě (20-40%). Tento typ sarkomu představuje asi 1% všech intestinálních nádorů [94, 95]. Až u 90% GIST případů je nalezena mutace v onkogenu *KIT*, který je lokalizován na 4q11. Tento gen kóduje transmembránový tyrozin-kinázový receptor pro SCF (stem cell factor), který je základem diagnostiky GIST, jelikož je SCF/CD117 exprimován u většiny GIST případů. Asi u 5% GIST se vyskytují mutace v genu *PDGFRA,* lokalizovaném na 4q12, který kóduje alfa podjednotku receptoru tyrozin-kinázy PDGF.

Pro léčbu *KIT* (*PDGFR*)-pozitivního inoperabilního nebo metastatického GIST lze indikovat imatinib, sunitinib nebo regorafenib, imatinib lze indikovat i v adjuvantní terapii. K průkazu KIT pozitivity se používá imunohistochemické stanovení exprese CD117 antigenu, případně molekulárně genetické vyšetření mutací *KIT* a *PDGFRA* genu. Mutace *KIT* se nejčastěji vyskytují v exonu 11 (~ 67%), méně často v exonech 9 (~ 10%), 13 (~ 1%) a 17 (~ 1%). Nádory s mutací v exonu 11 jsou nejvíce senzitivní k terapii imatinibem, nádory s mutací v exonu 9 jsou méně senzitivní, nicméně odpovídají na vyšší dávky imatinibu a na léčbu sunitinibem. Mutace *PDGFRA* genu se nejčastěji vyskytují v exonu 18,

raritně v exonech 12 a 14, přičemž mutace D842V v exonu 18 způsobuje rezistenci na imatinib a pacienti s touto mutací jsou vyřazeni z léčby TKI [30,94,96].

## 2. Experimentální část

### 2.1 Cíle práce

Předložená dizertační práce je zaměřena na prediktivní biomarkery u tří prognosticky nepříznivých skupin pacientů s karcinomem prsu, a to na pacienty s HER2-pozitivním karcinomem prsu, karcinomem prsu s polyzomií chromozomu 17 a pacienty s triple-negativním karcinomem prsu.

Cílem prvního projektu bylo navrhnout a optimalizovat metodiku vhodnou pro vyšetření počtu kopií genu *HER2* u pacientů, u nichž selhala *in situ* hybridizace. Tuto metodiku poté validovat na dostatečně velké kohortě pacientů s karcinomem prsu tak, aby byla použitelná v klinické praxi (kapitola 2.3.1) a navrhnutý prototyp vyšetřovacího kitu nabídnout ke komercionalizaci.

Cílem druhého projektu bylo objasnit výskyt polyzomie chromozomu 17 u pacientů s karcinomem prsu na základě vyšetření 6 markerů na chromozomu 17. Úkolem bylo nalezení vhodného markeru, který by byl použitelný pro rutinní diagnostiku a zhodnocení významu tohoto vyšetření pro diagnosticko-léčebnou rozvahu (kapitola 2.3.2).

Cílem poslední části práce bylo hledání biomarkerů, které by mohli predikovat účinnost adjuvantní chemoterapie u pacientek s triple-negativním karcinomem prsu. Práce byla zaměřena na stanovení exprese a počtu kopií jedenácti biomarkerů a jejich významu při predikci účinnosti adjuvantní antracyklinové terapie (kapitola 2.3.3).

## 2.2 Komentovaný úvod

Karcinom prsu je velmi heterogenní skupinou onemocnění na úrovni histologické, klinické, ale i biologické a genetické. I přes velkou heterogenitu jde o poměrně dobře léčitelné onemocnění, což dokazuje časový vývoj incidence a mortality, kdy i přes vzrůstající incidenci mortalita stagnuje. Pro lepší diagnostiku i úspěšnost screeningového programu svědčí fakt, že se vzrůstající incidencí vzrůstá současně i počet případů diagnostikovaných v raných stádiích I a II (~75%). Vzhledem k obrovské heterogenitě nádorů prsu je hlavním zájmem současné onkologie hledání specifických biomarkerů, které by pomohli objasnit prognózu či predikovat účinnost terapie u konkrétního pacienta. Velkým posunem v léčbě karcinomu prsu bylo zavedení vyšetření ER, PR a HER2 receptorů a na tyto receptory cílených terapeutik do klinické praxe. Řada dalších biomarkerů a cílených terapeutik je v současné době testována v klinických studiích.

Diagnostika HER2 pozitivity významně ovlivnila prognózu pacientů s HER2-pozitivním karcinomem prsu. Díky anti-HER2 terapii se z prognosticky velmi nepříznivé skupiny stala skupina pacientů s dobře léčitelným onemocněním. Kvalitní vyšetření HER2 receptoru je tedy naprosto esenciální pro selekci těch pacientů s karcinomem prsu, kteří mohou z léčby anti-HER2 inhibitory profitovat. Zlatým standardem je vyšetření pomocí imunohistochemie a *in situ* hybridizace. Tyto dvě metody jsou také doporučeny odbornými společnostmi ASCO/CAP. IHC, která detekuje expresi proteinu, je běžně používaná jako metoda screeningová. Případy, u nichž IHC poskytne nejednoznačný výsledek (IHC 2+), musí být retestovány pomocí ISH, která hodnotí *HER2* na genomické úrovni. Přestože anti-HER2 terapie cílí na buněčné úrovni HER2 protein, studie ukazují, že je vyšetření počtu kopií genu *HER2* lepším prediktorem léčebné odpovědi než vyšetření imunohistochemické. Přestože je amplifikace *HER2* jedinou prokázanou příčinou overexprese HER2 proteinu, byla diskrepance mezi IHC a FISH nalezena až u 20% případů. Diskrepance byla vysoká především při srovnání výsledků lokálních a centrálních laboratoří. Vysokou diskrepanci mezi centrální a lokální laboratoří potvrzují i výsledky srovnání našeho pracoviště s pracovišti lokálními, od kterých získáváme vzorky na verifikaci *HER2* statusu. HER2 diagnostikou by se měly jednoznačně zabývat pouze laboratoře s dostatečnou praxí a kvalifikací, jak je tomu i v České republice, kde je HER2 vyšetřováno v rámci sítě center prediktivní onkologie.

Falešně pozitivní/negativní IHC výsledky byly popsány především u vzorků se špatnou kvalitou způsobenou nesprávnou fixační procedurou či přítomností artefaktů. U vzorků se špatnou kvalitou dochází velmi často rovněž k selhání ISH. U ISH, na rozdíl od IHC, je díky chybějícímu signálu jednoznačně rozpoznatelné, že se jedná o nehodnotitelný vzorek. U IHC je naopak velmi obtížné rozlišit, že se jedná o arteficiální nepřítomnost barvení. Vzhledem k vysoké míře falešné pozitivity/negativity IHC a tomu, že se selhání ISH vyskytuje přibližně u 5% případů karcinomu prsu, je vhodné vyšetřit tyto pacienty nějakou alternativní metodou detekující počet kopií genu *HER2*.

Z tohoto důvodu byla zavedena a validována metoda založená na třech duplexních real-time PCR reakcích, detekující počet kopií genu *HER2* na základě srovnání se třemi kontrolními geny (*GCS1*, *DCK* a *EPN2*). Detekční limit metody byl experimentálně ověřen na diluční řadě buněčných linií, která potvrdila možnost detekce amplifikace *HER2* při minimálním zastoupení 5% vysoce amplifikovaných buněk. Metoda qPCR byla zvalidována na souboru 223 pacientů s karcinomem prsu, kde byla prokázána dostatečná senzitivita i specificita v porovnání s FISH i IHC (94,2%/95,1% a 100%/99,1%). Následně byla qPCR použita pro evaluaci *HER2* genu u 198/3696 vzorků, u nichž selhala FISH. Počet kopií genu *HER2* bylo možné vyšetřit téměř u 70% vzorků se špatnou kvalitou. Metoda qPCR je tedy dostatečně citlivou a specifickou metodou, kterou lze použít pro vyšetření genu *HER2* u vzorků, u nichž selhala *in situ* hybridizace a může tak u těchto vzorků doplnit výsledek imunohistochemie (kapitola 2.3.1).

Dalším problémem v diagnostice HER2 je polyzomie chromozomu 17, respektive zvýšený počet kopií centromerické oblasti chromozomu 17 (CEP17). Tato problematika je shrnuta v kapitole 1.2.3. Jak již bylo zmíněno, chromozom 17 je velmi často alterován a zmnožení signálu pro centromerickou oblast tohoto chromozomu bylo ve studiích popsáno až u 46% případů. Recentní studie ukázaly, že se nejedná o polyzomii chromozomu 17 jako takovou, nýbrž o zmnožení centromerické/ pericentromerické oblasti. Studie potvrdily, že je polyzomie chromozomu 17 pouze raritní událost, incidence pravé polyzomie či její prognostický či prediktivní význam však nebyl jednoznačně objasněn. Většina studií byla provedena pouze na relativně malém a často pre-selektovaném souboru pacientů. Ještě donedávna byl zvýšený počet kopií CEP17, u pacientů s nízkou amplifikací genu *HER2*, příčinou HER2 negativity (na základě negativního *HER2*/CEP17 poměru). Tito pacienti nebyli léčeni anti-HER2 terapií, přestože by z ní mohli, jak ukázaly retrospektivní studie, profitovat. Tento problém byl vyřešen v nových doporučeních ASCO/CAP, kde je u těchto případů pro enumeraci chromozomu 17 doporučeno použití alternativního markeru. V doporučeních však není specifikováno, která chromozomální oblast by měla být použita.

Pro nalezení vhodné enumerační sondy jsme testovali 3 oblasti na krátkém rameni chromozomu 17 (17p11.2, 17p12 a 17p13) u 67 pacientů s karcinomem prsu s počtem kopií CEP17≥2,5. Počet kopií sledovaných markerů významně klesal s rostoucí vzdáleností od centromery jako důsledek delece genu *p53*, částečně zasahující i do oblasti 17p12. Na základě tohoto zjištění byla, pro enumeraci chromozomu 17 u konsekutivního souboru 5477 pacientů s karcinomem prsu, vybrána sonda 17p11.2. Tato sonda byla použita pro potvrzení zvýšeného počtu kopií chromozomu 17 u 297 pacientů s CEP17≥3.0. Pomocí 17p11.2 byl zvýšený počet kopií centromerické oblasti chromozomu 17 potvrzen pouze u 67 případů. U tohoto pre-selektovaného souboru bylo celkově vyšetřeno 6 markerů, pokrývajících krátké i dlouhé rameno chromozomu 17. Pravá polyzomie chromozomu 17, respektive hyperdiploidie byla nalezena pouze u 0,48% případů. U těchto případů byl navíc nalezen významně

vyšší výskyt polyzomie chromozomu 8 a chromozomu 11. Na základě korekce počtu kopií chromozomu 17 pomocí sondy 17p11.2 bylo 21,6% (32/148) pacientů s původně negativním či hraničním výsledkem reklasifikováno do amplifikované kategorie a dostali tak možnost anti-HER2 léčby. Sonda 17p11.2 se ukázala jako vhodná pro enumeraci počtu kopií chromozomu 17 u pacientů s amplifikací CEP17 oblasti a může být použita pro HER2 diagnostiku karcinomu prsu. Pravá polyzomie chromozomu 17 byla nalezena u necelého půl procenta případů karcinomu prsu, prognostický či prediktivní význam této raritní aberace musí být objasněn (kapitola 2.3.2).

Poslední problematickou skupinou karcinomu prsu, kterou se zabývá předložená dizertační práce, je triple-negativní karcinom prsu. TNBC je nejvíce heterogenní skupinou karcinomu prsu s největším počtem somatických přestaveb. Vzhledem k negativitě ER, PR a HER2 receptorů a nedostatku cílené terapie je intenzivní výzkum zaměřen především na klasifikaci TNBC a identifikaci klinicky významných biomarkerů, které by mohly být terapeuticky ovlivněny. Tato problematika je shrnuta v kapitole 1.2.4. V současné době je hlavním problémem rezistence pacientů na konvenční adjuvantní chemoterapii a s tím související nedostatek biomarkerů, které by mohly účinnost terapie predikovat.
Ve spolupráci s Masarykovým onkologickým ústavem v Brně byl získán soubor 164 pacientek s TNBC, které byly léčeny adjuvantní chemoterapií založenou na antracyklinech. U těchto pacientek byla pomocí IHC vyšetřena exprese BCL2, EGFR, MYC, TOP2A a Ki-67 proteinů, počet kopií genů *EGFR*, *MYC*, *TOP2A* a chromozomů 7, 8 a 17 byl vyšetřen fluorescenční *in situ* hybridizací. Hodnocena byla doba přežití bez relapsu (RFS), breast cancer-specific survival (BCSS) a celkové přežití (OS). Vysoká exprese BCL2 proteinu byla nalezena jako statisticky významný prediktor špatné odpovědi na antracyklinovou terapii (RFS, BCSS), u OS se jednalo pouze o trend. V multivariační analýze byla navíc BCL2 exprese prokázána (společně s mírou postižení uzlin a velikostí nádoru) jako nezávislý prediktivní faktor účinnosti antracyklinové terapie (pro RFS, BCSS i OS). Podobné výsledky byly pozorovány i pro basal-like podskupinu TNBC. Z ostatních biomarkerů byla nalezena statisticky významná korelace zvýšené exprese EGFR s BCSS, u *MYC* amplifikace se jednalo pouze o trend (RFS a BCSS). U ostatních sledovaných biomarkerů nebyla nalezena žádná asociace s přežitím pacientek (RFS, BCSS ani OS), a to ani u genu *TOP2A*, který je považován za prediktor účinnosti antracyklinové terapie. Důvodem je pravděpodobně fakt, že je amplifikace genu *TOP2A* u TNBC velmi raritní (v naší studii pouze 2,1%) a počet pozitivních pacientek tak nebyl dostačující pro hodnocení prediktivního významu.
BCL2 exprese byla nalezena jako nezávislý prediktor přežití pacientek s TNBC (i basal-like TNBC) léčených adjuvantní antracyklinovou terapií. Výhodou BCL2 exprese jako biomarkeru je jeho snadná a levná stanovitelnost a jedná se tedy o biomarker, který je velmi vhodný pro použití v klinické praxi. Pro potvrzení prediktivní hodnoty tohoto biomarkeru a jeho zavedení do praxe je však zapotřebí velké prospektivní validační studie (kapitola 2.3.3).

## 2.3 Výsledky

### 2.3.1 Vyšetření genu *HER2* u pacientů s karcinomem prsu s nehodnotitelnou FISH

Evaluation of *HER2* gene status in breast cancer samples with indeterminate fluorescence *in situ* hybridization by quantitative real-time PCR method.

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# Evaluation of HER2 Gene Status in Breast Cancer Samples with Indeterminate Fluorescence *in Situ* Hybridization by Quantitative Real-Time PCR Method

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Administration of drugs targeting HER2 (official name *ERBB2*) is an important component of therapy for breast cancer patients with HER2 amplification/overexpression as determined by *in situ* hybridization (ISH) and immunohistochemistry (IHC). In approximately 5% of breast cancers, ISH assays fail. In these cases, HER2 protein expression is evaluated by IHC alone that may yield false negatives/positives for poor-quality samples. Therefore, we developed a method that was based on quantitative real-time PCR applicable for DNA from formalin-fixed, paraffin-embedded tissue samples. Its limit of detection was determined with breast cancer cell lines and validated with 223 breast cancer patient samples. On the basis of comparisons with fluorescent ISH (FISH) and IHC data, the sensitivity of the new method was 94.2% and 95.1%, its specificity was 100% and 99.1%, and overall concordance between results obtained with the quantitative real-time PCR method and FISH/IHC was 97.6% for both methods. The quantitative real-time PCR method was then used to evaluate the HER2 status of 198 of 3696 breast cancer tissues that yielded indeterminate FISH results. The HER2 copy number was successfully determined in 69.2% of these indeterminate samples. Thus, the DNA-based technique appears to be a specific, sensitive method for determining HER2 copy numbers when the FISH assay fails, which may complement IHC tests. *(J Mol Diagn 2015, ■: 1—10; http://dx.doi.org/10.1016/j.jmoldx.2015.03.007)*

The human epidermal growth factor receptor 2 gene (HER2, official name *ERBB2*) is located on chromosome 17q and amplified in 15% to 20% of breast cancer patients. HER2 is a prognostic biomarker associated with poor prognosis, early recurrence, and reduced progression-free survival.[1,2] HER2 expression is also a predictor of responses to drugs that target HER2 (including trastuzumab, lapatinib, and pertuzumab) that are currently approved by regulatory agencies for treating HER2-positive breast cancer patients.

Various methods can be used to determine the HER2 copy number or abundance of the corresponding protein in a tissue sample, including fluorescence (FISH), chromogenic, or silver *in situ* hybridization, immunohistochemistry (IHC), Southern or Western blot analysis, slot blot analysis, PCR, reverse-transcription PCR, and enzyme-linked immunosorbent assays.[3,4] IHC and ISH are widely accepted as the gold standards for evaluating HER2 status. IHC is the primary recommended screening method, with ISH being used to confirm IHC results. Patients with an IHC (HercepTest) score of 3+ are eligible for HER2-targeted therapy, whereas patients with 0 or 1+ scores do not overexpress HER2 and are therefore unsuitable for the


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**Table 1** Characteristics of HER2, GCS1, *DCK*, and *EPN2* Primers/Probes

| Gene | Primer/probe | DNA sequence | Product size (bp) |
|---|---|---|---|
| HER2 | Forward | 5′-AGAGTCACCAGCCTCTGCAT-3′ | 138 |
| | Reverse | 5′-GCAACTCCCAGCTTCACTTT-3′ | |
| | Probe | BHQ1-CTCCTTTTCACACTCTTGCCGACGTC-FAM | |
| GCS1 | Forward | 5′-CAGGTGACCCTGAAAATTCC-3′ | 133 |
| | Reverse | 5′-CTTCAGCATGGCTCTCCAG-3′ | |
| | Probe | BHQ1-AAATCAAGCCCTGCCAAGACTGGC-HEX | |
| *DCK* | Forward | 5′-CCGCCACAAGACTAAGGAAT-3′ | 109 |
| | Reverse | 5′-CGATGTTCCCTTCGATGGAG-3′ | |
| | Probe | BHQ1-AGAAGCTGCCCGTCTTTCTCAGCC-HEX | |
| *EPN2* | Forward | 5′-CCGCCACAAGACTAAGGAAT-3′ | 130 |
| | Reverse | 5′-CGATGTTCCCTTCGATGGAG-3′ | |
| | Probe | BHQ1-AGAAGCTGCCCGTCTTTCTCAGCC-HEX | |

treatment. Samples from patients with equivocal result (IHC 2+) must be retested for HER2 gene copy number by using the ISH assay.[2,5]

However, in approximately 5% of breast cancer samples, HER2 copy numbers cannot be evaluated with FISH because of poor tissue quality, which may be due to incorrect handling, especially in cases in which inappropriate fixation methods are used or degradation occurs.[6,7] Similarly, the use of different fixation methods can adversely affect IHC but not gene amplification and FISH results,[8] necessitating use of a DNA-based method to determine the HER2 gene copy number.

We therefore designed a novel method that is based on quantitative real-time PCR (qPCR) for determining HER2 gene copy numbers. The method involves three duplex qPCR amplifications to compare copy numbers of the HER2 gene with those of three reference genes: glucosidase I (GCS1; official name *MOGS*), deoxycytidine kinase (*DCK*), and epsin 2 (*EPN2*). These genes were selected because their copy numbers rarely change in breast cancers.[9]

## Materials and Methods

### Cell Lines

The CALU3 and MDA-MB-231 breast cancer cell lines used for determining the limit of detection (LOD) of qPCR method were purchased from ATCC (Rockville, MD). The HER2 gene is amplified and constitutively activated in the CALU3 cell line, which was used as a positive control. HER2 assays with the use of both FISH and IHC showed that cells of this line contained 20 copies of the HER2 gene per nucleus and had an IHC score of 3+. The MDA-MB-231 cell line was used as a negative control because it has a normal physiologic HER2 copy number (two copies) as determined by both FISH and IHC. CALU3 cells were diluted with MDA-MB-231 cells to create a dilution series with 1%, 5%, 10%, 15%, 20%, and 50% HER2-positive cell contents. Total DNA was extracted from samples of each suspension of the dilution series and analyzed by qPCR.

### Tissue Samples

A consecutive retrospective cohort of 181 formalin-fixed, paraffin-embedded (FFPE) invasive breast cancer samples collected in 2006 was used to assess the specificity and sensitivity of the qPCR method. For each sample, the HER2 gene copy number was determined by both FISH and qPCR, and the level of HER2 protein expression was determined by IHC. Samples with at least 10% of tumor tissue and both FISH and IHC data were included to the study. The consecutive retrospective cohort was further enriched for 60 samples with equivocal IHC result (2+) and complete FISH data collected in 2007. The final validation set consisted of 223 samples. The validation set was used for all statistical analyses.

Then, qPCR and IHC methods were used in parallel to analyze HER2 expression/copy number in 198 of 3696 patient samples prospectively collected between 2007 and 2012 that yielded indeterminate FISH results. The percentage of HER2-positive tumors in our study is much higher than other institutions and the literature. The reason is that mainly positive samples are sent from local laboratories to us for confirmatory testing, because our institution serves as a central/reference laboratory.

### IHC

HER2 protein was immunohistochemically detected in 4-μm FFPE sections by using the US Food and Drug Administration-approved HercepTest (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. The standard HercepTest scoring system was used in all cases to obtain scores of 0, 1+, 2+, or 3+, indicating no staining or incomplete faint membrane staining of $<10\%$ of tumor cells, incomplete faint membrane staining of $\geq 10\%$ of tumor cells, incomplete and/or moderate membrane staining of $\geq 10\%$ of tumor cells, and complete intense membrane staining in $\geq 10\%$ of tumor cells, respectively.[2]

### FISH

FFPE sections (4 μm) were baked overnight at 56°C on microscope slides, deparaffinized with xylene, dehydrated with


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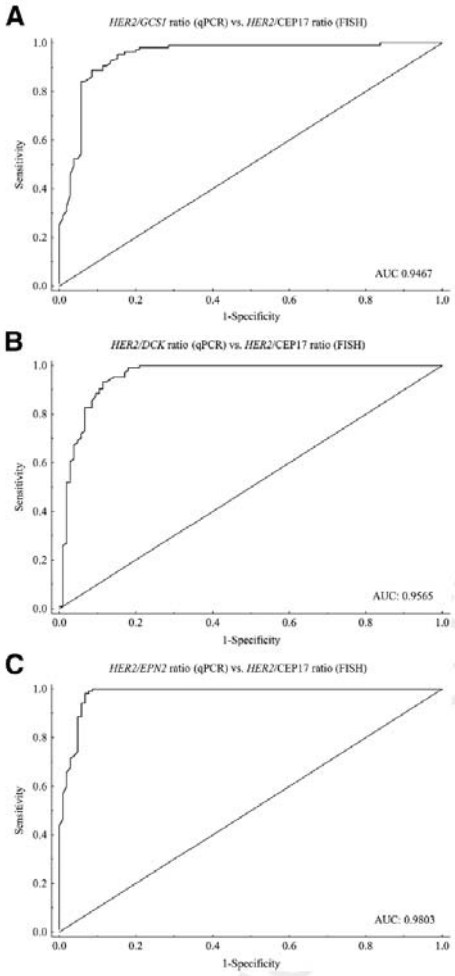



**Figure 1** ROC curves for HER2/reference gene ratios (qPCR) compared with gold standard FISH (HER2/CEP17 ratio) in the validation set. Shown are the ROC curves for the three reference genes separately: GCS1 (**A**), *DCK* (**B**), and *EPN2* (**C**). AUC, area under curve; CEP17, chromosome enumeration probe 17; FISH, fluorescence *in situ* hybridization; qPCR, quantitative real-time PCR; ROC, receiver operating characteristic.

ethanol, then chemically and enzymatically treated and co-denatured (2 minutes, 85°C) by using the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, IL) or Her-2/neu FISH Kit (IntellMed, Olomouc, Czech Republic), approved by the US Food and Drug Administration and/or Conformité Européenne, *In Vitro* Diagnostics. The sections were then incubated overnight at 37°C. Unannealed probe molecules were subsequently removed by washing the sections with 0.4× saline-sodium citrate followed by a 2× saline-sodium citrate solution that contained 0.1% Nonidet-P40. The samples' nuclei were then counterstained with DAPI.

HER2 and chromosome 17 signals were counted in 100 non-overlapping nuclei in each patient sample by using a fluorescent microscope (Olympus BX-51; Olympus America, Center Valley, PA). HER2 clusters of ≥20 signals were scored as 20 HER2 copies. The mean HER2/chromosome 17 ratio was calculated for each sample, using the scoring criteria recommended by the American Society of Clinical Oncologists and the College of American Pathologists. Samples were classified as amplified if the ratio was ≥2.0 and/or the mean HER2 copy number was ≥6, equivocal if the ratio was <2.0 and the mean HER2 copy number was 4 to 6, and negative if the ratio was <2.0 and the mean HER2 copy number was <4.

## DNA Extraction and qPCR

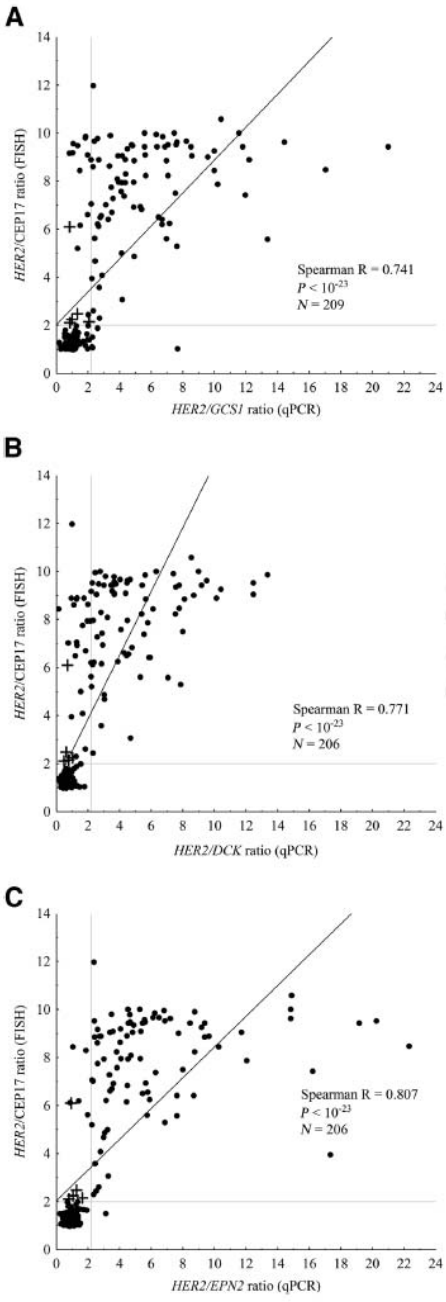
DNA was extracted from four 4-μm FFPE sections from each patient sample or from a million cells from each suspension of the CALU3/MDA-MB-231 cell line concentration series, using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. FFPE sections were deparaffinized with a routine series of xylene and ethanol washes before DNA extraction.

PCR was performed with 50 ng of total DNA and ThermoStart DNA polymerase (ThermoScientific, Waltham, MA) with PCR primers and probes listed in Table 1. PCR conditions consisted of 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

DNA isolated from FFPE normal (noncancerous) breast tissue samples that had physiologic HER2 gene copy number and showed no evidence of HER2 amplification and expression (as determined by FISH and the HercepTest) was used as a standard. The standard DNA was also tested by qPCR for no evidence of copy number changes of control genes (GCS1, *DCK*, and *EPN2*). From the standard DNA, a dilution series of 20, 30, 40, 50, 70, and 100 ng/reaction mixture was prepared to produce standard curves by plotting DNA concentrations against cycle threshold values. Relative gene copy numbers of HER2 and the reference genes were calculated from the standard curves, assuming that normal cells have two copies of the gene and approximately 7 pg of DNA.[10] The results obtained were expressed as ratios of the HER2 copy numbers to those of the reference genes. A cutoff value of 2.2 for amplification was selected on the basis of receiver operating characteristic curve analysis (Figure 1), and the HER2 gene was considered to be amplified in samples in which the ratio of the HER2 copy number to that of at least two of the three references gene exceeded this threshold.

## Statistical Analysis

Receiver operating characteristic curves were generated from data of validation set and to compare HER2 copy




number detected by qPCR with gold standard FISH (HER2/chromosome enumeration probe 17 ratio) (Statistica 12.0; StatSoft, Inc., Tulsa, OK). Cutoff values were calculated with receiver operating characteristic curve analysis (software R, package OptimalCutpoints) as points with 85% sensitivity. We selected cutoff 2.2 as the mean for all three reactions.[11] Nonparametric methods were used to analyze HER2 gene copy number data obtained from the qPCR and FISH experiments and to compare the HER2 gene/protein levels determined by qPCR and IHC. Spearman rank coefficients were applied to measure correlations between qPCR and FISH. These values as well as the *P* values of the significance test are presented in scatterplots (Figure 2). Post hoc multiple comparisons of mean ranks for all groups were performed, and *P* values (two-sided significance levels with a Bonferroni adjustment) associated with each comparison were calculated for significance by Kruskal-Wallis tests (Statistica 12.0; StatSoft, Inc.). Sensitivity, specificity, concordance, and Cohen κ values were calculated for data from all qPCR assays, using the FISH and IHC results as references (R Development Core Team, *http://www.r-project.org*, last accessed February 6, 2015). IHC score 3+ was considered positive indicator of HER2 expression, whereas scores of 0 and 1+ were considered negative indicators in all statistical analyses. IHC score 2+ was considered positive indicator of HER2 expression if the FISH was positive only.

## Results

### Detection Limit of the qPCR Method

The LOD for the method was determined with the CALU3/MDA-MB-231 cell line dilution series. The HER2 gene copy numbers in six samples with different amplified/nonamplified DNA contents were analyzed relative to those for the three reference genes by using qPCR. Six replicates were run for each reference gene. The qPCR method reliably detected HER2 gene amplification in samples that contained approximately 5% of strongly positive cells (Table 2).

### Sensitivity and Specificity Assessments of the qPCR Assay versus FISH and IHC Methods in the Validation Set

To validate the cell line data obtained with the novel qPCR method, a cohort of 223 invasive breast cancer samples (Table 3) was selected and used to assess its sensitivity and

**Figure 2** Correlation of HER2/reference gene ratios (qPCR) and HER2/CEP17 ratio (FISH) in the validation set. Scatterplots with regression line (orthogonal fit – total least squares regression) and reference lines for cutoffs ($x = 2.2$, $y = 2.0$) are shown for the three reference genes separately: GCS1 (**A**), *DCK* (**B**), and *EPN2* (**C**); $P < 10^{-23}$. Five false-negative samples (qPCR negative/FISH positive) are marked with a cross. CEP17, chromosome enumeration probe 17; FISH, fluorescence *in situ* hybridization; qPCR, quantitative real-time PCR.

**Table 2** Evaluation of the Detection Limit of the qPCR Method

| HER2 amplified cells (%)* | HER2/GCS1 gene ratio (95% CI)† | HER2/*DCK* gene ratio (95% CI)† | HER2/*EPN2* gene ratio (95% CI)† |
|---|---|---|---|
| 1 | 1.23 (1.118–1.342) | 0.78 (0.652–0.900) | 1.52 (1.220–1.829) |
| 5 | 2.43 (2.167–2.686) | 2.01 (1.880–2.133) | 4.83 (4.116–5.550) |
| 10 | 5.12 (4.096–6.149) | 6.06 (5.396–6.730) | 14.32 (12.994–15.638) |
| 15 | 5.03 (4.283–5.781) | 5.23 (4.939–5.515) | 13.56 (12.605–14.516) |
| 20 | 8.69 (7.766–9.609) | 12.25 (11.659–12.851) | 24.23 (22.570–25.892) |
| 50 | 16.94 (15.366–18.524) | 30.05 (27.198–32.902) | 58.99 (55.153–62.824) |

*The percentage of HER2-amplified cells refers to the percentage of CALU3 cells (>20 HER2 copies/nucleus) in suspensions of the dilution series.
†Average value from six replicates.
qPCR, quantitative real-time PCR.

specificity. The HER2 gene copy number and protein expression in all of these samples were measured in parallel by using FISH, IHC, and qPCR. HER2/GCS1, HER2/*DCK*, and HER2/*EPN2* products were successfully amplified in 212, 209, and 209 of the 223 samples, giving amplification successes of 95.1%, 93.7%, and 93.7%, respectively.

The amplification success/conformity (frequency of the identical positive/negative result) of HER2 status determined with the following combinations of reference genes HER2/GCS1 + *DCK*, HER2/GCS1 + *EPN2*, HER2/*DCK* + *EPN2*, and HER2/GCS1 + *DCK* + *EPN2* were 92.8% (207 of 223)/76.7% (171 of 223), 92.8% (207 of 223)/84.3% (188 of 223), 92.4% (206 of 223)/82.1% (183 of 223), and 91.9% (205 of 223)/74.4% (166 of 223), respectively. With the use of a single gene, we were able to analyze from 93.7% to 95.1% samples with sensitivity from 74.3% to 90.4%. However, the sensitivity improved substantially (91.7% to 93.2%) by using the combination of two reference genes with analyzable samples range from 76.7% to 84.3%. With the use of the combination of all three reference genes, the sensitivity was not superior to combinations of two genes (91.2%), nonetheless the number of analyzable samples decreased to 74.4% only.

On the basis of these data, we have established threshold criterion for qPCR HER2 positivity as a HER2/reference gene copy ratio ≥2.2 for at least two reference genes, to compromise high sensitivity of the qPCR assay with reasonable percentage of evaluable tissue samples. With the use of this criterion, we were able to determine the HER2 gene status of 210 of 223 samples (94.2%) from the validation set with sensitivity 94.2% and high concordance with FISH/IHC results (97.6%/97.6%). Sensitivity, specificity, and κ values for all combinations are summarized in Table 4.

### Comparison of FISH and qPCR Results

FISH data indicated that 102 of 223 patient samples (45.7%) were HER2 negative, 95 (93.1%) of which also gave negative qPCR results, whereas results for the other 7 samples (6.9%) were indeterminate because of qPCR amplification failure. Thus, all HER2 FISH-negative samples gave negative (or indeterminate) results in the qPCR analysis. FISH equivocal result was found in 12 cases (5.4%). Eleven cases (91.7%) were qPCR negative; one sample (8.3%) was indeterminate. Of the 223 patient samples tested, 109 (48.9%) were FISH positive and 98 of these 109 (89.9%) were also found to be positive by the qPCR analysis, whereas the qPCR results were indeterminate for six samples (5.5%), again because of amplification failure. Negative results were obtained from the qPCR method for 5 of the 109 FISH-positive samples (4.6%) (Table 5). In two of these false-negative cases, samples were heterogenic and yielded low amplification levels with three to eight gene copies per nucleus (HER2/chromosome 17 ratios were 2.11 and 2.25), together with strong complete immunohistochemical membrane staining in 10% and 15% of cells (IHC 3+), respectively.

Similarly, the other two false-negative samples yielded low amplification level with HER2/chromosome 17 ratio 2.15 and 2.48 and IHC score 1+ and 2+. In these borderline cases, the amplification levels were below the qPCR method's LOD. High-level amplification with 15 HER2 copies per nucleus, together with moderate membrane staining in 50% of tumor cells (IHC 2+) was found in the last false-negative case. qPCR method detected physiologic copy number of HER2 gene in all of the three qPCR reactions. The most probable reason for the qPCR failure could be the borderline percentage of tumor cells in the specimen (approximately 10%), leading to critical dilution of tumor DNA.

**Table 3** Histopathology of 223 Invasive Breast Cancer Samples Used to Validate the qPCR Method

| | | IHC (HercepTest), *n* | | | FISH, *n* | | |
|---|---|---|---|---|---|---|---|
| Tumor type | *N* (%) | 0/1+ | 2+ | 3+ | Nonamplified | Equivocal | Amplified |
| Ductal | 203 (91.0) | 51 | 64 | 88 | 84 | 12 | 107 |
| Lobular | 16 (7.2) | 14 | 1 | 1 | 15 | 1 | 0 |
| Mixed ductal/lobular | 4 (1.8) | 3 | 0 | 1 | 3 | 0 | 1 |

FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; qPCR, quantitative real-time PCR.

**Table 4** Specificity and Sensitivity of Duplex qPCR Amplifications of HER2 with GCS1, *DCK*, *EPN2*, and Their Combinations as Reference Genes, in Samples of the Validation Set

| Methods | Genes (qPCR) | Specificity | Sensitivity | κ (95% CI) | Analyzable samples, *N* |
|---|---|---|---|---|---|
| qPCR vs FISH | HER2/GCS1 | 0.963 | 0.829 | 0.792 (0.711–0.874) | 212 |
| | HER2/*DCK* | 1.000 | 0.743 | 0.742 (0.654–0.830) | 209 |
| | HER2/*EPN2* | 1.000 | 0.904 | 0.904 (0.847–0.962) | 209 |
| | HER2/GCS1 + HER2/*DCK* | 1.000 | 0.917 | 0.927 (0.870–0.984) | 171 |
| | HER2/GCS1 + HER2/*EPN2* | 1.000 | 0.932 | 0.936 (0.885–0.986) | 188 |
| | HER2/*DCK* + HER2/*EPN2* | 1.000 | 0.925 | 0.933 (0.880–0.986) | 183 |
| | HER2/GCS1 + HER2/*DCK* + HER2/*EPN2* | 1.000 | 0.912 | 0.924 (0.865–0.984) | 166 |
| | Min. 2 genes | 1.000 | 0.942 | 0.943 (0.898–0.988) | 210 |
| qPCR vs IHC | HER2/GCS1 | 0.963 | 0.845 | 0.811 (0.732–0.889) | 212 |
| | HER2/*DCK* | 0.991 | 0.748 | 0.741 (0.652–0.830) | 209 |
| | HER2/*EPN2* | 0.991 | 0.912 | 0.904 (0.846–0.962) | 209 |
| | HER2/GCS1 + HER2/*DCK* | 1.000 | 0.930 | 0.939 (0.887–0.992) | 171 |
| | HER2/GCS1 + HER2/*EPN2* | 1.000 | 0.943 | 0.946 (0.900–0.993) | 188 |
| | HER2/*DCK* + HER2/*EPN2* | 0.990 | 0.936 | 0.933 (0.879–0.986) | 183 |
| | HER2/GCS1 + HER2/*DCK* + HER2/*EPN2* | 1.000 | 0.925 | 0.937 (0.882–0.991) | 166 |
| | Min. 2 genes | 0.991 | 0.951 | 0.943 (0.898–0.988) | 210 |
| IHC vs FISH | | 1.000 | 0.982 | 0.982 (0.957–1.007) | 223 |

FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; Min. 2 genes, the criterion for amplification (a ratio of 2.2 in at least two of the three reference genes); qPCR, quantitative real-time PCR.

Although qPCR assessments for 5 of the 223 samples were not correct (according to FISH data), a highly significant positive correlation was found between the FISH results and all three duplex qPCR analyses ($P < 10^{-23}$). They all effectively detected elevated HER2 copy numbers, and the Spearman correlation coefficients for analyses by using GCS1, *DCK*, and *EPN2* as the reference genes were 0.74, 0.77, and 0.81, respectively (Figure 2). All of the qPCR analyses exhibited similar, high levels of specificity (96.3%, 100%, and 100%) and sensitivity (82.9%, 74.3%, and 90.4%). The overall sensitivity and specificity for the qPCR assay were 94.2% and 100%, respectively. The overall concordance between the qPCR and FISH results was 97.6%, with a κ value of 0.943 (95% CI, 0.898–0.988) (Table 4).

### Comparison of IHC versus Both DNA-based Methods (qPCR and FISH)

We also compared the performance (relative to IHC) of the IHC and FISH/qPCR methods, which assess HER2 status by using different principles. IHC gave negative indications (HercepTest scores of 0 or 1+) for 68 of 223 tested samples (30.5%). Of these 68, 59 (86.8%) were also FISH and qPCR negative, two (2.9%) were FISH equivocal and qPCR negative, five (3.0%) were FISH negative but qPCR indeterminate. One sample (1.5%) was qPCR negative and FISH positive. The qPCR results indicated that the remaining one IHC-negative sample (1.5%) was qPCR positive, and correspondingly strong HER2 amplification (>20 copies/nucleus) was found for this sample by using FISH analysis.

Strong IHC positivity (3+) was detected in 90 of all tested samples (55.2%). Both the FISH and qPCR methods gave positive results for 85 of these 90 samples (94.4%); qPCR failed for three FISH-positive samples (3.3%). Two of the IHC-positive samples (2.2%) were judged to be HER2 negative on the basis of the qPCR analysis alone. FISH positivity was detected in both samples. These samples were heterogeneous (see above). An equivocal IHC 2+ score was obtained for 65 samples (29.1%). Of these 65 IHC 2+ specimens, 36 (55.4%) were both FISH and qPCR negative, 9 (13.8%) were FISH equivocal/qPCR negative, and 12 (18.5%) were both FISH and qPCR positive. qPCR failed for three FISH-positive (4.6%), one FISH-equivocal (1.5%), and two FISH-negative (3.1%) samples. FISH positivity/qPCR negativity was found in two IHC 2+ cases (3.1%) (Table 5). One sample was heterogeneous; tumor DNA was probably diluted in the second false-negative case (see above).

Significant correlations were observed among all three qPCR ratios and the immunohistochemical scores ($P < 10^{-7}$)

**Table 5** HER2 Status of the 223 Breast Cancer Samples in the Validation Set

| | | qPCR | | |
|---|---|---|---|---|
| FISH | IHC | Positive | Negative | Indeterminate |
| Positive ($N = 109$) | 3+ | 85 | 2* | 3 |
| | 2+ | 12 | 2*† | 3 |
| | 0/1+ | 1 | 1* | 0 |
| Equivocal ($N = 12$) | 3+ | 0 | 0 | 0 |
| | 2+ | 0 | 9 | 1 |
| | 0/1+ | 0 | 2 | 0 |
| Negative ($N = 102$) | 3+ | 0 | 0 | 0 |
| | 2+ | 0 | 36 | 2 |
| | 0/1+ | 0 | 59 | 5 |

*False negative, heterogeneous sample.
†False negative, borderline percentage of tumor cells in the specimen.
FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; qPCR, quantitative real-time PCR.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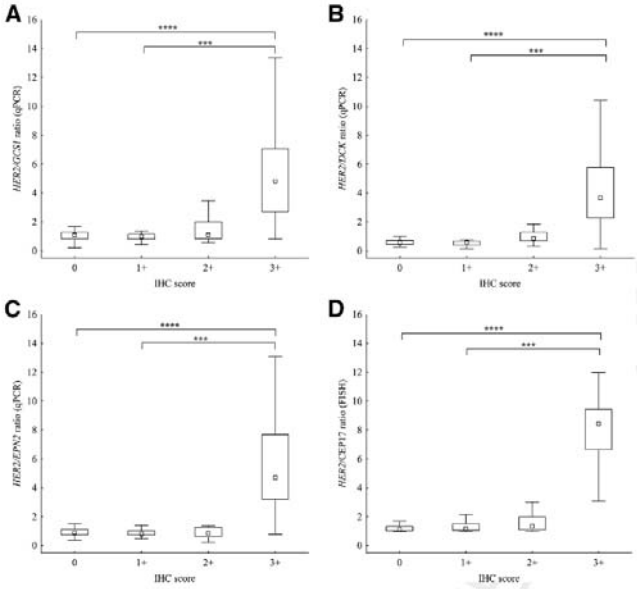


**Figure 3** Box plots show the distribution of the HER2 copy number detected by qPCR and FISH according to the immunohistochemical score in the validation set. Comparisons are shown for the HER2/reference gene ratios: GCS1 (**A**), *DCK* (**B**), and *EPN2* (**C**) detected by qPCR and HER2/CEP17 ratio (FISH) (**D**). Rectangle boxes indicate the 25% to 75% percentiles, small inner squares represent the median, whiskers show non-outlier range. Statistical differences between the pairs of groups (according to multiple comparisons) are indicated by asterisks; ***$P < 10^{-7}$ and ****$P < 10^{-23}$. CEP17, chromosome enumeration probe 17; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; qPCR, quantitative real-time PCR.

(Figure 3). All of the qPCR reactions exhibited similar, high levels of specificity (96.3%, 99.1%, and 99.1%) and sensitivity (84.5%, 74.8%, and 91.2%). The overall levels of sensitivity and specificity for the qPCR assay were 95.1% and 99.1%, respectively. The overall concordance between the FISH/qPCR and IHC results was 97.6%/97.6%, with a $\kappa$ value of 0.982/0.943 (the 95% CI for the $\kappa$ value was 0.957/0.898 to 1.007/0.988). No significant difference in concordance was found with the IHC data between the qPCR and FISH results (Table 4).

## HER2 Quantification in Poor-Quality Samples

A total of 3867 breast cancer tissue samples were prospectively collected between 2007 and 2012 for reference HER2-FISH testing in a central laboratory (joint facility of the Institute of Molecular and Translational Medicine and the Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic) to confirm their HER2 status (Figure 4). Of these specimens, 171 (4.4%) that were unsuitable for the analysis because of the absence of tumor cells in sections were excluded from the study. The HER2 gene and chromosome 17 copy numbers in the remaining 3696 samples were then determined by FISH. The FISH analyses were successful for 3498 of these specimens (94.6%); amplification was detected in 1523 (43.5%) of them, 159 (4.6%) were considered equivocal and 1816 (51.9%) gave negative results. High frequency of HER2-amplified breast cancers in the cohort was due to enrichment for positive tumors sent from local laboratories for confirmatory testing to the central laboratory. One hundred ninety-eight specimens (5.4%) could not be evaluated by FISH because of poor sample quality.

The qPCR method that used three reference genes was applied to determine the HER2 gene status of the 198 poor-quality tissue samples that yielded indeterminate FISH results. The qPCR amplification failed for 61 of 198 samples (30.8%) but was successful for the other 137 samples (69.2%). Among the 137 successfully amplified samples, 107 (78.1%) were found to have physiologic HER2 gene copy numbers. Of these 107, 72 (67.3%) were also IHC negative,

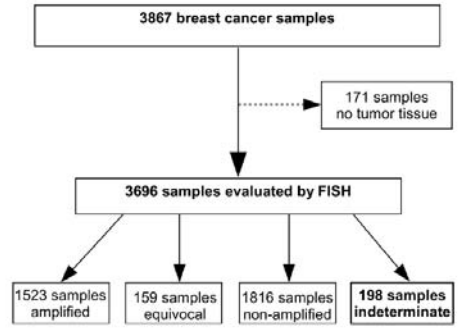


**Figure 4** Consort diagram of samples analyzed in the study. FISH, fluorescence *in situ* hybridization.


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**Table 6** HER2 Status of the 198 Poor-Quality Breast Cancer Samples in the Prospective Cohort

| | | IHC (HercepTest) | | | |
|---|---|---|---|---|---|
| HER2 status | *N* (%) | 3+ | 2+ | 0/1+ | Indeterminate |
| qPCR | | | | | |
| Positive | 30 (15.2) | 12 | 6 | 12 | 0 |
| Negative | 107 (54) | 16 | 17 | 72 | 2 |
| Indeterminate | 61 (30.8) | 6 | 16 | 36 | 3 |

IHC, immunohistochemistry; qPCR, quantitative real-time PCR.

17 (15.9%) were equivocal (IHC 2+), whereas the other 16 (15.0%) were IHC positive. IHC failed in two samples (1.9%). qPCR positivity was detected in 30 cases (21.9%) of which 12 (40%) also gave positive IHC result, 6 samples (20%) gave equivocal IHC result, whereas the other 12 samples (40%) were IHC negative. The results of qPCR and IHC differ in 28 of 137 cases (20.4%) (Table 6).

## Discussion

The assessment of HER2 status is fundamentally important for selecting therapies for breast cancer patients, because anti-HER2 agents significantly increase survival rates in both palliative and adjuvant settings. However, the therapeutic effect is limited to patients with tumors that overexpress or amplify the HER2 gene. Thus, assessment of HER2 status may serve as a paradigm for the role of laboratory medicine in the multidisciplinary management of cancer patients.[12]

The ISH and IHC assays are recommended by the American Society of Clinical Oncologists and the College of American Pathologists.[2,5] These methods are highly concordant because gene amplification is the most common mechanism of HER2 overexpression; to date, no strong evidence for other mechanisms of HER2 overexpression in breast cancer is presented.[8,13] However, the efficacy of trastuzumab therapy is linked to FISH HER2 positivity rather than IHC positivity,[14] and discrepancies between HER2 test results obtained with FISH and IHC are observed. Between 5% and 22% of all IHC 3+ breast cancers do not reportedly exhibit HER2 amplification, whereas between 2% and 11.5% of all IHC 0/1+ breast cancers show HER2 amplification.[8,14–17] False-positive or -negative IHC results are particularly common in samples with poor tissue quality.[7,18] False-positive IHC results arising from nonspecific antibody binding can occur in tissues with crush artifacts (needle biopsy specimens), tissue borders, or cautery artifacts.[8] False-negative IHC results can arise from delayed fixation and the use of inappropriate fixatives. In poor-quality samples, tissue damage that causes indeterminate FISH results also reduces the likelihood of IHC positivity.[8,19,20] Unlike the FISH assay, in which poor-quality samples can be readily identified because of the lack of a hybridization signal, it is often not possible to detect an artificial lack of staining because of poor sample quality when evaluating IHC results. This creates risks of obtaining false-negative results,[7] which may have serious consequences because a patient who could benefit from anti-HER2 treatment will not receive it.

Here, FISH failed in 5.4% of the tested specimens, similar to the rate reported in the literature.[8] Delays between sample resection and fixation lead to poor nuclear resolution, vague cellular outlines, and weak, nonuniform signals, thereby seriously compromising the quality of samples used in HER2 FISH assessments,[6] which are therefore evaluated with IHC alone. Thus, the FISH failures in the present study were probably because of poor sample quality arising from either inappropriate handling before fixation or use of an inappropriate fixation method. However, given the high rate of false positives and negatives observed with IHC, a robust alternative DNA-based method would be valuable for confirming HER2 copy numbers in samples that cannot be analyzed by FISH.

Therefore, we developed a method that can be applied to determine HER2 gene copy numbers in FFPE samples, using three duplex qPCR reactions in which the HER2 copy number are compared with those of three reference genes located on chromosomes 2, 4, and 17: GCS1, *DCK*, and *EPN2*, respectively. These reference genes were selected because their copy numbers rarely change in breast cancer. Both the sensitivity and $\kappa$ values increased with combinations of reference genes (Table 4). The use of three independent reference genes also decreased rates of false positives and negatives. The amplification success for each duplex reaction ranged from 93.7% to 95.1%. These results are wholly consistent with those presented in the literature, because the amplification efficacy for products of this length reportedly ranges from 69% to 100%.[21–23] However, the amplification success decreased with the number of parallel-evaluated genes. To maintain high specificity, sensitivity, and performance (amplification success) of the qPCR method, we finally evaluated samples as HER2 amplified if HER2-to-reference gene copy number ratios were $\geq$2.2 for at least two of the reference genes.

Surprisingly, although qPCR techniques are often used to detect HER2 mRNA,[24–26] DNA-based PCR is not widely used to determine HER2 gene status, except by the LightCycler HER2/neu DNA Quantification kit (Roche, Mannheim, Germany).[23,27–30] However, this kit only uses a single reference gene (not specified) located on chromosome 17, for which copy numbers frequently change in human cancers,[31] and use of a single reference gene can clearly impair the reliability of HER2 analysis and amplification performance.

Our qPCR method proved to be highly sensitive and specific on the basis of comparisons with FISH data (94.2% and 100%, respectively) and IHC data (95.1% and 99.1%, respectively) obtained for the validation set. The overall concordance of the FISH and qPCR results was 97.6%, and it was higher than levels reported for the LightCycler HER2/neu DNA Quantification Kit, which range from 80% to 92%.[27,28,30] The overall concordance of the qPCR and IHC results was 97.6%, also much higher than rates (80% to

91%) reported in previous studies.[27,28] We hypothesize that the high concordance of our qPCR method is due to the use of three (rather than one) more appropriate reference genes. Furthermore, in contrast to the FISH results, the qPCR analysis yielded no false-positive results and five false-negative results. In four of these cases, the amplification levels were probably below the method's LOD. The likely causes of failure were either a dilution effect that resulted from the presence of other tissue elements or tissue necrosis. The use of laser microdissection to isolate specific regions of interest within a sample may be beneficial in such cases.[23] The qPCR failed in the last false-negative case. Although there was high-level HER2 amplification detected by FISH and moderate membrane staining in 50% of tumor cells (IHC 2+), the qPCR assay was unsuccessful, most probably because of borderline percentage of tumor cells in the specimen.

Forty-nine samples were found to be qPCR negative but IHC positive. However, 47 of these were only equivocal IHC 2+, and no HER2 amplification is observed relatively often in such samples; the concordance between IHC and FISH results in 2+ cases ranges from 12% to 48%.[15,32–35] In two qPCR-negative/IHC 3+-positive samples the amplification levels were probably below the method's LOD as described above. One sample was found to be qPCR positive and IHC negative, but HER2 amplification was confirmed by FISH analysis in this sample, indicating that the immunohistochemical result was a false negative.

We then used our qPCR method to evaluate the HER2 copy number in 198 of 3696 tissue samples, obtained in a prospective study, for which FISH analysis failed. We were able to determine the HER2 gene status in 137 (69.2%) of these 198 samples. When the HER2 gene and protein detection by qPCR versus IHC were compared, the data differed in 20.4% cases (28 of 137). qPCR result was negative for HER2 amplification in at least 47% of the IHC 3+ tumors (16 of 34 cases). Conversely, 12 of 120 (10%) IHC 0/1+ tumors were qPCR positive. Despite the high disagreement with commonly used IHC, we do not assume that the problem is the qPCR method, considering the high concordance of qPCR with both IHC/FISH in the validation cohort. Most likely, the performance of the IHC was heavily impaired because of a high level of sample degradation that resulted in disruption of antigenic epitopes to cause false negativity, or a nonspecific antibody binding to damaged tissues to cause false positivity.[8] Unfortunately, we are not able to evaluate the results of qPCR in discrepant samples by other independent DNA technique because of FISH analysis failure. Nevertheless, we found six discordant/equivocal patients who were successfully tested by FISH with the use of different tissue block. Interestingly, five patients were both FISH/qPCR negative (three patients IHC 2+, two patients IHC 3+) and one was FISH/qPCR positive (IHC 2+). Although we were not able to prove directly the HER2 gene status in all qPCR versus IHC nonconforming samples, the data obtained from six patients having non-degraded parallel biopsies available suggest reliability of the qPCR method. Because no data obtained with the LightCycler HER2/neu DNA Quantification Kit for poor-quality samples are available, the presented qPCR method is the only technique reported to date that is capable of determining HER2 gene copy numbers in samples in which FISH fails.

Given the high concordance between our triple duplex qPCR technique and currently gold standard techniques, together with its high specificity and sensitivity demonstrated in tests with the validation set, we believe it is a promising method for determining HER2 gene copy numbers, especially in samples in which FISH fails. The main disadvantage of our method is its inability to distinguish HER2 status between invasive versus noninvasive components of tumors. The problem could be also tumor heterogeneity, which is, however, relevant also for ISH and IHC. Unlike the qPCR, the ISH and IHC techniques are capable to visualize molecular status in the context of tissue architecture, percentage of malignant cells, and presence of necrosis and to identify even small proportions of amplified cells. Samples analyzed by qPCR can fail in these circumstances. However, this is a relatively rare problem that can be easily resolved by tissue dissection. However, ISH and IHC are known to be affected by human factor and prone to subjective evaluation.

In summary, ISH is a gold standard technique that is generally capable of evaluating HER2 gene status in tumor architecture contexts. The triple duplex qPCR method presented here proved to be a highly concordant, specific, and sensitive tool for determining HER2 copy numbers in FFPE samples and could be recommended as an alternative DNA-based technique for samples in which the ISH fails.

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### 2.3.2 Četnost polyzomie chromozomu 17 u karcinomu prsu

Frequency of chromosome 17 polysomy in relation to CEP17 copy number in a large breast cancer cohort.
(draft manuskriptu, submitováno do časopisu Genes, Chromosomes and Cancer)

**Frequency of chromosome 17 polysomy in relation to CEP17 copy number in a large breast cancer cohort**

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**Abbreviated title**

Chromosome 17 polysomy in large breast cancer cohort

**Abstract**

Eligibility to anti-HER2 therapy for breast tumors strictly depends on demonstrating HER2 overexpression (by immunohistochemistry) or *HER2* gene amplification by *in situ* hybridization (ISH), usually defined by the ratio of *HER2* gene to chromosome 17 centromere (CEP17) copies. However, the CEP17 copy number increase (CNI) has been proven responsible for misleading *HER2* FISH results and recent small cohort studies suggest that chromosome 17 polysomy is actually very rare. Here we investigated by FISH the frequency of true chromosome 17 polysomy in a consecutive cohort of 5477 invasive breast cancer patients. We evaluated and selected the LSI 17p11.2 probe for chromosome 17 enumeration on a training cohort of 67 breast cancer samples (CEP17≥2.5). LSI 17p11.2 was used in the 297/5477 patients from the validation cohort displaying CEP17 CNI (CEP17≥3.0). Using *HER2*/17p11.2 scoring criteria, 37.3%/1.5% patients initially classified as equivocal/non-amplified were reclassified as amplified. For a more accurate assessment of chromosome 17 and ploidy in the samples, we tested six markers located on chromosome 17 and centromeric regions of chromosome 8 (CEP8) and 11 (CEP11) in 67 patients with CEP17 and LSI 17p11.2 CNI. True polysomy (hyperdiploidy) according to these markers was found in 0.48% of cases (24/5020). CEP8 and CEP11 CNI (≥3.0) was more frequent in the hyperdiploid than CEP17 non-polysomic group (55.6% vs. 6.1% and 25% vs. 2.3%, respectively). Our results suggest that chromosome 17 polysomy is a rare event found in less than 1% breast cancer cases and that polysomy of other chromosomes frequently occurs with chromosome 17 polysomy.

**Key words**

Breast cancer, HER2, CEP17, chromosome 17, polysomy, 17p11.2

**Introduction**

The amplification of the human epidermal growth factor receptor 2 gene (*HER2*, official name *ERBB2*) on chromosome 17q, a predictor of poor prognosis but good efficacy of anti-HER2 therapies (trastuzumab, pertuzumab, lapatinib), is reported in 15 to 20% of breast cancer cases. Immunohistochemistry (IHC) and *in situ* hybridization (ISH) are considered the gold standard tests for HER2 assessment in breast carcinoma (Wolff et al., 2013). IHC evaluates HER2 protein expression and ISH detects *HER2* copy number using fluorescent (FISH), chromogenic (CISH) or silver (SISH) detection systems or their combination by bright-field double ISH (BDISH) or dual-color dual-hapten ISH (DDISH) (Kurosumi, 2009; Kosa et al., 2013). *HER2* gene status may be evaluated by single-probe but more often is evaluated by dual-probe ISH together with a probe detecting the centromeric region of chromosome 17 (CEP17). Abnormalities of chromosome 17 are frequently reported in breast cancer, including both quantitative and qualitative aberrations, because several important breast cancer oncogenes, oncosuppressors and drug targets (like *TP53*, *NF1*, *HER2*, *TOP2A*, *STAT3*, *BRCA1*, *WNT3*, *PHB* and *RARA*) are located on this chromosome (Reinholz et al., 2009).

A copy number increase (CNI) of CEP17 has been described in 3% to 46% breast cancer cases (Hanna et al., 2014). The CNI was initially attributed to a polysomy of chromosome 17 until recent studies based on high-throughput technologies found that a CEP17 CNI more frequently reflects a gain or amplification of the centromeric region rather than a polysomy of the whole chromosome (Marchio et al., 2009; Yeh et al., 2009; Gunn et al., 2010; Moelans et al., 2010; Vranic et al., 2011). Marchiò et al evaluated 18 breast cancer patients with CEP17 CNI (CEP17 $\geq$ 3.0) and showed very complex changes of chromosome 17. Out of the 18 evaluated samples, true polysomy of chromosome 17 was found in one case only. The other cases displayed gains of 17q exceeding the centromeric region or focal centromeric gains or amplifications (Marchio et al., 2009). Other studies have also confirmed on limited patient cohorts (20-727 patients) that the polysomy of chromosome 17 is a rare event (Troxell et al., 2006; Varga et al., 2012).

The initial problem with *HER2* scoring in CEP17 CNI cases was resolved in the November 2013 update of the American Society of Clinical Oncology (ASCO) and the College of American

Pathologist (CAP) recommendation for *HER2* testing. According to their criteria, *HER2* should be reported as ISH positive if the *HER2*/CEP17 ratio is ≥ 2.0 or the mean *HER2* copy number ≥ 6.0. In rare cases of *HER2* copies between 4.0 and 6.0, a *HER2*/CEP17 ratio < 2.0 and CEP17 > 2.0, an alternative marker for chromosome 17 enumeration should be used for accurate *HER2*/chromosome 17 ratio evaluation (Wolff et al., 2013).

Even if the patient selection for anti-HER2 therapy is clear, the clinical significance of true chromosome 17 polysomy as well as the real percentage of truly polysomic cases remain unknown. The aim of this study was to evaluate the percentage of true polysomic cases in a large consecutive breast cancer patient cohort of 5477 individuals.

## Materials and methods

### *Study design*

A training cohort of 67 primary or metastatic breast cancer tissue samples with CEP17 ≥ 2.5 collected between 2004 and 2005 was used to select a suitable probe for chromosome 17 copy number enumeration.

A validation cohort of 5477 unselected consecutive samples was prospectively collected from patients with primary or metastatic breast cancer between 2004 and 2013 for reference *HER2*-FISH testing in a central laboratory (joint facility of the Institute of Molecular and Translational Medicine and the Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University in Olomouc, Czech Republic).

### *Immunohistochemistry (IHC)*

HER2 protein was immunohistochemically detected in 4-µm formalin–fixed, paraffin-embedded (FFPE) sections using the US Food and Drug Administration (FDA)-approved HercepTest (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. The standard HercepTest scoring system was used in all cases to obtain scores of 0 (no staining or incomplete faint membrane staining of < 10 % of the tumor cells), 1+ (incomplete faint membrane staining of ≥ 10 %

of the tumor cells), 2+ (incomplete and/or moderate membrane staining of ≥ 10 % of the tumor cells) or 3+ (complete intense membrane staining in ≥ 10 % of tumor cells) (Wolff et al., 2013).

***Fluorescence in situ hybridization (FISH)***

FISH was performed according to the manufacturers' instructions with the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, IL, USA) or Her-2/neu FISH Kit (IntellMed, Olomouc, Czech Republic), ON p53/SE17 (Kreatech diagnostics, Amsterdam, the Netherlands), LSI RARA, LSI TOP2A, LSI 17p11.2, LSI 17p12, CEP8 and CEP11 (all from IntellMed, Olomouc, Czech Republic). The pretreatment procedure was performed according to standard methods as previously published (Koudelakova et al., 2015).

The number of gene/centromere signals was counted in 100 non-overlapping nuclei in each patient sample using an Olympus BX-61 microscope. The ASCO/CAP scoring criteria were used for *HER2* evaluation. Samples were classified as amplified if the *HER2*/CEP17 ratio was ≥ 2.0 and/or the mean *HER2* copy number ≥ 6, equivocal if the *HER2*/CEP17 ratio was < 2.0 and the mean *HER2* copy number between 4 and 6 and negative if the *HER2*/CEP17 ratio was < 2.0 and the mean *HER2* copy number < 4 (Wolff et al., 2013). A mean marker copy number ≥ 3.0 for CEP17, 17p11.2, *RARA*, *TOP2A*, *TP53*, CEP8 and CEP11 was considered a CNI.

***Statistical analysis***

All statistical results were obtained with Statistica 12.0 (StatSoft, Inc., Tulsa, OK, USA). Nonparametric methods (a Wilcoxon exact paired test) were used to analyze differences in gene copy number for various probe locations. The Kruskal-Wallis test was used to analyze differences between distributions of chromosome 8 and 11 copy numbers in the breast cancer subgroups. P-values (two-sided significance levels) associated with each comparison were calculated and adjusted by the Bonferroni method. The subgroup of samples characterized by deletion of LSI p53 (copy number less than 1.8) was graphically presented by a line plot.

## Results

### *Selection of chromosome 17 enumeration probe*

To select an appropriate chromosome 17 enumeration probe, three locus specific identifier (LSI) probes on 17p (LSI 17p11.2, LSI 17p12 and LSI p53) were tested on a training set of 67 samples (CEP17 ≥ 2.5). The copy number of the tested markers significantly decreased with decreasing distance to *TP53* (from CEP17 to LSI p53; Figure 1A). The reason for the copy number decrease is probably the deletion of the 17p13 region (*TP53*) which could also include the 17p12 marker. In the training cohort, we found 5 samples with a *TP53* deletion (average copy number ≤ 1.8). A line plot (Figure 1B) illustrates the copy numbers of the markers and clearly demonstrates the *TP53* deletion in these samples. Because the 17p13 (*TP53*) region possibly includes the 17p12 marker, and *TP53* is frequently deleted in breast cancer, we chose the LSI 17p11.2 probe to identify samples with possible chromosome 17 polysomy.

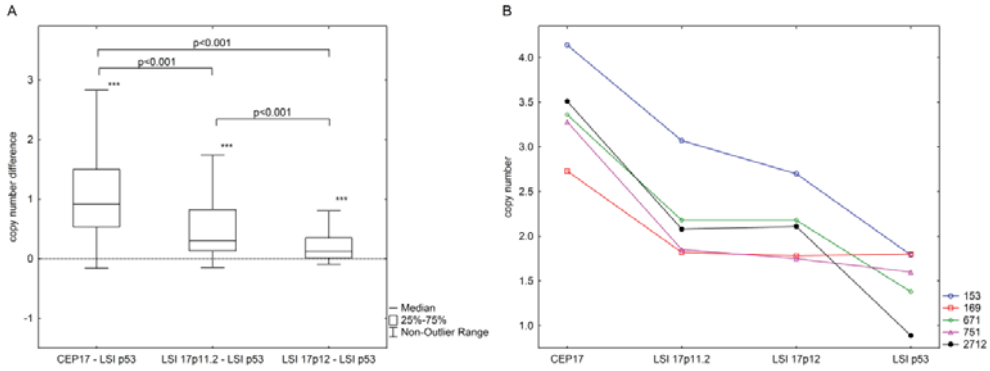



**Figure 1** Correlation of CEP17, 17p11.2, 17p12 and *TP53* copy numbers detected by FISH in the validation cohort (set of 67 samples with CEP17 ≥ 2.5). Boxplots (without outliers) are comparing the differences between the copy numbers of LSI p53 and CEP17, LSI 17p11.2, LSI 17p12 (**A**). Adjusted p-values (Bonferroni method) of Wilcoxon exact paired tests are given in the plot for each pair. Medians of all copy number differences from CEP17, respectively from LSI p53, are also significantly different from 0 (Wilcoxon exact test, p-value adjusted by Bonferroni method, *** means adjusted p-value < 0.001). **B** Line plot shows the behavior of the CEP17, 17p11.2, 17p12 copy numbers of samples with *TP53* deletion (copy number of LSI p53 less than 1.8).

***Detection of chromosome 17 polysomy***

A total of 5477 breast cancer samples were collected (Figure 2). Of these specimens, 212 (3.9%) that were unsuitable for the analysis due to the absence of tumor cells in sections and 245 (4.5%) with indeterminate FISH result were excluded from the study. In the remaining 5020 samples, *HER2* status was evaluated by IHC and FISH in parallel (summarized in Table 1). CEP17 CNI (CEP17 ≥ 3.0) was detected in 413 of the samples (8.2%). Of these, tissue was available for 297 samples which were tested for chromosome 17 polysomy with the LSI 17p11.2 probe. This probe revealed a 17p11.2 CNI in 67 of the samples.

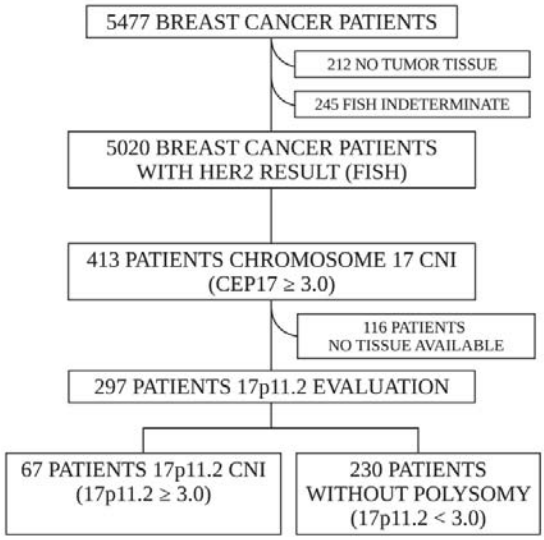



**Figure 2** Consort diagram of samples analyzed in the study.

To validate the chromosome 17 polysomy in these 67 patients, we used two markers on 17p (17p11.2 and *TP53*), CEP17 and three markers on 17q (*HER2*, *TOP2A* and *RARA*). Complete marker evaluation was not possible in 13 samples for which no tissue was available. A true polysomy, defined here by the increase of all evaluated markers, was found in 17 cases (17/54; 31.5%). Probably true polysomy with an isolated *TOP2A* deletion was found in 3 cases (5.6%) and probably true polysomy with an isolated *TP53* deletion in 14 cases (26%). Polysomy of chromosome 17 was not confirmed in the remaining 20 cases (37%). The distribution and extent of amplicons on chromosome 17 are

schematically illustrated in Figure 3. Taken together, true polysomy of chromosome 17 (hyperdiploidy) was identified in 0.48% breast cancer cases (24/5020) when we assume the same percentage of chromosome 17 polysomy in the 116 samples not tested for 17p11.2 CNI. The complete data are summarized in Supplementary Table 1.

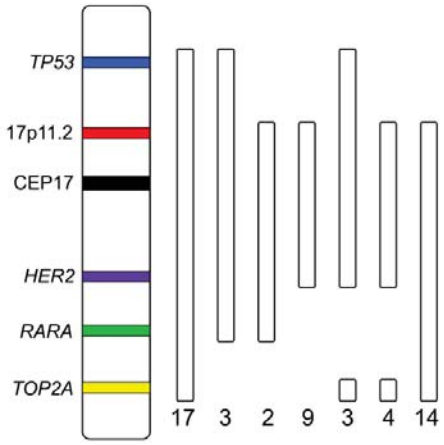



**Figure 3** Schematic illustration of gene region gains on chromosome 17 in patients with 17p11.2 CNI. The numbers below the bars indicate the number of patients corresponding to each subgroup.

***Change in HER2 status based on 17p11.2 evaluation***

Having identified different copy numbers of CEP17 and 17p11.2 in the set of 297 breast cancer samples, we next determined how this disparity affects the assessment of *HER2* amplification status using *HER2*/CEP17 and *HER2*/17p11.2 ratios. Using the recommended criteria for *HER2* evaluation (*HER2* copy number and/or *HER2*/CEP17 ratio), 65 (21.9%) samples were scored as non-amplified, 83 (27.9%) samples were scored as equivocal and 149 (50.2%) samples were scored as amplified. Of the 65 non-amplified patients, one (1.5%) was scored as amplified based on the *HER2*/17p11.2 ratio (≥ 2.0) (Table 2). This sample was negative by immunohistochemistry (IHC 1+). Of the 83 equivocal patients, 31 (37.3%) were reclassified as amplified based on the *HER2*/17p11.2 ratio. Of these 31 samples, 15 were negative by IHC (IHC 0/1+), 15 were IHC equivocal (IHC 2+) and 2 samples were IHC positive (IHC 3+).

**Table 1** *HER2* status of the whole study cohort.

| | | ***HER2* status (FISH)** | | | | |
|---|---|---|---|---|---|---|
| | | **Amplification (%)** | **Equivocal (%)** | **Physiologic (%)** | **Indeterminate (%)** | **Total (%)** |
| **HER2 status (IHC)** | **0/1+** | 63 (2.8) | 105 (36.0) | 1742 (70.8) | 157 (64.1) | **2067 (39.3)** |
| | **2+** | 207 (9.1) | 125 (42.8) | 578 (23.5) | 45 (18.4) | **955 (18.1)** |
| | **3+** | 1966 (86.7) | 56 (19.2) | 101 (4.1) | 35 (14.3) | **2158 (41.0)** |
| | **Indeterminate** | 32 (1.4) | 6 (2.1) | 39 (1.6) | 8 (3.3) | **85 (1.6)** |
| | **Total** | **2268 (43.1)** | **292 (5.5)** | **2460 (46.7)** | **245 (4.7)** | **5265 (100)** |

IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization

**Table 2** Change of *HER2* status based on *HER2*/17p11.2 ratio in 297 cases with CEP17 CNI.

| | | ***HER2* amplification based on *HER2*/17p11.2 ratio** | | |
|---|---|---|---|---|
| ***HER2* copy number/ *HER2*/CEP17 ratio** | **Total** | **Non-amplified → Amplified** | **Equivocal → Amplified** | **No change** |
| < 4.0 and < 2.0 | 65 | 1 (1.5%) | 0 (0%) | 64 (98.5%) |
| 4.0 - 6.0 and < 2.0 | 83 | 0 (0%) | 31 (37.3%) | 52 (62.7%) |
| ≥ 6.0 and/or ≥ 2.0 | 149 | 0 (0%) | 0 (0%) | 149 (100%) |

***Association of chromosome 17 polysomy with polysomy of chromosomes 8 and 11***

In order to differentiate between an isolated chromosome 17 polysomy and a hyperdiploid cancer genome, we evaluated the status of chromosome 8 and 11 in samples with or without 17p11.2 CNI. The copy number of chromosomes 8 and 11 was tested by centromeric probes CEP8 and CEP11 in the 67 patients with 17p11.2 CNI as well as in a subset of CEP17 non-polysomic and CEP17 CNI groups with available material. The distribution of samples with CEP8/11 CNI ($\geq$ 3.0) across CEP17 non-polysomic (CEP17 < 3.0), CEP17 CNI (CEP17 $\geq$ 3.0), 17p11.2 CNI (CEP17 $\geq$ 3.0, 17p11.2 $\geq$ 3.0) and hyperdiploid (all tested markers $\geq$ 3.0) groups was significantly different ($p<0.001$; Figure 4). The percentage of CEP8 CNI increased from 6.1% in the CEP17 non-polysomic group to 55.6% in the hyperdiploid group. Similarly for chromosome 11, CEP11 CNI in 2.3% samples of the CEP17 non-polysomic group rose to 25.0% of hyperdiploid patients. In all four CEP11 CNI cases, the CEP8 CNI was simultaneously found indicating possible polyploidy in these samples (Figure 5).

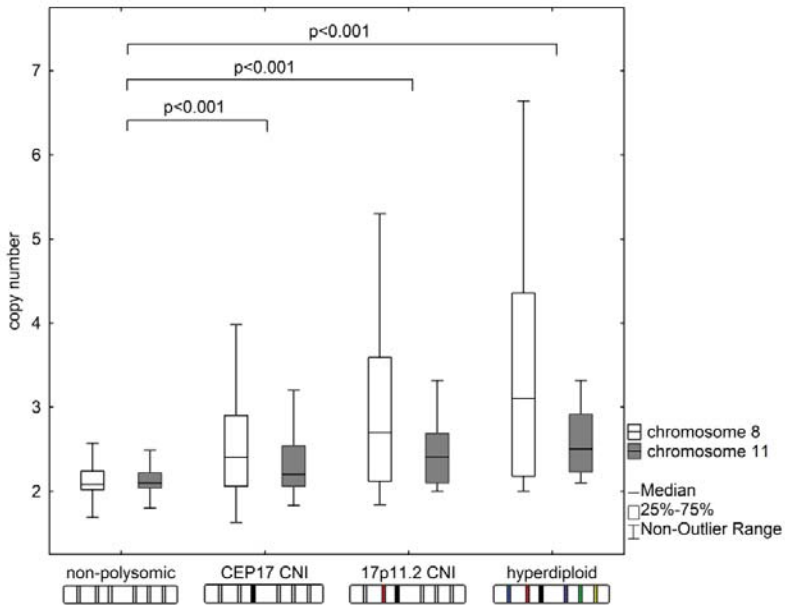
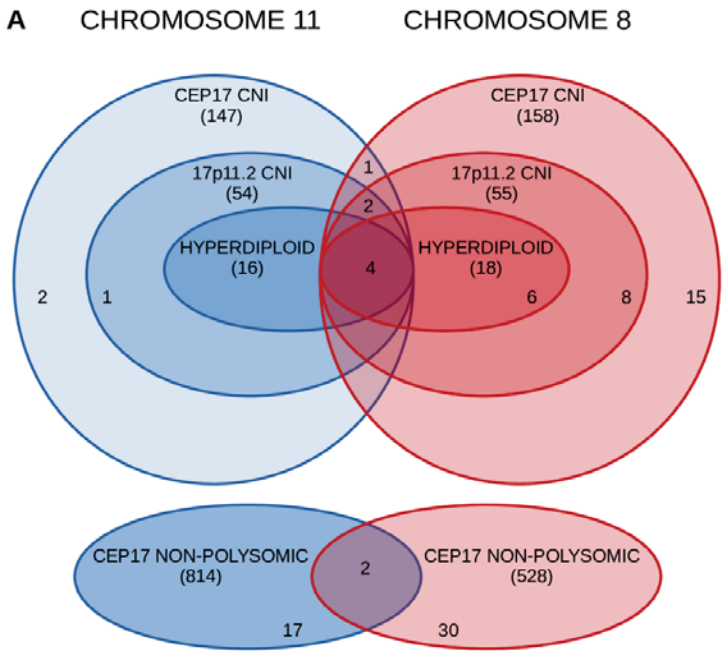



**Figure 4** Box plots showing the distribution of chromosome 8 (white) and chromosome 11 (grey) copy number detected by CEP8/CEP11 probes in breast cancer subgroups. Symbols in the legend indicate the chromosome 17 markers defining each subgroup (CEP17 non-polysomic, CEP17 CNI, 17p11.2 CNI, hyperdiploid).




**B**

| | CEP17 non-polysomy | CEP17 CNI | 17p11.2 CNI | Hyperdiploid |
|---|---|---|---|---|
| **CEP11 CNI** | 2.3% (19/814) | 6.8% (10/147) | 13.0% (7/54) | 25.0% (4/16) |
| **CEP8 CNI** | 6.1% (32/528) | 22.8% (36/158) | 36.4% (20/55) | 55.6% (10/18) |
| **CEP8+CEP11 CNI** | 0.4% (2/493) | 5.3% (7/132) | 12.0% (6/50) | 25.0% (4/16) |

CEP17, chromosome enumeration probe 17; CEP8, chromosome enumeration probe 8;
CEP11, chromosome enumeration probe 11; CNI, copy number increase

**Figure 5 A** An Euler diagram showing the distribution of chromosome 11/chromosome 8 (CEP11/CEP8) CNI in CEP17 non-polysomic, CEP17 CNI, 17p11.2 CNI and hyperdiploid subgroups. The total number of evaluated samples in each subgroup is indicated in brackets. **B** Table showing the percentage of CEP11 and CEP8 CNI in the studied groups.

**Discussion**

The copy number increase of CEP17 (usually called chromosome 17 polysomy) is a common finding described for 3% to 46% breast cancer cases (Hanna et al., 2014). In our study, CEP17 CNI was found in 8.2% cases (413/5020). Our finding is comparable to the 8% and 13% reported by studies on non-selected consecutive breast cancer populations (Downs-Kelly et al., 2005; Salido et al., 2005). True polysomy of chromosome 17 has been described as a very rare event by several studies using array-

based techniques, multiplex ligation-dependent probe amplification (MLPA) (Marchio et al., 2009; Yeh et al., 2009; Moelans et al., 2010; Gunn et al., 2010; Vranic et al., 2011) and extended FISH (Troxell et al., 2006; Tse et al., 2011; Varga et al., 2012). However, the majority of these studies evaluated chromosome 17 polysomy on relatively small and usually highly pre-selected sample sets (from 20 to 727 samples) (Gunn et al., 2010; Hanna et al., 2014) and the frequency of chromosome 17 polysomy in the overall population was therefore not clearly described. This study aimed to clarify the frequency of true chromosome 17 polysomy in a large consecutive cohort of more than 5000 patient samples. A sample of similar size has so far only been evaluated by Tse et al (Tse et al., 2011), albeit without further probing of hyperdiploidy.

To pre-select possible polysomic cases we tested the suitability of three probes on the chromosome 17p arm. We selected the short arm of chromosome 17 because the 17q arm exhibits a higher frequency of rearrangements and contains a number of breast cancer related genes that undergo frequent copy number changes (Reinholz et al., 2009). Upon testing the probes in the training cohort (CEP17 $\geq$ 2.5) we found that the copy number of evaluated markers decreased with increasing CEP17 to 17pter distance. The reason is probably the deletion of *TP53* which could involve the 17p12 marker. The *TP53* deletion is frequent in all cancer types and has been described in more than 50% of breast cancer cases (Sato et al., 1990; Varley et al., 1991; Persons et al., 1996; Sigurdsson et al., 2000). The *TP53* mutations or deletions occur mainly in triple negative and HER2-enriched breast cancer subtypes (Eroles et al., 2012). An independent deletion of 17p12 has also been described in breast cancer (Han et al., 2011). Based on these findings we selected the LSI 17p11.2 probe for chromosome 17 enumeration in 297 patients with CEP17 CNI. Similar probes targeting the 17p11.2 region have been used for chromosome 17 enumeration in other studies (Troxell et al., 2006; Tse et al., 2011; Varga et al., 2012). It should however be noted that the 17p11.2 region is not frequently deleted in breast cancer but in a minority of cases could be affected by the amplification of the centromeric region (Marchio et al., 2009).

Finally, we used six markers on chromosome 17 (*TP53*, 17p11.2, CEP17, *HER2*, *RARA* and *TOP2A*) to identify true polysomy in the subset of 67 samples with 17p11.2 CNI. Similar chromosome 17

enumeration markers have been evaluated in other studies (Troxell et al., 2006; Tse et al., 2011; Varga et al., 2012). Based on the aforementioned markers, true polysomy of chromosome 17 (hyperdiploidy) was found in 0.48% breast cancer cases (24/5020) if we assume the same representation of chromosome 17 polysomy in the 116 samples displaying CEP17 CNI but not tested for 17p11.2 copy number. A similar finding was published by Tse et al. who found that 171 samples out of 5683 tested had a CEP17 CNI. Of these 171, only 24 samples (0.42%) were found to be probably truly polysomic (based on 5 evaluated markers) (Tse et al., 2011). In our cohort, a gain of all markers except *TOP2A* was found in 3 cases. Considering the clearly confirmed *TOP2A* deletion in *HER2* positive cases (Engstrom et al., 2014; Almeida et al., 2014), we assume that these cases concern a polysomy of chromosome 17 with *TOP2A* deletion. Similarly, a gain of all markers except *TP53* was found in 14 cases. We assume that the polysomy of chromosome 17 could be also present at least in some of these cases since the deletion of the *TP53* gene is a common event in breast cancer as described above. Similarly the Tse et al study identified 23 samples (0.4%) positive for all markers except *TP53* (Tse et al., 2011). A slight difference between these two studies could be caused by a different representation of *HER2* positive breast cancer samples in the cohorts. The percentage of *HER2* positive tumors in our study is much higher compared to other institutions and the literature. The main reason is that positive samples are sent from local laboratories to us for confirmatory testing, since our institution serves as a central/reference laboratory. Although we did not wholly confirm true polysomy of chromosome 17 because the evaluated markers did not cover the entire chromosome 17 we could conclude that the true polysomy of chromosome 17 is very rare event occurring in less than 1% of breast cancer cases, which is in concordance with the literature (Moelans et al., 2014).

The precise evaluation of *HER2* status is fundamentally important for selecting therapies for breast cancer patients. Anti-HER2 therapy significantly increases survival rates in both palliative and adjuvant settings. In rare cases, the elevation of chromosome 17 copy number could affect the *HER2* amplification status. According to the recently upgraded criteria for *HER2* evaluation, the use of an alternative probe for chromosome 17 copy number evaluation is recommended in cases with an equivocal ISH result and CEP17 CNI (Wolff et al., 2013). In our study, the chromosome 17 copy

number of 297 patients with CEP17 CNI was evaluated with the LSI 17p11.2 probe. Out of these, 65 and 83 patients were initially classified as non-amplified and equivocal, respectively. Using *HER2*/17p11.2 scoring criteria, 37.3% (31/83) equivocal and 1.5% non-amplified (1/65) patients were reclassified as amplified. Even if the IHC score was negative or equivocal in the majority of these cases, *HER2* copy number was doubled compared to real chromosome 17 status and these 32 reclassified patients should benefit from anti-HER2 therapy. A benefit from trastuzumab therapy in IHC 0/1+ or IHC 2+ and FISH-positive cases has been previously described (Paik et al., 2008; Perez et al., 2010; Wolff et al., 2013). Moreover, benefit from trastuzumab therapy was observed even in IHC-negative/FISH-negative breast cancer patients (Kaufman PA et al., 2007; Perez et al., 2010) and did not depend on the level of *HER2* positivity. A similar benefit from trastuzumab was observed for patients with *HER2*/CEP17 ratios from 2 to 15 (Dowsett et al., 2009; Perez et al., 2010). The efficacy of trastuzumab in *HER2*-positive patients is unaffected by CEP17 CNI (Hofmann et al., 2008; Dowsett et al., 2009).

The copy number status of chromosome 8 and 11 centromeric regions was compared in patients with or without polysomy of chromosome 17. Interestingly, the significantly higher CNI of these two chromosomes was found in patients with chromosome 17 polysomy (55.6% vs. 6.1% for chromosome 8 and 25.0% vs. 2.3% for chromosome 11). Moreover, possible polyploidy was found in four cases with both CEP8 and CEP11 CNI. Polysomy of chromosome 8 is a frequent alteration in breast cancer reported in more than 30% of cases (Visscher et al., 1997; Roka et al., 1998; Bofin et al., 2003; Tagawa et al., 2003; Sneige et al., 2006; Corzo et al., 2006; Perez et al., 2011). Chromosome 8 polysomy was reported to be associated with poor prognosis and higher risk of breast cancer (Tagawa et al., 2003; Bofin et al., 2003; Sneige et al., 2006). Perez et al reported chromosome 8 polysomy as a predictor of trastuzumab efficacy in HER2-positive breast cancer tumors (Perez et al., 2011). Chromosome 11 polysomy is also a common finding in breast cancer reported in more than 30% of cases (Ichikawa et al., 1996; McManus et al., 1999; Tsukamoto et al., 2001; Takehisa et al., 2007) and associated with high histological grade and poor prognosis (Tsukamoto et al., 2001; Takehisa et al., 2007). Several breast cancer studies evaluated chromosome 11 and 17 (McManus et al., 1999),

chromosome 1, 11 and 17 (Ichikawa et al., 1996; Tsukamoto et al., 2001; Takehisa et al., 2007) or chromosome 1, 8, 11 and 17 status together (Sneige et al., 2006). Polysomy of all tested chromosomes (possible polyploidy) was observed in 15 to 29% cases (Tsukamoto et al., 2001; Sneige et al., 2006; Takehisa et al., 2007). All studies evaluated aneusomy by centromeric probes only and none of the studies analyzed these chromosomal copy number changes in breast cancer patients with true chromosome 17 polysomy. To our knowledge, this is the first study showing significantly higher representation of chromosome 8 and 11 centromeric region CNI (polysomy) in the group of breast cancer patients with true polysomy of chromosome 17.

In conclusion, true polysomy of chromosome 17 is a very rare event which should be evaluated by techniques covering the whole of chromosome 17. This approach is unrealistic in clinical practice because of its high cost and required technical equipment. Using an ISH technique with a set of surrogate chromosome 17 markers seems to be sufficient and practically applicable for chromosome 17 polysomy evaluation.

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**Supplementary Table 1** Detailed characterization of 67 cases with 17p11.2 CNI. Comparison of copy numbers of six markers on chromosome 17 and centromere regions of chromosome 8 and 11.

| Case No | Age at diagnosis | Tumor type | HER2 score (IHC) | *HER2* status (FISH) | CEP17 | 17p11.2 | *HER2* | *TOP2A* | *RARA* | *TP53* | CEP11 | CEP8 | Description |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 38 | ductal | 3+ | amplified | **4.14** | **3.07** | **14.81** | **3.68** | 2.05 | 1.79 | 2.84 | **5.88** | non-polysomic |
| 2 | 24 | ductal | 3+ | amplified | **3.06** | **3.25** | **16.94** | **11.85** | **17.85** | 2.57 | 2.56 | 2.19 | **polysomic**[a] |
| 3 | 62 | ductal | 3+ | amplified | **3.74** | **3.23** | **16.56** | 1.90 | **3.97** | **3.00** | 2.69 | 2.11 | **polysomic**[b] |
| 4 | 47 | ductal | 2+ | amplified | **4.05** | **4.40** | **8.01** | **8.56** | **7.65** | **3.75** | 2.11 | 2.18 | **polysomic** |
| 5 | 62 | ductal | 2+ | equivocal | **3.55** | **3.05** | **5.34** | **3.50** | **3.49** | 2.12 | 2.04 | no tissue | **polysomic**[a] |
| 6 | 55 | ductal | 3+ | amplified | **4.81** | **3.05** | **13.74** | 2.43 | no tissue | 1.98 | 2.18 | 2.03 | non-polysomic |
| 7 | 71 | ductal | 3+ | amplified | **3.05** | **3.44** | **22.50** | **3.02** | **3.75** | 2.03 | 2.06 | 2.18 | **polysomic**[a] |
| 8 | 63 | ductal | 3+ | amplified | **3.37** | **5.25** | **26.20** | **21.20** | **6.17** | 1.77 | 2.05 | 2.06 | **polysomic**[a] |
| 9 | 63 | ductal | 1+ | non-amplified | **3.14** | **3.56** | **3.46** | **3.13** | 2.52 | **3.30** | 2.23 | 1.99 | non-polysomic |
| 10 | 51 | ductal | 2+ | amplified | **4.04** | **5.27** | **8.09** | **10.07** | **10.08** | 2.47 | 2.50 | 2.54 | **polysomic**[a] |
| 11 | 69 | ductal | 3+ | amplified | **5.78** | **3.10** | **20.00** | 1.84 | 2.15 | 2.32 | 2.20 | 2.06 | non-polysomic |
| 12 | 66 | ductal | 3+ | amplified | **3.49** | **3.38** | **20.00** | **20.00** | **3.24** | **3.26** | 2.17 | 2.06 | **polysomic** |
| 13 | 63 | ductal | 1+ | equivocal | **5.23** | **3.28** | **5.96** | **5.37** | **5.87** | 2.18 | **4.41** | **4.51** | **polysomic**[a] **/polyploid**[c] |
| 14 | 45 | ductal | 1+ | non-amplified | **3.01** | **3.81** | **3.36** | **3.77** | 2.62 | 2.79 | 2.54 | 2.06 | non-polysomic |
| 15 | 67 | ductal | 3+ | amplified | **3.87** | **3.50** | **11.11** | **10.31** | no tissue | 2.32 | 2.03 | 2.97 | no tissue available |
| 16 | 49 | ductal | 1+ | equivocal | **4.16** | **3.76** | **4.22** | **3.86** | no tissue | **3.25** | 2.36 | 2.15 | no tissue available |
| 17 | 74 | ductal | 0 | equivocal | **4.23** | **4.12** | **4.89** | **3.53** | no tissue | 2.88 | 2.20 | 2.41 | no tissue available |
| 18 | 56 | ductal | 3+ | amplified | **3.30** | **3.02** | **20.00** | 2.93 | 2.48 | 2.98 | 2.48 | **3.11** | non-polysomic |
| 19 | 54 | ductal | 3+ | amplified | **4.04** | **4.00** | **14.48** | 1.84 | 2.70 | 1.30 | 2.00 | 2.00 | non-polysomic |
| 20 | 64 | ductal | 3+ | amplified | **3.08** | **3.09** | **15.00** | 2.35 | no tissue | no tissue | 2.62 | 2.95 | no tissue available |
| 21 | 46 | lobular | 3+ | amplified | **3.44** | **3.59** | **20.00** | **15.25** | 2.34 | **5.28** | 2.10 | 2.45 | non-polysomic |
| 22 | 55 | ductal | 3+ | amplified | **5.64** | **5.55** | **20.00** | **8.53** | 2.74 | 2.12 | 2.30 | no tissue | non-polysomic |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 23 | 69 | ductal | 2+ | equivocal | **4.05** | **3.10** | **4.16** | **3.04** | no tissue | no tissue | no tissue | no tissue | no tissue available |
| 24 | 52 | ductal | 3+ | amplified | **3.25** | **3.15** | **15.00** | **3.01** | 2.68 | 2.18 | no tissue | no tissue | non-polysomic |
| 25 | 50 | ductal | 3+ | amplified | **3.04** | **3.19** | **20.00** | 2.65 | no tissue | no tissue | no tissue | no tissue | no tissue available |
| 26 | 56 | ductal | 0 | equivocal | **4.37** | **3.64** | **4.54** | **3.01** | **3.82** | **3.80** | 2.26 | **3.16** | **polysomic** |
| 27 | 60 | ductal | 3+ | amplified | **3.71** | **3.25** | **20.00** | **6.87** | **3.50** | **4.00** | 2.51 | 2.16 | **polysomic** |
| 28 | 60 | ductal | 0 | non-amplified | **3.11** | **3.12** | **3.02** | **3.20** | **3.16** | 2.52 | 2.76 | **3.68** | **polysomic**[a] |
| 29 | 38 | ductal | 3+ | amplified | **3.28** | **10.45** | **20.00** | 1.93 | no tissue | 2.04 | no tissue | no tissue | non-polysomic |
| 30 | 58 | ductal | 3+ | amplified | **4.07** | **3.61** | **12.00** | **9.88** | **6.60** | 2,54 | 2.88 | **3.98** | **polysomic**[a] |
| 31 | 57 | ductal | 2+ | equivocal | **3.87** | **3.03** | **4.09** | **4.30** | no tissue | no tissue | no tissue | no tissue | no tissue available |
| 32 | 49 | ductal | 2+ | amplified | **4.09** | **3.74** | **10.00** | **3.52** | **3.50** | **3.24** | **4.82** | **8.84** | **polyploid** |
| 33 | 60 | ductal | 3+ | amplified | **3.22** | **3.03** | **19.80** | 2.45 | 2.40 | 2.68 | 2.07 | **3.64** | non-polysomic |
| 34 | 59 | ductal | 3+ | amplified | **3.84** | **3.73** | **20.00** | 2.34 | 2.26 | 2.20 | 2.05 | 1.84 | non-polysomic |
| 35 | 58 | ductal | 0 | amplified | **5.82** | **5.63** | **7.34** | **5.96** | **6.42** | **5.66** | 2.81 | 2.40 | **polysomic** |
| 36 | 56 | ductal | 3+ | amplified | **4.48** | **3.06** | **20.00** | **3.96** | **4.60** | 2.28 | 2.84 | 2.08 | **polysomic**[a] |
| 37 | 46 | ductal | 3+ | amplified | **3.75** | **3.24** | **20.00** | 2.06 | 2.70 | 2.84 | 2.24 | 2.94 | non-polysomic |
| 38 | 52 | ductal | 3+ | amplified | **3.22** | **3.04** | **20.00** | 1.88 | 2.70 | 2.50 | 2.52 | 2.90 | non-polysomic |
| 39 | 74 | ductal | 1+ | equivocal | **3.35** | **3.64** | **4.63** | **4.48** | **5.76** | **3.05** | no tissue | **3.05** | **polysomic/polyploid**[d] |
| 40 | 68 | ductal | 0 | equivocal | **3.78** | **3.87** | **4.17** | 2.35 | **4.42** | 2.00 | 2.10 | 2.54 | non-polysomic |
| 41 | 74 | ductal | 0 | non-amplified | **3.14** | **3.00** | **3.41** | 2.93 | 2.76 | 2.26 | 2.30 | **3.26** | non-polysomic |
| 42 | 63 | ductal | 0 | equivocal | **4.06** | **3.39** | **4.42** | 2.09 | **4.16** | **3.92** | no tissue | **5.30** | **polysomic**[b] |
| 43 | 57 | ductal | 2+ | amplified | **4.97** | **3.77** | **9.79** | **7.37** | **6.56** | **3.30** | no tissue | no tissue | **polysomic** |
| 44 | 38 | lobular | 1+ | non-amplified | **3.45** | **3.21** | **3.97** | **3.12** | **3.61** | **3.48** | 2.50 | 2.70 | **polysomic** |
| 45 | 62 | ductal | 1+ | equivocal | **3.39** | **3.75** | **5.35** | **5.77** | **5.30** | **4.88** | **5.86** | **6.64** | **polyploid** |
| 46 | 79 | ductal | 2+ | equivocal | **3.65** | **3.07** | **4.83** | **3.97** | **4.24** | 2.36 | 2.46 | no tissue | **polysomic**[a] |
| 47 | 46 | ductal | 3+ | amplified | **15.00** | **15.00** | **20.00** | 1.89 | no tissue | no tissue | 2.08 | 2.14 | no tissue available |
| 48 | 66 | ductal | 0 | amplified | **3.43** | **3.03** | **7.17** | **8.97** | no tissue | 2.18 | no tissue | **3.36** | no tissue available |
| 49 | 79 | ductal | 2+ | amplified | **4.18** | **4.07** | **7.04** | **4.37** | no tissue | no tissue | no tissue | no tissue | no tissue available |
| 50 | 57 | ductal | 2+ | equivocal | **3.72** | **3.09** | **4.79** | **3.84** | **3.04** | **5.68** | **3.32** | **4.20** | **polyploid** |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 51 | 70 | ductal | 1+ | non-amplified | **3.05** | **3.88** | **3.27** | **3.01** | **3.02** | **3.62** | **3.02** | **5.00** | **polyploid** |
| 52 | 58 | ductal | 2+ | amplified | **3.40** | **3.48** | **10.00** | **10.00** | **15.00** | 2.04 | 2.10 | 2.84 | **polysomic**[a] |
| 53 | 62 | ductal | 3+ | amplified | **5.14** | **3.40** | **20.00** | 2.52 | 2.54 | 2.78 | 2.46 | 2.78 | non-polysomic |
| 54 | 69 | ductal | 3+ | non-amplified | **3.26** | **3.49** | **3.44** | **3.20** | **3.00** | **3.08** | 2.10 | 2.00 | **polysomic** |
| 55 | 75 | ductal | 3+ | amplified | **5.29** | **3.42** | **25.00** | **3.24** | no tissue | no tissue | no tissue | 2.12 | no tissue available |
| 56 | 60 | ductal | 2+ | non-amplified | **3.13** | **3.10** | **3.82** | **5.62** | **5.62** | **3.01** | 2.30 | 2.36 | **polysomic** |
| 57 | 59 | ductal | 2+ | non-amplified | **3.19** | **3.64** | **3.85** | 2.85 | no tissue | no tissue | 2.00 | no tissue | no tissue available |
| 58 | 57 | ductal | 2+ | equivocal | **3.26** | **4.07** | **5.90** | **6.40** | no tissue | 2.76 | 2.04 | **3.59** | no tissue available |
| 59 | 74 | ductal | 1+ | non-amplified | **3.22** | **4.54** | 2.97 | 2.23 | 2.90 | 2.20 | **4.90** | **4.80** | non-polysomic |
| 60 | 78 | ductal | 1+ | equivocal | **4.02** | **4.28** | **5.96** | **6.24** | **5.20** | **3.80** | 2.20 | **4.36** | **polysomic** |
| 61 | 78 | ductal | 1+ | equivocal | **4.77** | **3.33** | **5.26** | **4.45** | **3.98** | **3.30** | no tissue | no tissue | **polysomic** |
| 62 | 72 | ductal | 2+ | non-amplified | **3.04** | **3.26** | **3.69** | 2.67 | **4.02** | **3.30** | 2.48 | **3.70** | **polysomic**[b] |
| 63 | 60 | ductal | 1+ | equivocal | **4.19** | **3.12** | **4.53** | **4.52** | **4.72** | 2.58 | **4.20** | 2.24 | **polysomic**[a] |
| 64 | 76 | ductal | 2+ | equivocal | **3.17** | **3.18** | **5.73** | 2.89 | **3.08** | 2.18 | 2.96 | 1.93 | non-polysomic |
| 65 | 37 | ductal | 2+ | equivocal | **3.64** | **4.50** | **4.16** | **4.30** | **3.10** | 1.52 | 2.48 | 2.03 | **polysomic**[a] |
| 66 | 59 | ductal | 2+ | equivocal | **3.73** | **3.89** | **4.33** | **3.55** | **3.84** | **3.02** | 2.60 | **3.54** | **polysomic** |
| 67 | 68 | ductal | 2+ | amplified | **3.28** | **3.23** | **6.49** | **5.50** | **4.20** | 1.66 | no tissue | 2.70 | **polysomic**[a] |

[a] Chromosome 17 polysomy with *TP53* deletion or 17q gain involving CEP17 and 17p11.2 regions.

[b] Probably polysomic case with *TOP2A* deletion.

[c] Probably polyploid case with *TP53* deletion.

[d] Probably polyploid case (1 marker was not evaluated).

Positive cases are shown in bold.

CEP8, chromosome enumeration probe 8; CEP11, chromosome enumeration probe 11; CEP17, chromosome enumeration probe 17; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

### 2.3.3 Prediktivní markery u triple-negativního karcinomu prsu

BCL2 is an independent predictor of outcome in basal-like triple-negative breast cancers treated with adjuvant anthracycline-based chemotherapy.

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RESEARCH ARTICLE

# BCL2 is an independent predictor of outcome in basal-like triple-negative breast cancers treated with adjuvant anthracycline-based chemotherapy

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**Abstract** Neither targeted therapies nor predictors for chemotherapy sensitivity are available for triple-negative breast cancer (TNBC). Our study included 187 patients with TNBC, 164 of whom were treated with anthracycline-based adjuvant chemotherapy. Eleven molecular biomarkers were analyzed. BCL2, epidermal growth factor receptor (EGFR), MYC, TOP2A, and Ki-67 protein expression was evaluated by immunohistochemistry. The status of the EGFR, MYC, and TOP2A genes and chromosomes 7, 8, and 17 was assessed using fluorescence in situ hybridization. High BCL2 expression predicted poor relapse-free survival (RFS) in patients treated with anthracycline-based adjuvant chemotherapy ($p$=0.035), poor breast cancer-specific survival (BCSS) ($p$=0.048), and a trend to poor overall survival (OS) ($p$=0.085). High levels of BCL2 expression predicted poor OS in basal-like (BL) TNBC patients treated with adjuvant anthracycline-based regimens (log-rank $p$=0.033, hazard ratio (HR) 3.04, 95 % confidence interval (CI) 1.04–8.91) and a trend to poor RFS (log-rank $p$=0.079) and poor BCSS (log-rank $p$=0.056). Multivariate analysis showed that BCL2

Results of this study were in part presented at the 2012 American Society of Clinical Oncology Annual Meeting, Chicago, Il, USA, 2012 [31].

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status, tumor size, and nodal status all had independent predictive significance for RFS (p=0.005, $p$=0.091, $p$=0.003, respectively; likelihood ratio test for the whole model, $p$=0.003), BCSS ($p$=0.012, $p$=0.077, $p$=0.01, respectively; likelihood ratio test for the whole model, $p$=0.016), and OS ($p$=0.008, $p$=0.004, $p$=0.004, respectively; likelihood ratio test for the whole model, $p$=0.0006). Similarly, multivariate analysis for BL TNBC showed BCL2, tumor size, and nodal status all had independent predictive significance for RFS (likelihood ratio test for the whole model, $p$=0.00125), BCSS ($p$=0.00035), and OS ($p$=0.00063). High EGFR expression was associated with poor BCSS ($p$=0.039) in patients treated with anthracycline-based adjuvant chemotherapy. Patients who underwent anthracycline-based adjuvant chemotherapy and exhibited CMYC amplification had a trend to worse BCSS ($p$=0.066). In conclusion, high BCL2 expression is a significant independent predictor of poor outcome in TNBC patients treated with anthracycline-based adjuvant chemotherapy, and this is the first study showing the BCL2 prediction in BL TNBC. BCL2 expression analysis could facilitate decision making on adjuvant treatment in TNBC patients.

**Keywords** Adjuvant chemotherapy · Anthracycline · BCL2 · EGFR · Predictive marker · Triple-negative breast cancer

## Introduction

Triple-negative breast cancer (TNBC) is a clinically and molecularly heterogeneous disease [1–3]. It has the highest frequency of somatic rearrangements among breast carcinomas (BCs) [4] and encompasses at least seven subtypes with different prevalences, gene expression profiles, and clinical outcomes [1, 2]. Although it is generally a fast-growing and highly malignant disease, its frequent sensitivity to chemotherapy makes it potentially curable if detected at a sufficiently early stage. On the other hand, experiences with neoadjuvant and adjuvant treatment indicate that approximately one third of TNBC cases are primarily chemoresistant, even when using regimes based on the most effective cytostatics such as anthracyclines, platinum derivatives, and taxanes. Because there is a lack of targeted treatments for TNBC in current clinical practice, there is an urgent need to identify molecular biomarkers that can be used to select appropriate chemotherapeutic regimens for a given cancer [1, 5–8].

BCL2 is a fundamental anti-apoptotic protein that plays an important role in physiological and pathological processes as a protooncogene and oncogene. It also performs other functions, such as promoting cell growth and proliferation [9–11]. Preclinical data showed that BCL2 expression was associated with resistance to anthracycline doxorubicin [12]. In keeping with this finding, the absence of BCL2 expression in prechemotherapy samples was associated with a higher probability of pathological complete response (pCR) to neoadjuvant doxorubicin-based chemotherapy [13–15]. In the context of adjuvant therapy, BCL2-negative patients treated with anthracyclines had better outcomes than those who received either cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) or no chemotherapy [15]. However, other studies have identified a positive association between prognosis and BCL2 protein expression in patients with breast cancer, including TNBC subgroups [9, 15]. It is not entirely clear why this is the case, but it may be partly due to changes in the expression of other proteins such as p27, HER3 and MDM4 in BCL2-positive cancers [15].

Epidermal growth factor receptor (EGFR, also known as HER1) plays roles in cell proliferation, migration, and protection against apoptosis [16]. Its overexpression appears to be a later event in tumorigenesis [17] and is frequently observed in TNBC, especially in metaplastic cancers where it occurs in up to 80 % of all cases [18]. The EGFR gene is amplified in 0–14 % of non-selected BC series and up to 28 % of metaplastic cancers [18]. However, the available data on the use of the EGFR oncogene/protein as a prognostic marker in BC are inconsistent and little is known about its potential utility as a marker in TNBC. Two studies found significant correlations between EGFR immunoreactivity (as a percentage of positive cells) and worse prognosis in both invasive ductal TNBC [19] and unselected TNBC [20].

The TOP2A gene encodes topoisomerase II alpha, the molecular target of the anthracyclines. Preclinical results indicate that TOP2A amplification may be predictive of anthracycline sensitivity [21]. Clinical evaluations of this relationship have yielded somewhat inconsistent results, but a large study of 4,943 patients identified TOP2A and HER2 coamplification as a predictor of responsiveness to anthracycline-based chemotherapy [22]. TOP2A gene amplification is frequently observed in HER2-amplified BC and also in a small proportion (3–9 %) of HER2 non-amplified BC [18, 23]. At present, little is known about the role of TOP2A in TNBC.

Of the many proteins that have been examined as markers for TNBC, Ki-67 and CMYC merit particular attention. Ki-67 is a marker of proliferating cells [24], and Ki-67 labeling values above 35 % were recently shown to be associated with an increased 5-year cumulative incidence of breast-related death in a study of node-negative TNBC patients treated with either adjuvant therapy (75 % were treated with CMF and 20 % received anthracycline therapy) or no chemotherapy (14 %) [25]. CMYC encodes a multifunctional nuclear phosphoprotein that contributes to cell cycle progression, apoptosis, and cellular transformation. The CMYC gene is frequently altered in BC [18], but its role in TNBC is not well defined.

Anthracycline-based regimens are most frequently used to treat TNBC. However, some patients do not benefit from this therapy and predictors of sensitivity are urgently needed. First,

TOP2A encodes topoisomerase alpha, a target of anthracyclines, and changes in TOP2A gene/protein could influence the sensitivity to anthracyclines. Second, EGFR, a potential predictor for targeted therapy with tyrosine kinase inhibitors, has been assessed with respect to its expression in high proportion of TNBC patients. Importantly, neither TOP2A nor EGFR has been reported as predictors in TNBC. Finally, in case of Ki-67, the aim was to validate data from the above-mentioned study [25]. The objective of this work was to determine whether BCL2 or the other markers discussed above can predict outcomes in TNBC patients treated with adjuvant anthracycline-based regimens.

## Materials and methods

### Patients

A retrospective study was performed according to the REMARK criteria [26]. A consecutive series of 335 TNBC patients was diagnosed or treated at the Masaryk Memorial Cancer Institute between the years 2004 and 2009. The study included 187 patients with TNBC, 178 of whom were treated with adjuvant chemotherapy and 9 of whom received no systemic chemotherapy. The characteristics of the patient population treated with anthracycline-based adjuvant therapies ($n$=164) are summarized in Table 1. Patients treated with other chemotherapies ($n$=17) received taxane therapy ($n$=11) or CMF treatment ($n$=3). Clinical data were reviewed retrospectively from medical records, and follow-up was assessed prospectively. Patient data obtained during routine diagnostics, treatment, and follow-up were archived and anonymized, including the following: date of birth, date of histological diagnosis, age at diagnosis, number and type of tumors, clinical stage, tumor serology markers at diagnosis, performance status according Eastern Cooperative Oncology Group (ECOG) criteria, type of surgery, onset and termination of treatment, chemotherapeutic regimen, number of chemotherapy cycles, use of radiotherapy, date of therapy effect evaluation and effect of primary therapy, date of relapse, relapse localization, further therapy, date of last follow-up control, clinical status at the time of last follow-up, and cause of death for dead patients. Data on pTNM; histological tumor type; grading; and the status of the estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) were obtained from histological assessments of the patients' primary tumors. The study was approved by the University Hospital Ethics Committee.

**Table 1** Patients' clinicopathological characteristics and BCL2 expression status

| Characteristic | | $N$ | % |
|---|---|---|---|
| T | 1 | 70 | 42.7 |
| | 2 | 85 | 51.8 |
| | 3 | 3 | 1.8 |
| | 4 | 5 | 3.0 |
| | NA | 1 | 0.6 |
| N | 0 | 99 | 60.4 |
| | 1 | 43 | 26.2 |
| | 2 | 10 | 6.1 |
| | 3 | 9 | 5.5 |
| | NA | 3 | 1.8 |
| Stage | 1 | 48 | 29.3 |
| | 2 | 93 | 56.7 |
| | 3 | 23 | 14.0 |
| Grade | 2 | 9 | 5.5 |
| | 3 | 155 | 94.5 |
| Histopathological type | IDC | 119 | 72.6 |
| | ILC | 3 | 1.8 |
| | Medullary IC | 22 | 13.4 |
| | Metaplastic IC | 6 | 3.7 |
| | IC other subtypes | 9 | 5.5 |
| | IC non-differentiated | 5 | 3.0 |
| BCL2 histoscore | High | 66 | 40.2 |
| | Low | 83 | 50.6 |
| | NA | 15 | 9.1 |
| Radiotherapy | 0 | 36 | 22.0 |
| | 1 | 128 | 78.0 |
| Chemotherapy | Anthracycline-based | 164 | 100.0 |
| Anthracycline-based chemotherapy and BCL2 histoscore | Without taxane and high BCL2 | 46 | 28.0 |
| | Without taxane and low BCL2 | 51 | 31.1 |
| | With taxane and high BCL2 | 20 | 12.2 |
| | With taxane and low BCL2 | 32 | 19.5 |
| | NA | 15 | 9.1 |

*NA* not available; *IC* invasive carcinoma; *IDC* invasive ductal carcinoma; *ILC* invasive lobular carcinoma; *IC* other subtypes: papillary, micropapillary, apocrine

### Specimen characteristics and assay methods

Both immunohistochemistry (IHC) evaluations and fluorescence in situ hybridization (FISH) were performed on formalin-fixed paraffin-embedded (FFPE) samples with a thickness of 4 μm as described previously [27, 28].

The expression of the HER2 protein was determined by the DAKO HercepTest (DAKO, Denmark) and scored on a qualitative scale from 0 to 3+ according to the DAKO manual and the guidelines for HER2 testing in BC published by the American Society of Clinical Oncology/College of American Pathologists. HER2 gene status was evaluated by FISH using the PathVysion HER2 kit (Abbott Laboratories, USA). The HER2 gene status was considered to be negative (FISH non-amplified) in cases where the HER2 gene/centromere of chromosome 17 ratio was less than 1.8. An IHC score of 0/1+ was

considered indicative of HER2 negativity at the protein level. The status of the estrogen receptor alpha (ERα) and progesterone receptor (PgR) was examined by IHC using SP1 and SP2 monoclonal rabbit antibodies (Lab Vision Thermo Fisher Scientific, USA). All but three of the cancers included in our study had 0 % expression of ERα and PgR.

Basal-like (BL) subgroup was defined according Nielsen et al. [29] as cytokeratin 5/6+ and/or EGFR+.

IHC was also used to study the expression of the following proteins (the sources of the antibodies used in each case are reported in parentheses): BCL2 (mouse, Biogenex, USA), EGFR (mouse, Neomarkers, USA), TOP2A (mouse, Dako, Denmark), Ki-67 (MIB1, Cell Signaling Technology, USA), and CMYC (mouse, Novocastra, UK). Proportion scores (0–100 %) and staining intensities (0–3) were evaluated, and histoscores were calculated as described previously. BCL2 expression was assessed as high (histoscore >10) or low (histoscore ≤10). EGFR expression was assessed as high (histoscore >120) or low (histoscore ≤120). The same cutoff was used for TOP2A protein expression. For CMYC, high and low expressions were indicated by histoscores of >190 and ≤190, respectively. Ki-67 positivity was analyzed using three different dichotomizing thresholds: 14 %, the median for the studied samples, and 35 %.

FISH was used to assess EGFR/HER1 gene amplification (Orange, 7p12, Intellmed) in relation to chromosome 7 (CEP 7, Green, Intellmed), TOP2A gene amplification (Orange, 17q21–q22, Intellmed) in relation to chromosome 17 (CEP 8, Green, Intellmed), and CMYC gene expression (Orange, 8q24, Intellmed) in relation to chromosome 8 (CEP 17, Green, Intellmed). Gene/chromosome ratios were calculated for each gene and chromosome pair. Ratios of ≥1.5 and <0.8 were considered indicative of gene amplification and deletion, respectively. Gene copy numbers of >4 were also considered indicative of gene amplification. Patients were grouped into four categories based on the percentage of cells in their tumors exhibiting gene amplification or deletion: those for whom >30 % of all tumor cells had one gene copy were assigned to group "−1," those in which >50 % of all tumor cells had three or more gene copies were assigned to the "+1" group, those having five or more gene copies were assigned to the "+2" group, and those in group "0" had normal gene status.

### Study design and statistics

All laboratory assessments were performed in a blind manner, and clinical and laboratory data were subsequently combined. The endpoints were relapse-free survival (RFS), breast cancer-specific survival (BCSS), and overall survival (OS). RFS was defined as the length of time from the surgery to any relapse or death, whichever occurred first. BCSS was assessed as the time from surgery to the date of death from breast cancer or the date of last follow-up. OS was determined as the time from surgery until the date of death (from any cause) or the date of last follow-up.

STATISTICA 12.0 and R [30] were used for the statistical analyses (Spearman coefficient, Kaplan-Meier survival analysis, log-rank test, multivariate backward stepwise Cox analysis).

## Results

### High BCL2 expression predicts poor anthracycline treatment outcome

Table 1 shows the distribution of patients with low and high levels of BCL2 expression as determined by IHC staining (Fig. 1). High levels of BCL2 protein expression predicted poor RFS (log-rank $p$=0.035, HR 2.37, 95 % CI 1.04–5.42) (Fig. 2a) and poor BCSS (log-rank $p$=0.048, HR 2.63, 95 % CI 0.97–7.12) (Fig. 2b) in patients treated with adjuvant anthracycline-based regimens. High BCL2 expression also predicted a trend to poor overall survival (OS) in these patients (log-rank $p$=0.085, HR 2.15, 95 % CI 0.88–5.27) (Fig. 2c). In addition, stage (RFS $p$=0.0004, OS $p$=0.0002), tumor size (RFS $p$=0.003, OS $p$=4e−7), and nodal status (RFS $p$=0.018, OS $p$=0.016) were associated with outcome in a univariate analysis of anthracycline-treated patients. Low BCL2 expression had high negative predictive values (NPV) for relapse-free survival (89.16 %; 95 % CI 80.66–94.19 %) and OS (90.36 %; 95 % CI 82.12–95.03 %). Multivariate analysis revealed that BCL2 expression, tumor size, and nodal status all had independent predictive significance for RFS ($p$=0.005, $p$=0.091, and $p$=0.003, respectively; likelihood ratio test for the whole model, $p$=0.003), BCSS ($p$=0.012, $p$=0.077, $p$=0.01, respectively; likelihood ratio test for the whole model, $p$=0.016), and OS ($p$=0.008, $p$=0.004, $p$=0.004, respectively; likelihood ratio test for the whole model, $p$=0.0006). The initial multivariate analysis model included BCL2 status, age, tumor size, nodal status, and histopathological grade as variables (Supplementary Table S1A). Detailed information on age and follow-up is shown in Supplementary Table S2.

### High BCL2 expression predicts poor anthracycline treatment outcome in BL TNBC

High levels of BCL2 protein expression predicted poor OS in BL TNBC patients treated with adjuvant anthracycline-based regimens (log-rank $p$=0.033, HR 3.04, 95 % CI 1.04–8.91) (Fig. 2f). High BCL2 expression also predicted a trend to poor RFS (log-rank $p$=0.079, HR 2.57, 95 % CI 0.86–7.68) (Fig. 2d) and poor BCSS (log-rank $p$=0.056, HR 2.78, 95 % CI 0.93–8.3) (Fig. 2e). Multivariate analysis revealed that BCL2 expression, tumor size, and nodal status all had

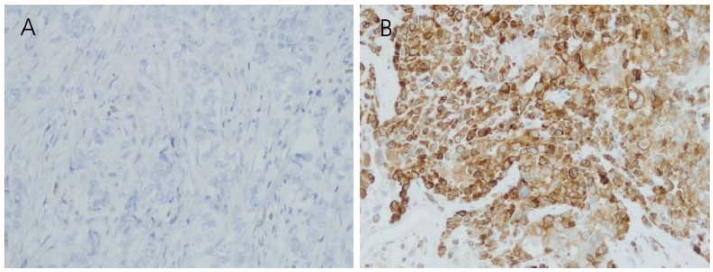



**Fig. 1** Immunohistochemical staining (IHC) of the BCL2 protein (mouse antibody, Biogenex, USA) in formalin-fixed paraffin-embedded (FFPE) triple-negative breast cancer tissue samples. **a** BCL2 low (invasive ductal carcinoma, grade 3, histoscore 0, magnification ×200). **b** BCL2 high (medullary carcinoma, grade 3, histoscore 180, cytoplasmic staining, magnification ×200)

independent predictive significance for RFS ($p$=0.029, $p$=0.013, and $p$=0.003, respectively; likelihood ratio test for the whole model, $p$=0.00125), BCSS ($p$=0.017, $p$=0.0106, $p$=0.002, respectively; likelihood ratio test for the whole model, $p$=0.00035), and OS ($p$=0.007, $p$=0.0236, $p$=0.0029, respectively; likelihood ratio test for the whole model, $p$=0.00063). The initial multivariate analysis model included BCL2 status, age, tumor size, nodal status, and histopathological grade as variables (Supplementary Table S1B). The non-BL TNBC group was too small to obtain significant associations (Supplementary Table S1C).

## EGFR protein expression and survival

High EGFR expression was present in 20 of the 148 (13.5 %) patients treated with anthracycline-based chemotherapy and 21 of all 166 (12.7 %) patients in our series. High EGFR protein expression was associated with a high probability of breast cancer-associated death in patients treated with anthracycline-based adjuvant chemotherapy (log-rank $p$=0.039, HR 2.87, 95 % CI 1.01–8.15) (Fig. 3). However, in a multivariate model including tumor size, nodal status, age, grade, and EGFR expression as variables, only nodal status had borderline independent predictive significance ($p$=0.044) for BCSS (log-rank test for the whole model, $p$=0.055). High EGFR protein expression was associated with a trend to worse BCSS and OS in all patients (log-rank $p$=0.077 and $p$=0.086, respectively) (Supplementary Figs. S1A and S1B). Again, EGFR expression was not an independent factor for BCSS or OS in this multivariate analysis. EGFR status was not associated with RFS in either anthracycline-treated patients or the whole series.

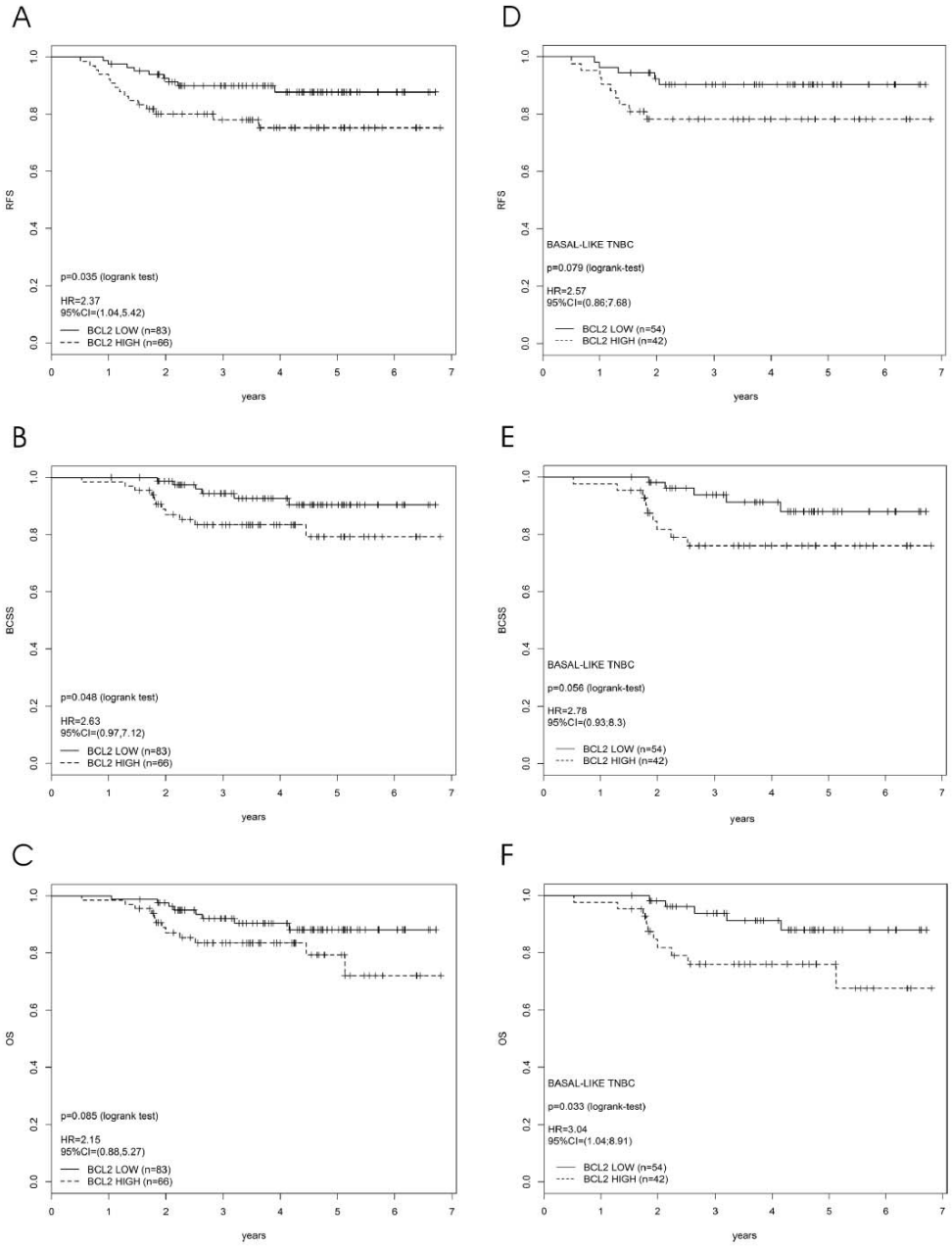
## Other markers in prognosis and prediction

Patients were considered to exhibit CMYC amplification if they had a CMYC/chromosome 8 ratio of ≥1.5 and a CMYC copy number of ≥4. Ratios and copy numbers indicative of CMYC amplification were identified in 13/143 (9.1 %) and 23/143 (16.1 %) of anthracycline-treated patients, respectively. CMYC copy numbers of ≥4 were associated with a trend to worse RFS ($p$=0.066) and BCSS ($p$=0.082) in anthracycline-treated patients (Supplementary Figs. S2A and S2B). The levels of CMYC amplification in all patients were similar to those for the anthracycline-treated cohort: 14/160 (8.8 %) exhibited a CMYC/chromosome 8 ratio of ≥1.5, while 27/160 (16.9 %) exhibited a CMYC copy number of ≥4. The association between CMYC amplification and a trend to worse BCSS ($p$=0.087) was also detected in the complete set of patients (Supplementary Fig. S2C).

Patients were considered to exhibit TOP2A amplification if they had a TOP2A/chromosome 17 ratio of ≥1.5 and a TOP2A copy number of ≥4. Ratios and copy numbers indicative of TOP2A amplification were found in 3/143 (2.1 %) and 8/143 (5.6 %) of anthracycline-treated patients, respectively. The corresponding figures for all patients were 4/160 (2.5 %) and 9/160 (5.6 %), respectively. TOP2A amplification was not associated with any of the considered outcomes (RFS, BCSS, or OS). Only one tumor exhibited a TOP2A deletion, indicated by a TOP2A/chromosome 17 ratio of <0.8.

EGFR gene amplification, indicated by an EGFR/chromosome 7 ratio of ≥1.5 and an EGFR copy number of ≥4, was rare in both anthracycline-treated patients and all patients. Of the anthracycline-treated group, 6/143 (4.2 %) exhibited a high EGFR/chromosome 7 ratio and 11/143 (7.7 %) exhibited a high EGFR copy number. The corresponding values for the whole series were 7/160 (4.4 %) and 12/160 (7.5 %), respectively. Neither EGFR amplification nor changes in the numbers of chromosomes 7, 8, or 17 were associated with any survival outcome.

All but two of the patients exhibited >14 % staining for the proliferation marker Ki-67, demonstrating the high proliferative capacity of TNBC. Ki-67 staining was analyzed as both a categorical and continual variable. However, neither analysis revealed any association between Ki-67 staining and survival


A
RFS
p=0.035 (logrank test)
HR=2.37
95%CI=(1.04,5.42)
BCL2 LOW (n=83)
BCL2 HIGH (n=66)
years
D
RFS
BASAL-LIKE TNBC
p=0.079 (logrank-test)
HR=2.57
95%CI=(0.86;7.68)
BCL2 LOW (n=54)
BCL2 HIGH (n=42)
years
B
BCSS
p=0.048 (logrank test)
HR=2.63
95%CI=(0.97,7.12)
BCL2 LOW (n=83)
BCL2 HIGH (n=66)
years
E
BCSS
BASAL-LIKE TNBC
p=0.056 (logrank-test)
HR=2.78
95%CI=(0.93;8.3)
BCL2 LOW (n=54)
BCL2 HIGH (n=42)
years
C
OS
p=0.085 (logrank test)
HR=2.15
95%CI=(0.88,5.27)
BCL2 LOW (n=83)
BCL2 HIGH (n=66)
years
F
OS
BASAL-LIKE TNBC
p=0.033 (logrank-test)
HR=3.04
95%CI=(1.04;8.91)
BCL2 LOW (n=54)
BCL2 HIGH (n=42)
years

◀ **Fig. 2** Kaplan-Meier curves for BCL2. High BCL2 expression significantly associates with poor relapse-free survival (RFS) (**a**), with poor breast cancer-specific survival (BCSS) (**b**), and with a trend to worse overall survival (OS) (**c**) in the whole cohort of TNBC treated with anthracycline-based adjuvant chemotherapy. A trend to worse RFS (**d**), BCSS (**e**), and significant association with poor OS (**f**) was found for high BCL2 expression in basal-like TNBC subgroup treated with anthracycline-based adjuvant chemotherapy. Low BCL2 expression was used as a reference for hazard ratio in all graphs (HR 1.00)

outcomes (RFS, BCSS, or OS). Similarly, the expression of the TOP2A and CMYC proteins was also not associated with any survival outcome.

## Discussion

We found an association between high BCL2 protein expression and poor outcome in TNBC patients treated with adjuvant anthracycline-based chemotherapies (this study and [31]). This treatment probably induces apoptotic cell death in tumors with low BCL2 expression. However, high levels of BCL2 expression block apoptosis, allowing the tumor cells to survive. This conclusion is supported by the observation that anti-BCL2 therapy (ABT-737) induced characteristics of apoptosis in vitro when applied in conjunction with the topoisomerase II poison etoposide [11]. An association between low levels of BCL2 expression and an enhanced anthracycline response (pCR) in neoadjuvant settings has also been reported by others [13, 15, 32]. In vitro, anti-BCL2 therapy involving treatment with a BCL2 antisense oligonucleotide known as G3139 (oblimersen) increased the MDA-MB-231 cell line's chemosensitivity to anthracyclines and taxanes [12]. However, phase I/II clinical studies examining G3139 in combination with doxorubicin and docetaxel yielded unfavorable results [33]. The authors suggested that this failure may have been due to inadequate drug delivery to the tumor. BCL2 messenger RNA (mRNA) enrichment was observed in the mesenchymal stem-like TNBC subtype [1]. In a subsequent study, only 23 % (3/13) of patients with this TNBC subtype exhibited a pCR after neoadjuvant anthracycline/taxane chemotherapy [7].

We identified low BCL2 protein expression as an independent predictor of good outcome in patients treated with anthracycline-based adjuvant chemotherapy on the basis of a multivariate analysis using an initial model whose variables included age, tumor size, nodal status, and histopathological grade. Conversely, Abdel-Fatah et al. [15] found an association between BCL2 negativity and worse survival in early primary TNBC ($n$=635). This association was independent of chemotherapy (assessed as yes or no, where yes means that the patient was receiving some form of chemotherapy and no means that they were not), traditional pathological prognostic factors (lymph node stage, tumor size, tumor grade), and other potential confounders (lymphovascular invasion, mitotic index). Interestingly, the patients studied by Abdel-Fatah et al. were treated with a range of different chemotherapies including anthracycline-based regimens ($n$=177) and CMF ($n$=104). In addition, many ($n$=354) had no chemotherapy because they either declined systemic therapy or were diagnosed and treated before TNBC was identified as an aggressive

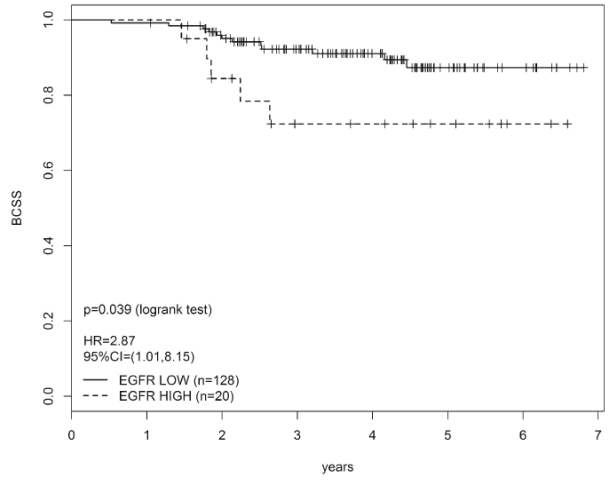



**Fig. 3** Kaplan-Meier curve for EGFR. High EGFR protein expression associates with poor breast cancer-specific survival (BCSS) in TNBC patients treated with anthracycline-based chemotherapy

tumor entity or before adjuvant chemotherapy became the standard of care. Importantly, BCL2-negative patients treated with anthracycline-containing adjuvant therapies had better survival than those treated with adjuvant CMF or no systemic chemotherapy. However, there was no significant difference in survival between patients with tumors having high and low BCL2 expressions. On the other hand, positive BCL2 protein expression was associated with good outcomes in TNBC patients treated with CMF [15, 34]. Recently, similar data were published in unselected BC [35].

BCL2 as a predictor of outcome in non-BL TNBC was recently reported by Choi et al. [36]. We could not confirm this association, probably due to small number of non-BL TNBC patients. On the other hand, Choi et al. did not find BCL2 as a predictor in BL group. Inconsistent results could be caused also by different populations studied and TNBC heterogeneity itself. Moreover, another BCL2 antibody (clone 100) was used by Choi et al., while we used the same clone as in vast majority of publications (clone 124, for more details, please see our recent review [37]). Choi et al. [36] also used more stringent criteria for BCL2 positivity which resulted in low proportion of BCL2-positive cases (9.6 %).

The advantage of BCL2 protein expression as a predictive marker is its easy detection. Routine immunohistochemical analyses of BCL2 expression in FFPE tissue samples could easily be performed at the time of diagnosis in any pathology laboratory with standard facilities.

High EGFR expression was associated with poor BCSS in the present series, which is consistent with the findings of Viale et al. [19]. Such patients may benefit from anti-EGFR-targeted therapies and should be included in relevant clinical trials.

The proportion of EGFR gene amplification observed in this work (7.5 %) was similar to that reported by Shah (5 %) [3]. The EGFR-positive tumors identified in both studies exhibited high levels of amplification; however, the low frequency of EGFR amplification probably precluded any significant association with outcomes in TNBC.

We observed a trend to worse survival among patients with CMYC gene amplification, which is consistent with previously published results for an unselected cohort of BC patients [18]. CMYC protein expression was found in the majority of the TNBC cohort examined in this work. A recent study demonstrated that xenografts with elevated MYC protein expression regressed following treatment with cyclin-dependent kinase inhibitors [38], suggesting that clinical investigations using these drugs may reveal new treatment possibilities for this particular subgroup of TNBC patients.

TOP2A amplification is also a potential predictor of responsiveness to anthracycline-based therapy [18, 21, 22, 39, 40]. However, TOP2A amplification is rare in TNBC, occurring in only 2.1 % of our anthracycline-treated cohort when detected based on gene/chromosome ratios or 5.6 % when detected based on the TOP2A gene copy number. The low frequency of TOP2A amplification probably precluded the identification of any association with outcome in this work. A study comparing cyclophosphamide, epirubicin, and 5-fluorouracil (CEF) and cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) treatments in unselected BC patients demonstrated that both TOP2A amplification and TOP2A deletion have important effects on outcome [39–41]. The anthracycline-containing CEF regimen was found to be superior to the CMF treatment for all tumors with TOP2A aberrations (including both TOP2A amplifications and deletions). This phenomenon has not yet been fully explained. However, TOP2A deletion may be a surrogate marker for chromosomal and genomic instability, which would explain the sensitivity of TOP2A-deleted tumors to the DNA-damaging anthracyclines [42]. The roles of TOP2A amplification and deletion as predictors should be analyzed in a larger prospective study.

A recent study identified TOP2A protein overexpression as a predictor of pCR based on a multivariate analysis [15]. However, our analysis of TOP2A protein expression (based on a histoscore threshold of >190) revealed no association with survival in adjuvant settings. This may be due to the intratumoral heterogeneity of TOP2A expression or variation in its expression during the cell cycle. As such, further investigation is required to determine the utility of TOP2A protein expression as a predictor of outcomes in TNBC patients treated with anthracycline-based chemotherapies. Similarly, we found no association between Ki-67 expression and survival. Munzone et al. reported that Ki-67 labeling in excess of 35 % was associated with negative survival in node-negative TNBC [25]. On the other hand, others [43–45] have reported an association between strong Ki-67 labeling and pCR in unselected BC patients undergoing preoperative chemotherapy involving mitoxantrone with methotrexate; CMF; epirubicin/docetaxel; or 5-fluorouracil, epirubicin, and cyclophosphamide. However, many of these studies used different Ki-67 labeling thresholds and the predictive capacity of Ki-67 labeling has yet to be clinically validated.

A so-called anthracycline-based score (A-score) has been developed by analyzing gene expression in breast cancers and was shown to predict anthracycline responsiveness and resistance [46]. The score is calculated by considering three gene expression signatures: a TOP2A gene signature and two signatures relating to tumor invasion and immune response. Calculation of the A-score thus requires the extraction of mRNA from the tumor and microarray analysis of gene expression, which is not possible in all pathology labs. Moreover, the method is expensive to perform and requires the availability of a specialized team. Finally, it is not an in situ method and has not been validated for use with adjuvant therapies in TNBC. Conversely, BCL2 protein expression analysis is a simple and inexpensive in situ method.

## Conclusion and further directions

To our best knowledge, this is the first study showing BCL2 protein expression as an independent predictor of outcome in basal-like (BL) TNBC treated with adjuvant anthracycline-based chemotherapy. If validated in prospective study, BCL2 protein expression could improve decision making on adjuvant chemotherapy in clinical practice [47, 48]. In addition, in vitro or in vivo studies using TNBC cell lines or xenografts should be conducted to provide mechanistic insights into the predictive role of BCL2 expression analysis.

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**Conflicts of interest** MH and RT are owners of Intellmed stock.

**Authors' contributions** KB conceived, designed, and directed the study; analyzed the data; and wrote the manuscript. MS obtained clinical data. GK performed IHC (BCL2, TOP2A, EGFR, CMYC), and JV and LR performed the statistical analyses. JB had oversight of the study's progression and contributed to the data analysis and the writing of the manuscript. RT and VK performed the FISH experiments. KC and MH contributed to the editing of the manuscript. ZK contributed to the writing of the manuscript. All authors approved the manuscript.

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# 3. Souhrn

Současná onkologie se zaměřuje především na hledání nových prognostických a prediktivních biomarkerů, nových terapeutik a léčebných strategií se zaměřením na personalizaci léčby. Velmi významným objevem byl u karcinomu prsu biomarker HER2 a na něj cílená monoklonální protilátka trastuzumab, která zásadně změnila prognózu přibližně pětiny pacientů s tímto onemocněním.

Prvním cílem předkládané práce bylo navrhnout, optimalizovat a validovat metodu, použitelnou pro detekci počtu kopií genu *HER2* u vzorků karcinomu prsu, u nichž selhala fluorescenční *in situ* hybridizace (FISH). Byla zavedena metodika kvantitativní real-time PCR, která srovnává počet kopií genu *HER2* a tří kontrolních referenčních genů (*GCS1*, *DCK* a *EPN2*). Detekční limit byl stanoven pomocí diluční řady buněčných linií CALU3 a MDA-MB-231. Senzitivita a specificita metody byla stanovena na validačním souboru 223 pacientů s invazivním karcinomem prsu. Metodika byla poté požita pro vyšetření 198/3696 vzorků karcinomu prsu, u nichž selhala FISH.

Dále jsme se zaměřili na ověření polyzomie chromozomu 17 u pacientů s karcinomem prsu. Ze tří sond, lokalizovaných na 17p a testovaných na souboru 67 pacientů s karcinomem prsu, byla vybrána sonda lokalizovaná do oblasti 17p11.2, která byla použita pro enumeraci chromozomu 17 u 297 pacientů se zvýšeným počtem kopií CEP17 (CEP17≥3). Zvýšený počet kopií 17p11.2 byl nalezen u 67 pacientů, u nichž bylo pro vyšetření chromozomu 17 použito celkově 6 markerů, pokrývajících krátké i dlouhé rameno chromozomu 17. Pravá polyzomie chromozomu 17 byla nalezena pouze u 0,48% případů. Na základě korekce počtu kopií chromozomu 17, pomocí 17p11.2 sondy, byl HER2 status reklasifikován u pětiny původně HER2 negativních/hraničních pacientů s karcinomem prsu, čímž u nich došlo ke změně doporučení léčebného režimu.

U pacientů s triple-negativním karcinomem prsu jsme se zaměřili na hledání biomarkeru, který by mohl predikovat účinnost adjuvantního antracyklinového režimu. Ze všech jedenácti testovaných markerů byla nalezena vysoká exprese BCL2 proteinu jako nezávislý prediktor špatné odpovědi na antracyklinovou terapii, a to u RFS, BCSS i OS.

V nedávné době byla identifikována celá řada biomarkerů, jejichž validita musí být, stejně jako u BCL2 exprese, potvrzena nezávislou prospektivní studií. Význam dostatečně validovaných biomarkerů je pro dnešní personalizovanou onkologii obrovský, jak je vidět z příkladu HER2. I přesto, že je tento biomarker rutinně používán přes desetiletí, je stále řada problémů, které je třeba v rámci rutinní diagnostiky řešit.

## 4. Summary

Current oncology is mainly focused towards the search of novel prognostic and predictive biomarkers, new therapeutics and therapeutic strategies leading to the development of personalized medicine. HER2 biomarker and HER2-targeted monoclonal antibody, trastuzumab was one of the most important finding in breast cancer which fundamentally influenced the prognosis of approximately 20% breast cancer patients.

The first goal of the thesis was to design, optimize, and validate the method for *HER2* copy number detection in breast cancer samples with indeterminate *in situ* hybridization result. For the purpose, the quantitative real-time PCR (qPCR) comparing HER2 copy number with the copy number of the reference genes (*GCS1*, *DCK* and *EPN2*) was designed. The detection limit was determined using the dilution series of CALU3 and MDA-MB231 cell lines, and the sensitivity and specificity of the method was validated using 223 breast cancer patient samples. The qPCR method thus developed was then used to evaluate the HER2 status of 198/3696 breast cancer tissues that yielded indeterminate FISH results.

The second objective was focused towards the verification of the chromosome 17 polysomy in breast cancer patients. Three probes localized on different parts of 17p were tested on a set of 67 breast cancer patients. The locus specific identifier (LSI) 17p11.2 probe was selected for chromosome 17 enumeration in 297 patients with CEP17 copy number increase (CEP17 $\geq$ 3.0). A total of six markers located on both long and short arms of chromosome 17 were tested in 67 breast cancer patients with 17p11.2 CNI. The true polysomy was found in 0.48% breast cancer cases. Using 17p11.2 correction of chromosome 17 copy number, the HER2 status was reclassified in 21.6% of breast cancer patients which were initially classified as non-amplified / equivocal, and the patients were considered eligible for anti-HER2 therapy.

At last, the third part of the thesis was focused on the search of biomarker, suitable for predicting the efficacy of adjuvant anthracycline therapy in triple-negative breast cancer patients. A total of eleven markers were tested. The high level of BCL2 expression was found to be the independent predictor of poor overall survival, relapse-free survival as well as breast cancer-specific survival in patients treated with anthracycline-based adjuvant therapy.

Recently, a number of biomarkers have been identified, however, they must be validated by independent prospective study, including BCL2 expression. On the other hand, the properly validated biomarkers, such as HER2, have huge significance in current personalized oncology. But, despite the fact that HER2 is being used in routine diagnostics for more than a decade, there are still many questions which remain unanswered.

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# 7. Přílohy

**Příloha 1**

**Genetic Markers in Triple Negative Breast Cancer** (draft manuskriptu; submitováno do časopisu The Breast)

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ABSTRACT

Triple negative breast cancer (TNBC) is an aggressive histological subtype characterized by negative immunohistochemical staining for estrogen receptor (ER), progesterone (PR) receptor and human epidermal growth factor receptor 2 (HER2). This heterogeneous disease is diagnosed in approximately 15-20 % of breast cancer cases. TNBC has a poor prognosis, minimal response to current chemotherapy and no possibility of targeted therapy. The common feature of all TNBC subtypes is the higher probability of relapse and lower overall survival in the first years after diagnosis. For this reason, intensive research is focused on finding new molecular targets and designing personalized therapeutic approaches. Despite its high heterogeneity, several genetic markers of TNBC have been identified. The objective of this review is to summarise the clinically relevant genetic markers in TNBC that may lead to the development of personalized targeted therapy.

KEYWORDS

Triple negative breast cancer, genetic marker, p53, PIK3CA, BRCA1/2, androgen receptor.

## INTRODUCTION

Breast cancer is the leading cause of cancer death in women worldwide and triple negative breast cancer (TNBC) accounts for approximately 15 - 20 % of all new cases. All TNBC subtypes share a basic gene expression pattern – the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, ERBB2) expression [1,2]. Despite these common features, TNBC is a very heterogeneous disease and could be divided into many distinct subgroups according to its clinical, histopathological and molecular profile [3]. TNBC patients are typically young (<40 years) [4-8], African-American [4,9-11] and have shorter progression-free survival (PFS) and overall survival (OS) compared to non-TNBC breast cancer patients [4,11,13-17]. The disease follows a more aggressive course with higher relapse rates (RR) and worse prognosis in comparison to hormone receptor-positive tumours [4,11-13]. The insidiousness of TNBC lies in the high prevalence at diagnosis of grade 3 tumours with a high proliferation rate [18]. Another feature of TNBC is the peak risk of recurrence between the first and third year (hazard ratio HR=2.6; $P<0.0001$) and the majority of deaths within 5 years after therapy (HR=3.2; $P<0.0001$) in comparison to non-TNBC phenotypes [7]. The recurrence of TNBC is associated with a high risk of metastasis in lung and central nervous system and a lower risk of metastasis in bone [7,19-23].

Considering the high RR and poor survival of TNBC patients, the urgency for establishing personalized therapy is indisputable and current investigations seek to identify genes as therapeutic targets for TNBC. Furthermore, no specific marker exists for predicting TNBC response to standard or targeted therapy. This review focuses on genetic alterations in TNBC which may serve as predictive markers of prognosis and chemotherapy benefit.

## INTRINSIC SUBGROUPS OF BREAST CANCER

Breast cancer is a heterogeneous group of diseases which may be classified according to gene expression profiles into luminal A, luminal B, basal-like, normal-like and HER2-enriched subgroups [13,24]. The PAM50 assay, a 50-gene subtype predictor, was developed based on these expression profiles [25]. These so-called ‘intrinsic’ subgroups of breast cancer display differences in incidence, age at diagnosis, prognosis and response to treatment [24,26-30].

Basal-like tumours and TNBC were grouped together until Bertucci and colleagues [31] drew attention to their different molecular signatures. At the morphological level, TNBC and basal-like breast cancer (BLBC) are similar [32] in terms of larger tumour size, higher grade, presence of geographic necrosis, pushing borders of invasion and stromal lymphocytic infiltrate [7,23,33]. However, gene expression profiles of only 71 % of TNBC samples clustered as basal-like. On the other hand only 77 % of the basal-like tumours bore TNBC signatures [31]. Prat et al. [34] confirmed this observation by molecularly characterizing 412 TNBC and 473 basal-like (based on PAM50 subtype prediction) breast cancer samples. Using this approach, 21.4 % of TNBC were not assigned as BLBC and 31.5 % of BLBC did not display a TNBC profile. Out of 412 TNBC samples, 78.6 % were identified as BLBC, 7 % were normal-like, 7.8 % were HER2-enriched, 4.4 % were luminal B and 2.2 % were luminal A.

TNBC SUBTYPES

Faced with TNBC's vast molecular heterogeneity, subsequent research focused on classifying TNBC subtypes by disease prognosis or probability of response to systematic therapy. Lehmann and colleagues [35] identified six different TNBC gene expression profile subtypes using a top-down approach of hierarchical clustering to group 587 TNBC cases identified in 21 gene expression datasets. The subtypes (summarized in Table 1) were named according to their expression patterns: basal-like 1 and 2 (BL1/2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and luminal androgen receptor (LAR). Using this classification approximately 30 TNBC cell lines were identified as models of distinct subtypes for pharmacological strategies.

Both BL1 and BL2 subtypes are sensitive to DNA-damaging agents (such as cisplatin) and display elevated levels of cell cycle and DNA damage response genes. While BL1 is characterized by high levels of cell division genes and elevated Ki67 expression, BL2 displays upregulated growth factor pathway, glycolysis and gluconeogenesis genes.

Both M and MSL subtypes are characterized by decreased distant metastasis-free survival (DMFS) and a positive response to PI3K/mTOR inhibitors and dasatinib. M and MSL subtype gene expression profiles overlap with that of chemoresistant metaplastic breast cancer and display an upregulation in epithelial-mesenchymal transition (EMT), cell motility and cellular differentiation genes. Unlike the M subtype, the MSL subtype upregulates genes involved in

angiogenesis and growth factor pathways, and downregulates proliferation genes. The MSL subtype molecularly overlaps with the previously described claudin-low subtype by displaying reduced claudin 3, 4 and 7 expression [36].

The IM subtype displays upregulated immune signalling genes (immune cell and cytokine signalling, antigen processing and presentation, core immune signalling pathways). The IM expression profile is similar to the signature of medullary breast cancer with good prognosis.

The final subtype is LAR, which displays an enrichment of genes involved in hormone signalling, steroid synthesis and androgen/estrogen metabolism. Patients of the LAR subtype have shorter relapse-free survival (RFS). This subtype overlaps with the previously described molecular apocrine group [37]. A possible therapy regime is based on the AR antagonist (bicalutamide). LAR subtype cell lines are sensitive to PI3K inhibitors due to a mutation in the PIK3CA kinase domain [2].

Masuda and colleagues analysed the predictive value of expression profiles in 130 TNBC patients who have undergone standard neoadjuvant anthracycline/taxane-based therapy [38]. The study revealed a different pathologic complete response (pCR) among TNBC molecular subtypes ($P$=0.044) and found TNBC subtype to be an independent predictor of pCR status ($P$=0.022) by a likelihood ratio test. BL1 showed the highest pCR rate (52 %), BL2 and LAR the lowest (0 % and 10 % respectively). This study confirmed the clinical relevance of TNBC subtypes in a personalized treatment strategy.

## GENETIC MARKERS IN TNBC

To date, two large studies have focused on the genetics of TNBC [39,40] and reported a diversity of genetic alterations at low frequency. The most common mutations were described for the *TP53* tumour suppressor gene. However, TNBC groups generally displayed genetic heterogeneity because the vast majority of mutations occurred only at low frequency. Moreover, only a minority of mutations (36 %) are transcribed into mRNA [39].

Shah and colleagues performed exome sequencing, RNA sequencing, high resolution single nucleotide polymorphism arrays and targeted deep resequencing on 104 primary TNBC samples grouped into various subsets. The most frequent copy number aberrations were identified for the *PARK2* (6 %), *RB1* (5 %), *PTEN* (3 %) and *EGFR* (5 %) genes. *TP53* mutations were found to be the most common event observed in 53.8% cases. Other frequent

mutations were in the *PIK3CA* (10.2 %), *USH2A* (9.2 %), *MYO3A* (9.2 %), *PTEN* and *RB1* genes (7.7 %) [39].

The Cancer Genome Atlas Group analysed samples from 463 patients by genomic DNA copy number arrays, DNA methylation, exome sequencing, mRNA arrays, microRNA sequencing and reverse-phase protein arrays. In the group of 93 basal-like tumours (76 TNBCs), the most commonly mutated genes were *TP53* (80 %), *PIK3CA* (9 %), *MLL3* (5 %), *AFF2* (4 %), *RB1* (4 %) and *PTEN* (1 %). Copy number alterations were observed in several chromosomal regions or genes: amplification or gain of *MYC* (40 %), *MDM2* (14 %), *Cyclin E1* (9 %), 1q, 10p and loss of *PTEN*, *RB1*, *INPP4B* (30 %), 8p and 5q. High expression of *CDKN2A*, low expression of *RB1* and high genomic instability were also found to be typical features of a BLBC profile [40].

The discovery of fusion gene *EML4-ALK* in non-small cell lung cancer fuelled interest in finding such a structural rearrangement in breast carcinoma, particularly in TNBC. Structural rearrangements occur mainly in ER-negative subtypes and TNBC, albeit in very low numbers. An enrichment of a MAGI3-AKT3 translocation [41] and rearrangements involving the *NOTCH1/2* and *MAST* genes [42] were identified in TNBC by whole exome sequencing.

*TP53*

*TP53* is one of the most important genes maintaining homeostasis and genome integrity though cell cycle arrest, DNA repair and apoptosis. Aberrations of *TP53* have been described in all types of human cancers [43]. Expression of mutant p53 in tumours is associated with a high proliferation rate, early disease recurrence and early death in node-negative breast cancer [44]. Aberrant expression of p53 is also linked to all breast cancer subtypes – a missense mutation is predominantly associated with the luminal subtype, and non-sense and frame-shift changes have been demonstrated in basal-like tumours [40]. In TNBC, *TP53* is the most frequently mutated gene, occurring in 62 % of basal-like and 43 % non-basal TNBC [39]. Mutations in *TP53* result in increased genetic instability, specific cytogenetic changes and higher probability of loss of heterozygosity in TNBC. Coates and colleagues [45] described the association of aberrant p53 expression with different outcomes (disease-free survival - DFS, overall survival - OS) depending on the tumour tissue's ER status. ER-negative patients with p53 expression (TNBC and HER2-positive subtypes) had better prognosis, while p53 expression in ER-positive patients was related to a worse prognosis [45,46]. Considering the

fact that *TP53* status was evaluated by IHC only, the results may be misrepresentative due to the potentially different nature of mutations within ER-positive/negative groups. Other studies found *TP53* mutations to be a poor prognostic factor causing chemoresistance [47-49]. The predictive and prognostic significance of *TP53* mutations in TNBC (as well as other types of breast cancer) differs across the spectrum of mutation types. A prospective validation study is needed to clarify the clinical relevance of these published findings.

*BRCA1/2*

The *BRCA1* and *BRCA2* genes play roles in activation and transcriptional regulation of DNA reparation, control of cell cycle, cellular proliferation and differentiation [50,51]. BRCA1/2 proteins play an essential role in DNA-double strand break repair (homologous recombination) and maintenance of DNA stability [52]. Over 80 % of hereditary *BRCA1*-mutated breast cancers are classified as TNBC and/or BLBC [3,53-57]. Sporadic cancers with the same characteristics as *BRCA1/2*-mutation carriers were described as displaying BRCAness [58]. BRCAness is present among TNBC subtypes, particularly the basal-like ones [2], which display increased DNA damage response gene expression and a higher response to cisplatin regimes and neoadjuvant anthracycline and taxane therapy [38]. Breast cancers with *BRCA1* mutations often express basal markers [30,55-57,59-61]. A promising therapeutic tool for BRCAness in TNBC has been found in poly(adenosine triphosphate-ribose) polymerase (PARP) inhibitors [62]. A large number of early phase clinical trials have investigated PARP inhibitor activity (olaparib, veliparib, niraparib, rucaparib, BMN-673) in breast cancer patients either as a single agent or in combination with chemotherapy with promising results [63-68].

Given the DNA repair defects in TNBC it was hypothesized that alkylating agents such as platinum may be used as a treatment regime. This was shown in preclinical studies of TNBC patients with *BRCA1*-defective tumours and high pCR rates for platinum based-therapy were also reported in subsequent studies [69-71]. However, the Spanish Breast Cancer Research Group (GEICAM) 2006-03 randomised phase II study which involved adding carboplatin to anthracycline/taxane-based therapy in BLBC did not report pCR improvements [72]. The effectivity of platinum in combination with standard adjuvant chemotherapy should be clarified in ongoing randomised clinical trials.

BRCA1 is a relatively large protein and hundreds of deleterious mutations have been identified to date. Not only loss of BRCA function but also mutant germline variants could have an impact on TNBC phenotype. *BRCA1* mutation 4153delA compared to 5382insC was associated with poorer OS [73,74] and epigenetically inactivated *BRCA1* (by promoter methylation) is associated with poorer prognosis as well. On univariate analysis, African-American race, node positivity, stage, and *BRCA1* promoter methylation was associated with worse RFS and OS after anthracycline or taxane based therapy. Promoter methylation of *BRCA1* was significantly associated with worse RFS and OS also based on multivariable analysis [75].

*PIK3CA*

The *PIK3CA* gene encodes the p110α catalytic subunit of PI3K, a family member of lipid kinases that mediate important cell functions such as survival, differentiation and proliferation [76]. *PIK3CA* mutations are associated with ER-positive breast cancer. The frequency of *PIK3CA* mutations in TNBC is about 10 % with enrichment in the LAR subtype. In TNBC, high activity of the PI3K/Akt/mTOR pathway is common and therapeutically targetable [77,78]. The PI3K pathway is also frequently activated through loss of *PTEN*, loss of *INPP4B*, translocation involving *AKT3* and amplification of *PIK3CA* [39,41]. Many ongoing clinical trials are evaluating mTOR, PI3K, AKT and mTOR/PI3K inhibitors alone or in combination with other therapies (cisplatin, PARP and AR inhibitors). Preclinical data have demonstrated a higher sensitivity of TNBC tumours to combination therapy [79-81].

*Tyrosine kinase receptors*

The tyrosine kinase receptors EGFR, FGFR and vascular endothelial growth factor receptor (VEGFR) have been reported as potential TNBC treatment targets. While EGFR overexpression has been described in approximately 60 % of TNBC, an *EGFR* amplification or high copy number has been reported in only 5-30 % of cases [82-85]. High *EGFR* copy number correlates with EGFR overexpression and copy number is associated with poor clinical outcome in TNBC. This fact predestines *EGFR* copy number status as a predictor in patients with TNBC for response to anti-EGFR therapy [84]. However, the clinical trials investigating cetuximab in combination with carbo/cisplatin or ixabepilone showed disappointing results with no significant effect of anti-EGFR therapy [86-88]. A recently

published neoadjuvant phase II study confirmed the efficacy of panitumumab in combination with anthracycline/taxane-based chemotherapy. Several predictive biomarkers (such as high EGFR and low cytokeratin 8/18 expression and high density of CD8+ tumour-infiltrating lymphocytes) were identified [89].

A mutation in *FGFR1* or *FGFR2* may work as a driver mutation and occurs in approximately 9 % and 4 % of TNBC, respectively. Turner and colleagues confirmed the constitutive activation of *FGFR2* in *FGFR2* amplified cell lines. The *FGFR1/2* amplified cell lines were also highly sensitive to FGFR inhibitors (brivanib/PD173074) [58,90].

VEGFR, another potential therapeutic target, promotes angiogenesis [91]. Even if the *VEGFR* amplifications or mutations are very rare in TNBC, a number of clinical trials have confirmed that addition of bevacizumab to chemotherapy significantly elevates pCR rates in TNBC [92-95]. Other studies have investigated the efficacy of multi-targeted tyrosine kinase receptor inhibitor sunitinib. Despite its promising effect in preclinical models [96] sunitinib did not improve efficacy in clinical trials [97,98]. Clinical trials which may clarify the efficacy of bevacizumab and tyrosine kinase inhibitors (sunitinib, sorafenib) in TNBC are underway.

*Androgen receptor*

The androgen receptor (AR) as well as the ER and PR belong to the nuclear steroid hormone receptor family. Steroid hormone receptors have an important role in cell signalling; their essential function lies in regulating gene expression [99-101]. The expression of AR has been found in approximately 70 % of breast cancers and is associated with ER positivity. In breast cancer, positive expression of AR is generally correlated with decreased risk of recurrence and better OS and DFS [100,102]. In TNBC, AR is expressed in approximately 10 to 20 % of cases [2] and AR positivity was associated with better clinical outcome in the majority of studies including one meta-analysis [100,103-105]. Other studies did not confirm AR-positivity as predictor of better outcome [8,106]. A recent study described a significantly lower response to neoadjuvant chemotherapy (pCR 10 % vs. overall pCR 28 %) in AR positive patients of the LAR subtype. Despite the low pCR rate, the OS and DMFS were better compared to other TNBC groups [38]. AR is a promising and easily detectable marker which can identify subgroups of TNBC patients that will derive no clinical benefit from standard chemotherapy. These patients could probably profit from targeted therapy based on

AR antagonists alone or in combination with PI3K inhibitors. Clinical studies with AR-positive TNBC are underway.

## CONCLUSIONS

Current research is focused on finding genes that may serve as therapeutic targets for all or one particular TNBC subtype. High-throughput analysis tools (such as DNA methylation, exome sequencing, mRNA arrays, genomic DNA copy number arrays etc.) help to understand the nature of TNBC; however, the gross datasets require deciphering. The main problem of TNBC is its immense heterogeneity which blights the identification of markers. To find a proper, universal gene or marker which could predict therapy response or patient prognosis is unrealistic in TNBC. Nevertheless, several promising markers and targeted therapeutics have been found and have to be verified in well-defined prospective clinical studies. As in the clinical trials of AR-positive TNBC patients, the marker-selected population should be preferentially evaluated.

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Table 1 TNBC subtypes [35].

| Subtype | Signalling pathways | Important markers | Chemosensitivity | Potential therapy |
|---|---|---|---|---|
| BL1 | Cell cycle, proliferation and DNA damage pathways | ATR, BRCA, MYC, NRAS, Ki-67 | very good | Cis-platin, PARP inhibitors |
| BL2 | Cell cycle, proliferation, growth factor signalling, glycolysis, gluconeogenesis | EGFR, MET, EPHA2, TP63 | very poor | Cis-platin; PARP and growth factor inhibitors |
| IM | Immune signalling | JAK1/2, STAT1/4, IRF1/7/8, TNF | medium | - |
| M | EMT, cell motility, differentiation, proliferation | Wnt, ALK, TGF-β | medium | PI3K/mTOR, Src inhibitors |
| MSL | EMT, cell motility, differentiation, growth factor signalling, angiogenesis | EGFR, PDGFR, ERK1/2, VEGFR2 | medium | PI3K/mTOR, Src inhibitors |
| LAR | Androgen/estrogen metabolism, steroid synthesis, porphyrin metabolism | AR, FOXA1, KRT18, XBP1 | poor | AR antagonist; PI3K, Hsp90 inhibitors |

**Příloha 2**



# Personalizovaná medicína a biomarkery v onkologii

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**Článek popisuje možnosti současné personalizované medicíny v onkologii, a to konkrétně prediktivní význam biomarkerů pro účinnost cílené terapie. Autoři se věnují rutinně používaným biomarkerům stejně jako testovaným biomarkerům, jejichž verifikace a následné zavedení do klinické praxe je v současné době předmětem intenzivního výzkumu.**

**Klíčová slova:** biomarker, personalizovaná medicína, cílená léčba, onkologie.

## *Personalized medicine and biomarkers in oncology*

**This paper describes current options of personalized medicine in oncology, particularly the importance of predictive biomarkers for targeted treatment response. The authors discuss the routinely used biomarkers as well as tested biomarkers. Verification and subsequent usage of these biomarkers in clinical practice is currently the subject of intensive research.**

**Key words:** biomarker, personalized medicine, targeted therapy, oncology.

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## Úvod

Současná onkologie zaznamenala v posledních několika letech velký posun v oblasti studia diagnostických, prognostických a prediktivních biomarkerů. Řada výsledků intenzivního výzkumu v této oblasti je v současné době implementována do diagnosticko-léčebné praxe. Do popředí zájmu se dostávají především protinádorová léčiva, specifická pro konkrétní typ nádoru, a biomarkery, které s dostatečnou pravděpodobností definují skupinu pacientů, kteří z této léčby budou profitovat. Dochází tak k personalizaci onkologické léčby na základě genetických a/nebo proteomických charakteristik samotného nádoru.

Charakteristika nádorového biomarkeru je velmi obtížná, jedná se o heterogenní skupinu měřitelných znaků, kterými lze spolehlivě odlišit nádorovou buňku od jejího nenádorového progenitoru. Nádorové biomarkery jsou v současné době používány v oblasti diagnostiky, prognózování nemocí, predikci léčebné odpovědi a monitorování průběhu onemocnění (1). Vzhledem k rozsáhlosti této oblasti je následující text věnován pouze prediktivním biomarkerům.

## Biomarkery, používané pro predikci účinnosti terapie

K prvním biomarkerům, které byly rutinně zavedeny pro individualizaci léčby, patří vyšetření exprese estrogenových/progesteronových receptorů. Nadměrná exprese těchto receptorů se vyskytuje až u 70% pacientů s karcinomem prsu a je prediktorem dobré léčebné odpovědi na hormonální terapii (2). Velmi významný byl i objev fúzního proteinu BCR-ABL, který znamenal zvrat v léčbě chronické myeloidní leukemie (CML). Přítomnost BCR-ABL fúze, která se vyskytuje u 95% pacientů s CML, je prediktorem dobré léčebné odpovědi na multikinázový inhibitor imatinib (nilotinib, dasatinib). Léčba imatinibem je rovněž velmi účinná u gastrointestinálních nádorů (GIST), kde blokuje kinázovou aktivitu mutovaného onkogenu c-KIT (3, 4). V posledních několika letech byly objeveny další prediktivní biomarkery, které našly široké uplatnění v klinické praxi (tabulka 1).

### HER2

HER2 kóduje transmembránový protein p185, patřící do rodiny receptorů pro epidermální růstový faktor (EGFR/ErbB). Za fyziologických podmínek reguluje buněčný růst a diferenciaci. Amplifikace způsobující nadměrnou expresi proteinu HER2 bývá nalezena přibližně u 15–20% pacientů s karcinomem prsu, nejčastěji u duktálního typu s negativitou hormonálních receptorů. HER2 pozitivní nádory prsu jsou agresivnější, často metastazují do CNS, jsou méně citlivé na standardní chemoterapii. HER2 amplifikace/nadměrná exprese je nezávislým negativním prognostickým faktorem (5). Pacientům s HER2 pozitivním karcinomem prsu lze indikovat humanizovanou monoklonální protilátku IgG1 trastuzumab (Herceptin), a to jak v paliativním, tak neo-/adjuvantním režimu. Trastuzumab blokuje receptorem aktivovanou signalizaci a navozuje protinádorovou imunitní odpověď (ADCC). Trastuzumab je podávaný intravenózně v monoterapii či kombinaci s taxany, inhibitory aromatázy, vinorelbinem či kapecitabinem (6, 7). Při progresi onemocnění lze HER2 blokovat lapatinibem (Tyverb), duálním inhibitorem EGFR a HER2, v kombinaci s kapecitabinem (8). Velmi slibně vypadají i výsledky studií, kombinující trastuzumab s lapatinibem, které prokázaly synergní antiproliferační efekt této kombinace, a to i v neo-adjuvantním podání (9, 10).

Trastuzumab je v kombinaci s cisplatinou a kapecitabinem nebo 5-fluorouracilem (5-FU) indikován pacientům s metastatickým HER2 pozitivním adenokarcinomem žaludku. HER2 nadměrná exprese/amplifikace se u nádorů žaludku vyskytuje přibližně u 20% případů a je asociována s intestinálním typem nádoru (11, 12).

### EGFR

EGFR kóduje transmembránový tyrosinkinázový receptor s obdobnou funkcí jako HER2. U karcinomu plic byly popsány aktivační mutace EGFR genu, jejichž výskyt je přibližně 30% u Asiatů a 15% v kavkazské populaci. Mutace se vyskytují téměř výhradně u plicního adenokarcinomu. Nejčastější mutace, delece v exonu 19 (báze 746–753) a substituce argininu za leucin (L858R) v exonu 21 bývají nalezeny u 90% případů (13). U pacientů s aktivační mutací EGFR byla jednoznačně prokázána účinnost nízkomolekulárních ATP-kompetitivních inhibitorů, gefitinibu (Iressa), resp. erlotinibu (Tarceva), které lze indikovat pacientům s pokročilým plicním karcinomem a prokázanou aktivační mutací genu EGFR v první, resp. druhé a třetí linii léčby. Prokázána je rovněž lepší odpověď na erlotinib/gefitinib u pacientů se zvýšeným počtem kopií genu EGFR (14, 15).

**Tabulka 1.** Rutinně používané biomarkery cílené terapie

| Biomarker | Lokalizace | Druh mutace | Metoda vyšetření | Diagnóza | Léčivo | Typ | Léčba | Způsob podání | Dávkování |
|---|---|---|---|---|---|---|---|---|---|
| c-KIT | 4q12 | mutace | IHC | GIST | imatinib | ATP kompetitivní NIB | paliativní, adjuvantní | orálně | 400 mg denně nebo 800 mg 2× denně |
| HER2 | 17q21 | amplifikace/ nadměrná exprese | IHC, ISH | BrCa | trastuzumab | humanizovaná mAb | paliativní, adjuvantní, neoadjuvantní | intravenózně | úvodní – 4 mg/kg hmotnosti; dále – 2 mg/kg týdně |
| | | | | | | | | | úvodní – 8 mg/kg hmotnosti; dále – 6 mg/kg á 3 týdny |
| | | | | | lapatinib | ATP kompetitivní NIB | paliativní | orálně | 1250 mg/denně |
| EGFR | 7q31 | mutace/ delece | PCR | NSCLC | erlotinib | ATP kompetitivní NIB | paliativní | orálně | 150 mg/denně |
| | | | | | gefitinib | ATP kompetitivní NIB | paliativní | orálně | 250 mg/denně |
| KRAS | 12p12 | mutace | PCR | CC | cetuximab | chimerická mAb | paliativní | intravenózně | úvodní – 400 mg/m$^2$; dále – 250 mg/m$^2$ týdně |
| | | | | | | | | | 500 mg/m$^2$ á 2 týdny |
| | | | | | panitumumab | humanizovaná mAb | paliativní | intravenózně | 6 mg/kg hmotnosti á 2 týdny |
| BRAF | 7q34 | bodová mutace | PCR | melanom | vemurafenib | ATP kompetitivní NIB | paliativní | orálně | 960 mg/2× denně |
| ALK | 2p23 | inverze (translokace) | IHC, ISH | NSCLC | crizotinib | ATP kompetitivní NIB | paliativní | orálně | 250 mg/2× denně |

GIST – gastrointestinální tumor; BrCa – karcinom prsu; NSCLC – nemalobuněčný karcinom plic; CC – karcinom kolorekta; NIB – nízkomolekulární inhibitor; mAb – monoklonální protilátka; IHC – imunohistochemie; ISH – in situ hybridizace; PCR – polymerázová řetězová reakce

Zvýšená exprese/počet kopií genu EGFR je rovněž, společně s wild-type KRAS, prediktorem dobré odpovědi na léčbu cetuximabem (Erbitux)/panitumumabem (Vectibix) u metastatického kolorektálního karcinomu (viz kapitola KRAS) (16).

## KRAS

KRAS gen kóduje na membránu vázaný protein s GTPázovou aktivitou, který zprostředkovává signální přenos mezi EGFR a dalšími proteiny proliferační kaskády Ras/Raf/MEK/ERK. Přibližně u 40 % pacientů s kolorektálním karcinomem bývá nalezena mutace genu KRAS, nejčastěji se jedná o bodovou mutaci kodonu 12 (záměna glycinu za valin/asparagin), následovanou mutacemi v kodonu 13 a 61 (17). U KRAS mutovaných případů je onkogenní signál, vzhledem k jeho umístění v signální kaskádě, nezávislý na EGFR aktivaci a u karcinomu kolorekta je proto mutovaný KRAS prediktorem špatné odpovědi na blokátory EGFR (cetuximab/panitumumab) (16, 18). Cetuximab je chimérická protilátka IgG1, která blokuje extracelulární doménu EGFR, stimuluje degradaci EGFR a protinádorovou imunitní odpověď. Cetuximab je indikován pacientům s metastatickým kolorektálním karcinomem s expresí EGFR genu a wild-type KRAS v první i druhé linii léčby, a to jak v monoterapii (po selhání léčby oxaliplatinou a irinotekanem), tak v kombinaci s chemoterapií. Panitumumab je plně humanizovaná protilátka IgG2, blokující signalizaci EGFR, která je indikována pacientům s metastatickým kolorektálním karcinomem v monoterapii (po selhání léčby fluoropyrimidinem, irinotekanem a oxaliplatinou) či v kombinaci s chemoterapií (17).

Mutace genu KRAS se vyskytují přibližně u 20 % případů nemalobuněčného plicního karcinomu (NSCLC). KRAS mutace se vyskytují častěji u pacientů s adenokarcinomem, v kavkazské populaci a u kuřáků. U pacientů s NSCLC byl prokázán negativní prediktivní účinek při léčbě gefitinibem/erlotinibem. Nicméně, EGFR a KRAS mutace jsou u NSCLC mutačně exkluzivní, klinický význam vyšetřování KRAS pro predikci účinnosti biologické léčby je tedy minimální (19).

## BRAF

BRAF gen kóduje serin/threonin kinázu přenášející signál od proteinu KRAS, jejíž mutace bývá nalezena u 40–70 % melanomů. Z více než 90 % se jedná o substituci valinu za glutamát v kodonu 600 (V600E), která vede k aktivaci kinázové aktivity a přenosu mitogenního signálu. Pacientům s prokázanou mutací V600E a neresekovatelným či metastazujícím melanomem lze v první i dalších liniích léčby indikovat v monoterapii specifický BRAF inhibitor vemurafenib (Zelboraf) (20, 21). Vemurafenib cílené způsobuje programovanou buněčnou smrt pouze u buněk melanomu s V600E mutací. U pacientů s melanomem bez V600E mutace se zdá, že vemurafenib paradoxně podporuje buněčný růst (22).

## ALK

ALK gen kóduje transmembránový tyrosinkinázový receptor, jehož fyziologická funkce není zcela objasněna a za fyziologických podmínek je exprimován pouze v tenkém střevě, mozku a varlatech. Patologická exprese, způsobená inverzí malé části krátkého ramene chromozomu 2 za vzniku fúzního proteinu EML4-ALK, bývá nalezena u 2–7 % pacientů s NSCLC. Tato chromozomální přestavba se vyskytuje u mladších pacientů s adenokarcinomem a nekuřáků bez současných mutací KRAS či EGFR (23). Pacientům s metastatickým či pokročilým plicním karcinomem a prokázanou přestavbou genu ALK lze v monoterapii indikovat crizotinib (Xalkori), duální inhibitor C-MET a ALK, který v druhé fázi klinického testování ukázal velmi slibné výsledky (24, 25).

## Biomarkery toxicity léčby

Molekulární biomarkery lze použít i pro personalizaci běžně užívané chemoterapie. Metabolická dráha 5-FU zahrnuje geny, jejichž mutace vedou k systémové toxicitě způsobené hromaděním toxických metabolitů. Mutace a snížená exprese genu DPYD pro dihydropyrimidin dehydrogenázu (DPD), resp. snížená aktivita DPD zodpovědné za odbourávání 5-FU, vede k hromadění aktivního metabolitu v těle pacienta, a tím extrémní senzitivitě na běžné dávky 5-FU. Bylo nalezeno více jak 50 mutací tohoto genu, které jsou u přibližně 3–5 % populace aso-

cionány s toxicitou léčby 5-FU (26, 27). Stanovení aktivity enzymu DPD, které lze provést na úrovni mRNA z periferní krve, však v současnosti stále není součástí rutinního screeningového vyšetření před zahájením léčby 5-FU, přestože jde o závažný farmakogenetický problém s fatálními následky (28). Zvýšená exprese thymidylát syntázy (TS) způsobená mutací genu TYMS je spojena taktéž se zvýšenou citlivostí na léčbu 5-FU vedoucí až k toxicitě (29, 30). Podobně mutace v genu pro enzym thiopurin-S-metyltransferázu (TPMT) predikují citlivost k léčbě thiopuriny a mohou vést až ke snížené toleranci běžně užívaných dávek (31).

Homozygotní varianta alely UGT1A1 (UGT1A1*28) je přítomna přibližně u 10 % populace a způsobuje výrazné snížení aktivity enzymu UDP-glukuronyltransferázy, který katalyzuje biotransformaci významného protinádorového léčiva – irinotekanu. Významné snížení enzymatické aktivity vede k závažným toxickým postižením. Vyšetření mutace genu je doporučováno FDA od roku 2005 (32), v ČR je dostupné, nicméně není součástí rutinní praxe.

Projevy toxicity mohou být i dobrým prediktorem pro účinnost terapie, jak je tomu u EGFR-cílených terapií (33). Kožní toxicita, zapříčiněná vazbou léčiva na EGFR a inhibicí jeho signální dráhy v keratinocytech, koreluje s dobrou účinností léčby u kolorektálního a plicních karcinomů (1, 34).

## Testované biomarkery

Díky intenzivnímu výzkumu posledních let a s rozvojem nových technologií byla identifikována řada markerů, které souvisí se samotným vznikem nádoru či jeho rezistencí na použitá cytostatika. Tyto markery jsou v současné době testovány jako potenciální cíle protinádorové terapie (tabulka 2).

### C-MET

C-MET proto-onkogen kódující hepatocytární růstový receptor (HGFR) s tyrozin kinázovou aktivitou je amplifikován u celé řady nádorových onemocnění. U NSCLC se získanou rezistencí na gefitinib/erlotinib bývá amplifikace C-MET nalezena přibližně u 20 % případů (35). V současnosti je u NSCLC gen C-MET jedním z nejnadějnějších prediktivních markerů, současná blokace EGFR a C-MET vypadá jako slibná strategie pro léčbu pacientů se získanou rezistencí na gefitinib/erlotinib. Nízkomolekulární inhibitor ARQ197 (tivantinib) se nachází ve fázi III klinického testování. Výsledky fáze II (NCT00777309) potvrdily výrazné zlepšení PFS i OS u pacientů s lokálně pokročilým nebo metastatickým neskvamózním plicním karcinomem léčených kombinací tivantinibu a erlotinibu oproti samotnému erlotinibu (36). Dalším testovaným inhibitorem je crizotinib, indikovaný NSCLC pacientům s ALK přestavbou, který vykazuje slibné účinky u pacientů s NSCLC a de novo amplifikací C-MET (37). Crizotinib se zdá být účinný rovněž u pacientů s NSCLC s nedávno objevenou translokací ROS1 (38). Účinnost monoklonální protilátky MetMAb (onartuzumab) proti C-MET je testována v klinické studii fáze III (NCT01456325) v kombinaci s erlotinibem u C-MET pozitivních pacientů s pokročilým či metastatickým NSCLC (39).

### PI3K/AKT/mTOR

Molekuly účastnící se signální dráhy PI3K/AKT/mTOR patří mezi nejvíce studované v souvislosti se vznikem různých druhů nádorových onemocnění (40–46). PIK3CA mutace a ztráta exprese PTEN je spojena s rezistencí na trastuzumab, respektive s horší prognózou onemocnění při léčbě karcinomu prsu (47, 48) a s rezistencí na léčbu cetuximabem u kolorektálního karcinomu (49). Dvě hlavní aktivační mutace PIK3CA (E545K a H1047R) a mutace PTEN, vedoucí ke ztrátě funkce, jsou spojeny s rezistencí na lapatinib u pokročilého HER2 pozitivního prsního karcinomu (50). Cestou ke zvrácení rezistence je duální inhibitor PI3K/mTOR NVP-BEZ235 (51), který je v současnosti testován v klinických studiích I a II fáze (NCT00620594). U celé řady nádorových onemocnění se v klinických studiích testují také další nízkomolekulární inhibitory PI3K jako BKM120 – panPI3K inhibitor (NCT01501604) či BYL719 – selektivní inhibitor PI3K (NCT01219699).

U pacientů s karcinomem prsu a mutací AKT byla prokázána rezistence na tamoxifen. AKT mutantní karcinomy jsou cílem nových inhibitorů účastnících se klinických studií jako např. panAKT inhibitor MK-2206 (52). Inhibitory mTOR jsou používány pro léčbu různých typů malignit (53, 54, 55). V současnosti je studium zaměřeno zejména na odhalení prediktorů účinnosti mTOR cílené léčby.

### BRCA1/2

Cílená léčba karcinomů prsu a ovarií s BRCA1/2 mutacemi je zaměřená na využití tzv. „synthetic lethality" principu za použití inhibitorů poly (ADP-ribóza) polymerázy (PARP). Předpokladem je zablokování excizní opravy DNA zajišťovanou PARP, která společně s BRCA1/2 mutacemi, poškozujícími opravy dvouvláknových zlomů, vede k usmrcení buněk (56). Klinické studie fáze II u pokročilého karcinomu prsu a ovarií přinesly slibné výsledky v léčbě olaparibem (KU-59436, AZD2281) (57, 58). Taktéž klinické studie druhé a třetí fáze zaznamenaly úspěchy v léčbě triple-negativního karcinomu prsu iniparibem (BSI-201) v kombinaci s gemcitabinem a karboplatinou (59, 60). Součástí klinického testování jsou i další PARP inhibitory jako

***Tabulka 2.*** Testované biomarkery cílené terapie

| Biomarker | Lokalizace | Druh mutace | Diagnóza | Léčivo | Typ | Léčba | Způsob podání |
|---|---|---|---|---|---|---|---|
| ROS1 | 6q22 | translokace | NSCLC | crizotinib | ATP kompetitivní NIB | paliativní | orálně |
| C-MET | 7q31 | amplifikace | NSCLC | tivantinib | ATP kompetitivní NIB | paliativní | orálně |
| | | | | onartuzumab | humanizovaná mAb | paliativní | intravenózně |
| PI3K | 3q26.3 | mutace | BrCa | NVP-BEZ235 | ATP kompetitivní NIB | paliativní | orálně |
| | | | | BKM120 | ATP kompetitivní NIB | paliativní | orálně |
| | | | AST | BYL719 | ATP kompetitivní NIB | paliativní | orálně |
| AKT | 14q32.32 | mutace | AST | MK-2206 | alostérický inhibitor | paliativní | orálně |
| BRCA1/2 | 17q21/ 13q12.3 | mutace | BrCa a ca ovárií | olaparib | ATP kompetitivní NIB | paliativní | orálně |
| | | | TNBC | iniparib | ATP kompetitivní NIB | paliativní | orálně |
| MGMT | 10q26 | hypermetylace | nádory CNS | temozolomid | alkylační činidlo | paliativní | orálně |
| TOP2A | 17q21.2 | amplifikace | BrCa | antracykliny | inhibitor topoizomerázy | paliativní, adjuvantní | intravenózně |

BrCa – karcinom prsu; NSCLC – nemalobuněčný karcinom plic; TNBC – triple negativní karcinomy prsu; AST – pokročilé solidní nádory; CNS – centrální nervová soustava; NIB – nízkomolekulární inhibitor; mAb – monoklonální protilátka

AG-014966, ABT-888, MK-4827 (61). Uplatnění principu „synthetic lethality" a účinek PARP inhibitorů se předpokládá také u tzv. „BRCAness" nádorů, které sice nenesou BRCA1/2 mutace, ale systém opravy DNA mají poškozený jiným způsobem. Příkladem může být poškození genu PTEN, jež podle posledních poznatků vede ke genomové nestabilitě (62, 63) a které ve studii na *in vitro* a xenograftových modelech zvyšuje účinek PARP inhibitorů na nádorové buňky (64).

### MGMT

Nové biomarkery se testují i na epigenetické úrovni. Umlčení MGMT genu pro O-6-metylguanin-DNA metyltransferázu, prostřednictvím hypermetylace promoteru, vede ke snížení exprese MGMT, což je spojeno s lepší odpovědí na léčbu alkylačními činidly (65), resp. temozolomidem u gliálních nádorů mozku (66). Zavedení testování MGMT do rutinní diagnostiky brání především nejednotnost používaných analytických metod pro určení míry metylace.

## Závěr

Intenzivní výzkum v oblasti klinické onkologie přinesl za poslední desetiletí celou řadu nových léčiv a prediktorů léčebné odpovědi, což umožnilo personalizovat terapii na základě genetických/expresních změn nádoru. Markery jako HER2, EGFR, KRAS, BRAF a ALK, jejichž prediktivní význam byl prokázán řadou klinických studií, jsou v současné době nedílnou součástí rutinní diagnostiky, jejich testování má bezesporu zásadní vliv na volbu adekvátního terapeutického postupu a zkvalitnění života konkrétního pacienta.

V probíhajících klinických studiích je sledována řada dalších biomarkerů, jejichž alterace jsou u různých nádorových onemocnění často nacházeny a stávají se tak potenciálními cíly pro protinádorovou terapii. Velkým problémem stále zůstává nejednotnost diagnostických metod a hodnotících kritérií jednotlivých studií a v řadě případů rovněž minimální množství materiálu, které je limitující pro kvalitní stanovení všech potenciálních markerů. Příslibem pro identifikaci nových cílů protinádorové terapie jsou moderní techniky jako sekvenování nové generace a čipové technologie umožňující získání kompletní informace o celém genomu/transkriptomu pacienta v rámci jednoho vyšetření.

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**Příloha 3**

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# Non-small cell lung cancer - genetic predictors

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**Background.** Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer that is the leading cause of cancer-related mortality worldwide. Several predictive markers have been found in NSCLC patients to date but only a few are currently used for tailored therapy.
**Methods and Results.** PubMed and Web of Science online databases were used to search review and original articles on the most important predictive markers in NSCLC.
**Conclusion.** EGFR activating mutations (exons 18 to 21) and EML4-ALK rearrangement are clinically important markers able to select NSCLC patients which benefit from EGFR or ALK tyrosine kinase inhibitors (gefitinib, erlotinib, crizotinib). Other markers, such as KRAS mutation, EGFR T790M mutation and C-MET amplification, are responsible for resistance to these inhibitors. Overcoming of this resistance as well as discovery of new potential markers and inhibitors is the main goal of ongoing research and clinical trials in NSCLC.

**Key words:** NSCLC, EGFR, KRAS, ALK, C-MET, ROS1, tyrosine kinase inhibitors, resistance

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## INTRODUCTION

Lung cancer is the most frequent cause of cancer-related deaths worldwide and it is responsible for more than 1 million deaths annually[1,2]. The main reason is high tumor aggressivity and high metastasis potential. Non-small cell lung cancer (NSCLC) is diagnosed in approximately 85% of lung cancer cases and includes the adenocarcinoma, squamous cell carcinoma and large cell carcinoma subtypes[3]. The intensive research has been made in the past few years on genetic, transcriptional, translational and epigenetic levels and the remarkable discoveries have been found. At least nine important driver mutations causing NSCLC have been described, mainly in adenocarcinoma subtype. Several markers are already used for best treatment strategy selection. Developing new drugs targeting the markers, clarification of predictive value of these markers as well as new markers discovering is still the subject of intensive research[4]. In this review, the clinically most important genetic alterations in NSCLC, such as EGFR, KRAS, C-MET, EML4-ALK and ROS1 are summarized.

## EGFR

The epidermal growth factor receptor (EGFR) gene is located on 7p11 and encodes a tyrosine-kinase receptor from the HER family which is involved in development, progression, angiogenesis and metastasis of various cancer types. After ligand binding (EGF, TGF-α, amphiregulin), the receptor hetero-/homodimerizes, autophosphorylates tyrosine residues and activates two main downstream signaling pathways – RAS/MAPK and PI3K/AKT (ref.[5]). Three mechanisms of EGFR activation in tumor cells have been described, including EGFR mutations, amplification/gene copy number gain (CNG) and overexpression.

### Amplification/overexpression

EGFR overexpression is found in up to 80% of NSCLC cases and EGFR CNG/amplification is found in almost 60% of them, while these events often occur concurrently[6-12]. Increased EGFR expression was considered to be a poor prognostic factor in NSCLC patients[12,13] but a meta-analysis combining 18 studies of 2972 patients did not confirm the prognostic significance of EGFR expression (HR=1.14; 95% CI 0.97-1.34; $P$=0.103) (ref.[14]).

The predictive value of EGFR amplification/overexpression for responsiveness to EGFR tyrosine kinase inhibitors (EGFR TKIs) was tested in several studies. Initial studies, including the large trials BR.21 and ISEL, found clear association between increased EGFR copy number and good response to EGFR TKIs(ref.[9,15-17]). Other studies have not confirmed this finding[18,19]. In a recent meta-analysis[20] which combined 22 independent studies (2005-2009) including 1821 NSCLC patients treated with EGFR TKIs monotherapy, EGFR CNG was significantly associated with increased overall survival (OS) (HR=0.77; 95% CI 0.66-0.89; $P$=0.001), progression-free survival (PFS) (HR=0.60; 95% CI 0.46-0.79; $P$<0.001) and time-to-progression (TTP) (HR=0.50; 95% CI 0.28-0.91; $P$=0.02). The following studies published by Brugger et al.[21] and Hirsch et al.[22] did not confirm the predictive significance of EGFR FISH positivity to erlotinib. The clinical relevance of EGFR amplifications is difficult to decipher

because about 50% of EGFR-mutated cases show the co-existence of increased EGFR copy number. The predictive value of EGFR copy number could be therefore affected by the occurrence of simultaneous EGFR mutation[8,15,23]. At the present, EGFR copy number testing is not recommended in the selection of treatment in NSCLC.

**Activating mutations**

In 2004, two independent research groups[24,25] sequenced EGFR in advanced NSCLC patient samples. The aim was to evaluate the possible predictive value of EGFR mutations for EGFR TKIs therapy. In 14 out of 15 patients who were good responders to gefitinib therapy, small in-frame deletions or amino acid substitutions were identified. No EGFR mutations were found in gefitinib non-responders. In these studies, EGFR activating mutations were identified[24,25].

Activating mutations of EGFR, occurring in exon 18 to 21 in the ATP-binding pocket part of the tyrosine-kinase domain, have been reported in 5 to 30% NSCLC cases depending on study population (app. 15% incidence in Caucasians compared to 30% in Asians). These mutations lead to a ligand-independent EGFR activation and are preferentially found in never/former smokers, women, East Asians and patients with adenocarcinoma histology. More than 3000 somatic EGFR mutations have been described to date[26]. Deletions in exon 19 (including residues 746 to 753) and arginine to leucine substitution (L858R) in exon 21 constitute about 90% of them[6,8,27]. Substitution of glycine to serine, alanine or cysteine in codon 719 (G719X) of exon 18 occurs in an additional 4% of cases and other missense mutations and small in-frame duplications/insertions in exon 20 account for the rest[6].

**Targeted therapy**

The most effective inhibitors of EGFR tyrosine kinase signalization are the small anilinoquinazoline derivatives, that act as reversible ATP-competitive inhibitors, erlotinib (Tarceva®, Genentech) and gefitinib (Iressa®, AstraZeneca).

After successful preclinical[28] and phase I clinical studies[29,30], gefitinib progressed to phase II studies. Objective response rates between 10 and 20% were reported in two double-blind, randomized phase II trials (IDEAL 1 and 2) which enrolled 210 and 221 NSCLC patients previously treated with one or two regimes[31,32]. Based on these results, gefitinib was FDA approved for advanced NSCLC patient treatment in May 2003. Based on results from unsuccessful ISEL study, in June 2005, FDA limited the use of gefitinib. However, the IPASS trial confirmed the benefit of patients with EGFR mutations of gefitinib therapy and European Medicines Agency (EMA) approved gefitinib for the treatment of locally advanced or metastatic NSCLC patients with EGFR activating mutation in June 2009 (ref.[33,34]).

The low-molecular weight inhibitor, erlotinib, showed antitumor activity in preclinical and phase I clinical studies[35]. Erlotinib was FDA approved in November 2004 based on the results of phase III randomized trial BR.21 which included 731 NSCLC patients treated by erlotinib or placebo in second or third line setting. The OS of the treated group was 2 months longer than the placebo group (6.7 months vs. 4.7 months). The 1-year OS was 31% for the erlotinib group compared to 22% for the control group[36].

The predictive role of EGFR mutations to EGFR TKIs therapy sensitivity was revealed by different studies and confirmed by large meta-analysis including 59 studies of 3101 NSCLC patients. EGFR mutations were predictive of response to single agent EGFR TKIs with sensitivity and specificity of 0.78, resp. 0.86 (ref.[37]). Many other studies elucidating EGFR TKIs efficiency in different settings and biomarker-selected populations were recently reviewed[38,39]. In general, EGFR TKIs treatment significantly improves the survival of NSCLC patients with EGFR mutations compared to chemotherapy.

Several clinical trials, clearly reviewed by Patil et al.[40], are evaluating the efficacy of cetuximab (Erbitux®, Merck KGaA) in combination with various types of treatment and assessing the predictive role of EGFR, KRAS and other potential biomarkers. Predictive value of EGFR mutations, amplification or overexpression and KRAS mutations for cetuximab therapy was not confirmed to date[41,42].

**EGFR TKIs and *de novo* resistance**

The best described mechanism of *de novo* resistance to EGFR TKIs is mutation in the KRAS oncogene which is present in 20 to 30% of lung cancer patients. KRAS and its importance for NSCLC therapy management is discussed below. Another cause of *de novo* resistance is the occurrence of insertion mutations in exon 20 of EGFR. *In vitro* studies have demonstrated that insertion in EGFR exon 20 causes both oncogenic transformation and resistance to EGFR TKIs (ref.[43]). Experiences with patients harboring EGFR exon 20 insertions corresponds with preclinical data. Clinical data showed very few responses to EGFR TKIs in these patients[15,44,45]. Substitution of methionine to threonin at position 790 (T790M) of the EGFR exon 20 was reported in 2.7-40% of TKI-naïve cases[44,46,47]. Patients with this mutation were found to have poorer outcome on EGFR TKIs therapy[47-49]. A secondary T790M mutation is more frequent and is associated with acquired resistance (described below). *De novo* resistance to EGFR TKIs therapy was also found in NSCLC patients with HER2 exon 20 insertions. Cancer cells presenting this mutation remain sensitive to HER2 targeted therapies but show resistance to EGFR TKIs (ref.[50,51]).

**TKIs and acquired resistance**

Acquired resistance to EGFR TKIs is a serious problem because the majority of initially responsive, EGFR TKIs-treated patients develop resistance within 12 months[52]. Resistance to EGFR TKIs may be caused by presence of cancer stem cell-like cells which are selected during EGFR TKIs therapy[53]. Generally, two crucial mechanisms of acquired resistance have been described, secondary T790M EGFR mutation and C-MET amplification. T790M mutation was described as the first mechanism of EGFR TKIs acquired resistance in 2005 by Kobayashi and Pao et al.[54,55]. Both groups studied NSCLC

patients with EGFR activating mutation (L858R or exon 19 deletion), who progressed on the gefitinib or erlotinib therapy. The T790M mutation was identified by comparison of pre- and post-progression samples and confirmed on NSCLC cell lines *in vitro*. A secondary T790M mutation is localized in the ATP-binding pocket of the kinase domain and is present in approximately 50% of NSCLC patients with acquired resistance[27,56-58]. Substitution in codon 790 increases ATP binding affinity of EGFR tyrosine kinase domain and EGFR TKIs are not able to bind. T790M mutated cells lose sensitivity to gefitinib and erlotinib but not to irreversible TKIs (e.g. pan-HER inhibitor PF0299804) (ref.[59]). A second mechanism of acquired resistance, C-MET amplification, is discussed below.

## KRAS

KRAS (Kirsten rat sarcoma viral oncogene homolog) gene localized on 12p12 encodes membrane-bound GTPase protein which, as well as other members of the RAS protein family (NRAS and HRAS), plays an important role in EGFR-mediated signal transduction. EGFR activates KRAS through the adaptor protein Grb-2 (growth factor receptor-bound protein 2) and guanine nucleotide-exchange factor (GEF) molecules which are responsible for exchange of GDP to GTP. GTP-KRAS binds target proteins (e.g. RAF), activates them and GTPase activating proteins (GAP) stimulate GTP hydrolysis. KRAS-mediated signaling regulates several cellular processes, such as proliferation, differentiation and survival.

### Activating mutations

Pathologic KRAS activation resulting from mutations in the KRAS gene has been found in many cancer types including NSCLC. KRAS mutations occur in approximately 20% of lung cancer cases[60,61]. The majority (about 90%) of found point mutations occur in exon 2 (codon 12 and 13), less frequent are mutations in exon 13 (codon 61) (ref.[26,61]). Point mutation leads to amino acid substitution and GAP insensitivity resulting in constitutively active GTP-binding KRAS signal transduction. KRAS mutations are more frequently found in Caucasian population, adenocarcinomas, males and current smokers[60,62,63]. In never smoking patients with adenocarcinoma, KRAS mutation is probably associated with transition mutation (G to A) compared to transversion (G to T or G to C) in current smokers[60]. Recent meta-analysis has shown KRAS mutations occurring in 26% of former or current smokers vs. 6% in never smokers[64]. The majority of studies have shown that KRAS and EGFR mutations are mutually exclusive[63,65-68]. Co-existence of both mutations was reported by Han et al. only[69].

### Prognostic role

Several studies have evaluated the importance of KRAS mutations for survival, recurrence and metastasis. In 2005, Mascaux et al.[70] published the results of meta-analysis comparing KRAS prognostic significance in 28 independent retrospective studies with a total number of 3620 patients included. This meta-analysis showed a worse survival of KRAS mutated patients with HRs of 1.30 (95% CI, 1.20-1.49; $P$=0.01). In subgroup analysis, KRAS was a statistically significant prognostic factor in adenocarcinomas (HR=1.52; 95% CI, 1.30 to 1.78; $P$=0.02) but not in squamous cell carcinomas. Following studies did not confirm KRAS mutations as an independent prognostic factor[67,68]. The prognostic importance of KRAS mutations in NSCLC remains controversial and needs to be confirmed on prospective well-defined NSCLC patient cohorts.

### Resistance to EGFR TKIs

Although the prognostic role of KRAS mutations is not clearly described, the predictive significance of EGFR TKIs therapy response was confirmed in several studies. KRAS mutations have been reported to be associated with *de novo* resistance to EGFR inhibitors in NSCLC patients in several studies[15,16,19,58,65,69]. Recently Mao et al. published meta-analysis of 22 studies analyzing 1470 NSCLC patients, KRAS mutation was detected in 16% (231/1470). Objective response rate (ORR) of KRAS mutated patients was 3% compared to 26% ORR in patients with wt-KRAS. This analysis confirmed that KRAS mutations are negative predictors of tumor responsiveness to EGFR TKIs therapy in NSCLC (ref.[64]). However, due to the mutual exclusivity of EGFR and KRAS mutations, the clinical importance of KRAS assessment in NSCLC remains low.

## C-MET

The C-MET protooncogene is localized on chromosome region 7q31 (ref.[71]) and codes a tyrosine kinase receptor - hepatocyte growth factor receptor (HGFR). HGF/SF (hepatocyte growth factor/ scatter factor) is the only known ligand of this receptor. HGF binding results in phoshorylation of C-MET tyrosine residues[72], recruitment of adaptor proteins Grb2, Gab1, SHC and activation of downstream MAPK, PI3K-Akt and STAT signaling pathways[73-75]. C-MET and HGF are required for normal tissue development and therefore they are widely expressed in a various cell types. C-MET/HGF dysregulation and pathogenic activation is described in almost all cancer types[76-78] and has been identified as a promising therapeutic target. The first reported oncogenic C-MET activation resulting from translocation of chromosome 1 and 7 was found in an osteosarcoma cell line. Fusion TRP-MET protein has constitutive tyrosine kinase transforming activity[76]. C-MET can be activated by many other mechanisms, such as amplification, overexpression of receptor or ligand and point mutation[57,79,80].

### Amplification/overexpression

C-MET amplification leads to receptor overexpression and constitutive HGF-independent activation[81]. C-MET amplification has been reported in range from 3 to 21% of EGFR TKI-naïve NSCLC patients and is associated with poor prognosis, increased proliferation, tumor invasiveness and angiogenesis[82-87]. The greatest percentage of

reported C-MET FISH-positive cases results from chromosome 7 polysomy. True C-MET amplification is rare event in NSCLC, occurs in 3 to 7% cases[84-86,88,89]. Some studies have reported association between C-MET and EGFR amplification[83,84]. Chromosome 7 polysomy is probably responsible for significant correlation between EGFR and C-MET FISH positivity. Higher copy number/overexpression of C-MET was found in brain metastasis compared to primary lung tumor tissues. C-MET-activated tumor cells have probably higher potential to migrate and create metastasis[90,91].

**Resistance to EGFR TKIs**

The importance of C-MET copy number evaluation rapidly increased when Engelman et al. found that the cause of acquired resistance to gefitinib in an NSCLC cell line (HCC827) is amplification of chromosomal region 7q31.1–7q33.3 where C-MET is localized. Consequently, C-MET-driven EGFR TKIs resistance was confirmed on 18 NSCLC patient samples[57]. C-MET amplification has been described in approximately 20% of NSCLC patients with acquired resistance[57,88,92-94]; in some cases T790M mutation of EGFR occurs simultaneously. Engelman et al. found that the bypass mechanism of C-MET signaling activation in resistant cells is through ERBB3-mediated PI3K-Akt signaling pathway[57].

Turke et al. theorized that NSCLC cells become C-MET amplified and therefore resistant during EGFR TKIs treatment by selection of a preexisting small C-MET amplified clone[95]. This study was performed on EGFR TKIs-sensitive NSCLC cell line HCC827 and 27 paired NSCLC patient samples (pre- and post-therapy). In the cell line study, a small subpopulation of C-MET amplified cells increased 300x over 19-days EGFR TKIs exposure. In tumor samples, C-MET-driven resistance was observed in 4 out of 27 cases, rare subpopulation (< 1%) of C-MET amplified cells was found in pre-treatment specimens in all 4 cases. These data suggest that acquired C-MET-driven resistance can be suppressed by dual EGFR and C-MET inhibition.

**Targeted therapy**

Several strategies of C-MET inhibition based on the mechanism of HGF/C-MET activation have been reported. In C-MET amplified/overexpressed tumors, selective blockade of active receptor by small-molecule inhibitors or monoclonal antibodies seem to be effective. Several C-MET TKIs such as PHA665752 (ref.[81,96]), PF-02341066 (crizotinib, Xalkori®, Pfizer), SGX523 (ref.[97,98]), ARQ197 (tivantinib, ArQule) (ref.[99,100]) and XL184 (cabozantinib, Exelixis) (ref.[101]) as well as monoclonal antibody MetMAb (onartuzumab, Genentech) (ref.[102]) were tested in a preclinical setting on NSCLC cell lines and xenograft models.

Cabozantinib, dual inhibitor of VEGFR2 and C-MET, has reached clinical testing in several cancer types. In NSCLC, cabozantinib is investigated in combination with erlotinib compared to erlotinib alone in phase I/II clinical study (NCT00596648) (ref.[103]). This inhibitor seems to be an effective inhibitor of tumor angiogenesis and metastasis in C-MET-deregulated NSCLC cases[101].

One of the most promising molecules is the non-ATP-competitive selective C-MET inhibitor tivantinib which passed phase I and II clinical trials. Sequist et al.[104] reported results of double-blind randomized phase II trial (NCT00777309) including 167 randomly assigned previously treated, EGFR TKI-naïve NSCLC patients. Patients who obtained erlotinib combined with tivantinib (ET) were compared to patients obtaining erlotinib with placebo (EP). Median PFS was 3.8 months for ET compared to 2.3 months for EP (HR=0.81; 95% CI, 0.57-1.16; $P$=0.24). ET-treated patients had significantly longer time to development of new metastasis (7.3 vs. 3.6 months, $P$<0.01). Significantly better response to ET therapy was observed in KRAS mutated patients compared to KRAS mutated in the EP regime (HR=0.18; 95% CI, 0.05 to 0.70; $P$=0.006). In this study, only 2 patients had true C-MET amplification, increased copy number (≥ 4 copies/cell) was found in 37 patients. C-MET positive patients tend to benefit from the ET regime and this benefit rises with increasing cut-off of C-MET copy number. Tivantinib in combination with erlotinib can prolong PFS, OS and time to metastasis in NSCLC patients compared to erlotinib alone. Ongoing clinical trials combining tivantinib and erlotinib in different setting are summarized in Table 1.

Crizotinib, a dual inhibitor of ALK and C-MET kinases is approved for treatment of NSCLC patients with ALK rearrangement. Nevertheless, response to crizotinib was shown in non-ALK rearranged NSCLC cell lines, xenograft model[105] as well as patient with *de novo* amplification of C-MET (ref.[106]). Anti-tumor activity of crizotinib is studied in randomized phase I/II trial (NCT00965731) in NSCLC patients treated by erlotinib alone versus erlotinib in combination with crizotinib[103]. The results from this study could clarify the inhibitory effect of crizotinib in C-MET amplified cases as it was shown on xenograft models[107].

MetMAb (onartuzumab) in combination with erlotinib have been evaluted in randomized, double-blind, phase II trial (NCT00854308). PFS was 2.2 vs. 2.6 months for patients obtained erlotinib + MetMAb (EM) vs. erlotinib + placebo (EP). In subgroup of C-MET positive NSCLC patients, PFS was 2.9 for EM vs. 1.5 months for EP. Efficiency of MetMAb in NSCLC should be confirmed by ongoing clinical trials combining MetMAb with erlotinib (NCT01456325), bevacizumab/pemetrexed (NCT01496742) and paclitaxel + platinum (NCT01519804) (ref.[103]).

## ALK

The ALK (anaplastic lymphoma kinase) protein is a transmembrane tyrosine kinase receptor normally expressed only in the small intestine, testis and brain[108] but not in normal lung tissue[109]. Translocation of the ALK gene t(2;5) leading to NPM1-ALK fusion was firstly reported by Morris et al.[108] in anaplastic large cell lym-

**Table 1.** Trials are listed on the US National Institute of Health database[103].

| Study | Phase | Treatment schedule | Stage | Biomarker selection | EGFR TKIs pretreated | Outcome | Estimated enrollement |
|---|---|---|---|---|---|---|---|
| NCT01251796 | Phase I | erlotinib + tivantinib | IIIB,IV | CYP2C19 poor metabolism | possible | Primary: DLT<br>Secondary: T and E farmacokinetics, antitumor activity | ND |
| NCT01580735 | Phase II | erlotinib + tivantinib | IIIB,IV | EGFR mutation | yes | Primary: ORR<br>Secondary: PFS, OS, DCR; safety profile | 40 |
| NCT01395758 | Phase II randomised | Arm 1: erlotinib + tivantinib<br>Arm 2: pemetrexed, docetaxel, or gemcitabine | IVA,IVB | KRAS mutation | no | Primary: PFS<br>Secondary: OS, ORR, safety of T+E combination | 98 |
| NCT01244191 | Phase III randomised | Arm 1: erlotinib + tivantinib<br>Arm 2: erlotinib + placebo | IIIB,IV | no | no | Primary: OS<br>Secondary: PFS, OS in wt-EGFR pts. | 988 |
| NCT01377376 | Phase III randomised | Arm 1: erlotinib + tivantinib<br>Arm 2: erlotinib + placebo | IIIB,IV | wt-EGFR | no | Primary: OS<br>Secondary: PFS, ORR, adverse events | ND |

CYP2C19-cytochrome P450 2C19; DLT-dose-limiting toxicity; PFS-progression free survival; OS-overall survival; ORR-objective response rate, T-tivantinib; E-erlotinib; pts.-patients; DCR-dissease control rate; wt-wild type; ND-not defined

phoma (ALCL). Alterations of ALK gene were also identified in neuroblastomas[110] and inflammatory myofibroblastic tumors[111]. In 2007, Soda and colleagues identified a small inversion in the short arm of chromosome 2, inv(2)(p21p23) in NSCLC patients. This inversion leads to fusion of the N-terminal part of the echinoderm microtubule associated protein like-4 (EML4) with kinase domain of ALK (ref.[112]).

**EML4-ALK fusion**

EML4-ALK rearrangement is being found in approximately 2-7% NSCLC cases[113-115]. The fusion leads to protein redistribution to cytoplasm[112] and protein dimerization via coiled-coil domains of EML4 resulting in phosphorylation and highly oncogenic ALK kinase activation[116,117]. More than 13 variants of EML4-ALK have been identified to date containing different parts of EML4; the coiled-coil domain is preserved in all variants. Exon 13 (variant 1), resp. exon 6a/b (variant 3a/b) of EML4 fused to the ALK exon 20 are the two most frequent variants which are present in more than 50% cases[117-120]. Tree other rare fusion partners of ALK are known in NSCLC, KIF5B (ref.[121]), TFG (ref.[122]) and KLC1 (ref.[123]). The incidence of these fusion partners is less than 1% (ref.[121,122,124]). Heuckemann et al. showed that protein stability and sensitivity to treatment depend on EML4-ALK variant and fusion partner type[125].

Except for ALK rearrangement, ALK amplification/CNG have been reported[120,126]. Increased ALK copy number was associated with EGFR FISH positivity but no association with prognosis was found[126]. The significance, if any, of ALK CNG for response to therapy, prognosis or histopathologic features, needs to be analyzed.

A subgroup of EML4-ALK patients has typical clinical and histological features. ALK rearrangement is typically found in adenocarcinoma with signet ring cell subtype, younger patients[113,114,116,117] with never or light (10 packs per year) smoking history[114,127]. No other association with gender or ethnicity has been found. ALK fusion is mutually exclusive in most NSCLC cases[114,128,129], concurrent EGFR and KRAS mutations were described in only few cases[130-134].

**Targeted therapy**

EML4-ALK fusion is a therapeutic target for the ATP-competitive TKI crizotinib. In preclinical analyses, the inhibitory effect of crizotinib was confirmed on ALK rearranged cell lines derived from a variety of human cancers[135,136]. Based on these studies, crizotinib entered multicenter, open-label phase I trial (NCT00585195). In this study, crizotinib showed significant antitumor activity in enrolled 82 advanced, ALK-positive NSCLC patients. The ORR to crizotinib was 57% at mean treatment duration of 6.4 months. The estimated probability of 6 month progression-free survival was 74% (ref.[113]). In the retrospective data analysis from this study, reported by Shaw et al.[115], the 1-year OS was 74% and 2-year OS was 54%. ALK-positive patients treated by crizotonib had similar OS compared to EGFR TKIs-treated EGFR-mutant patients ($P$=0.786) but significantly better OS than ALK-positive crizotinib-untreated group. Moreover, ALK-positive crizotinib-treated patients had significantly better OS ($P$=0.020) than controls (wt-EGFR, ALK-negative) treated by conventional chemotherapy. Based on the results of phase I study and ongoing phase II studies (255 patients; NCT00932451), crizotinib (Xalkori®, Phizer) was FDA approved in August 2011 and EMA approved in October 2012 for treatment of locally advanced or metastatic ALK-positive NSCLC patients. Ongoing clinical trials evaluating efficiency of crizotinib in different setting are summarized in Table 2.

**Resistance to crizotinib**

Similar to other TKIs therapies, *de novo* as well as acquired resistance to crizotinib have already been reported. Two mutations in ALK kinase domain, C1156Y and L1196M, were identified as potential mechanisms of resistance to crizotinib therapy in 28-years old NSCLC patient[137]. Both mutations as cause of acquired resistance to crizotinib were confirmed in following studies[134,138] and other resistance-related mutations, L1152R, G1269A/S and S1206R, have been described[134,139,140]. Some other potential mechanisms of resistance, such as EML4-ALK CNG, KRAS and EGFR concurrent mutations, were described by Doebele et al.[134].

Several treatment strategies overcoming crizotinib resistance are tested on cell lines and xenografts models[141,142]. The Hsp90 inhibitors which show the most promising results are tested in number of clinical trials. Inhibitors IPI-504 (Phase II; NCT01228435), AP26113 (Phase I/II; NCT01449461), CH5424802 (Phase I/II; NCT01588028), X396 (Phase I; NCT01625234) are tested in advanced lung cancer patients in monotherapy[125,143] whereas STA-9090 (Phase I/II; NCT01579994) and AT13387 (Phase I/II; NCT01712217) inhibitors are tested in combination with crizotinib (detailed in Table 2) (ref.[103]).

## OTHER CLINICALLY IMPORTANT BIOMARKERS

HER-2 (17q) overexpression has been described in approximately 20% NSCLC cases, whereas insertion in HER-2 exon 20 is the rare event (2%). These mutations occur mainly in adenocarcinoma, non-smokers and Asians and are associated with resistance to EGFR TKIs (ref.[50]). This resistance can be overcome by dual TKIs inhibition by lapatinib or BIBW 2292 (ref.[51,144]).

Translocation of ROS1 gene (6q) was identified as potential driver mutation in NSCLC cell lines[122]. ROS1 gene rearrangement has been described in approximately 2% NSCLC cases and tree fusion partners, CD74, SLC34A2 and FIG, have been identified to date[145,146]. Patients with ROS1 rearrangement have similar features as patients harboring EGFR mutation or ALK rearrangement, ROS1 rearranged patients are more likely Asian, younger and never smokers with adenocarcinoma histology[145]. ROS1 rearrangement leads to constitutive kinase activity and sensitivity to TKIs *in vitro*[135]. Bergethon et al.

**Table 2.** Trials are listed on the US National Institute of Health database[103].

| Study | Phase | Treatment schedule | Stage | Biomarker selection | Pretreatment | Outcome | Est. enrollment |
|---|---|---|---|---|---|---|---|
| NCT01637597 | Exploratory | crizotinib | IIIB,IV | ALK positive | yes (Pt-CHT) | Primary: ORR (comparing different methods of ALK evaluation)<br>Secondary: PFS, OS | 42 |
| NCT01712217 | Phase I | crizotinib + AT13387 | IIIB,IV | ALK positive | yes (crizotinib) | Primary: DLT<br>Secondary: pharmacokinetics, antitumor activity, CTCs, PFS, OS | 228 |
| | Phase II randomised | Arm 1: crizotinib<br>Arm 2: crizotinib + AT13387 | IIIB,IV | ALK positive | yes (crizotinib) | Primary: ORR<br>Secondary: safety, PFS, OS, overall RR | |
| | Phase II randomised | Arm 1: AT13387<br>Arm 2: crizotinib + AT13387 | IIIB,IV | ALK positive | yes (crizotinib) | Primary: objective overall RR<br>Secondary: safety, PFS, OS | |
| NCT01441128 | Phase I | Cohort 1: crizotinib + PF-00299804<br>Cohort 2: PF-00299804 untill progression; then crizotinib + PF-00299804 | IIIB,IV | no | yes (CHT or targeted) | Primary: overall safety profile<br>Secondary: pharmacokinetic parametrs, ORR; predictive biomarkers | 22 |
| NCT01579994 | Phase I/II | ganetespib (STA-9090) + crizotinib | IIIB,IV | ALK positive | no (possible CHT) | Primary: MTD, efficacy<br>Secondary: OS, overall RR, safety profile | 55 |
| NCT01500824 | Phase II | crizotinib | IIIB,IV | ALK positive | yes (CHT) | Primary: ORR<br>Secondary: PFS, OS, DCR, DR, TTR | 50 |
| NCT00932451 | Phase II | crizotinib | IIIB,IV | ALK positive | yes (CHT) | Primary: ORR<br>Secondary: DR, DCR, OS, HRQoL, plasma concetration of crizotinib, type of EML4-ALK variant, protein expression, PFS, TTR, QTc | 1100 |
| NCT01639001 | Phase III randomised | Arm 1: crizotinib<br>Arm 2: pemetrexed+cisplatin/carboplatin | IIIB,IV | ALK positive | no | Primary: PFS<br>Secondary: ORR, DR, OS, TTD, HRQoL | 200 |
| NCT00932893 | Phase III randomised | Arm 1: crizotinib<br>Arm 2: docetaxel<br>Arm 3: pemetrexed | IIIB,IV | ALK positive | yes (1x Pt-CHT) | Primary: PFS<br>Secondary: ORR, AE, DR, DCR, OS, HRQoL, plasma concetration of crizotinib and ph.biomarkers, type of EML4-ALK variant, TTR, QTc | 318 |
| NCT01154140 | Phase III randomised | Arm 1: crizotinib<br>Arm 2: pemetrexed+cisplatin/carboplatin | IIIB,IV | ALK positive | no | Primary: PFS<br>Secondary: ORR, AE, plasma concetration of crizotinib, type of EML4-ALK variant, patients reported outcome, OS | 334 |
| NCT01597258 | Phase IV | (all patients treated by crizotinib) | ND | ND | ND | Primary: incidence of adverse drug reactions<br>Secondary: ORR | 2000 |

DLT-dose-limiting toxicity; CTCs-circulating tumor cells; PFS-progression free survival; OS-overall survival; ORR-objective response rate; overall RR-overall response rate; CHT-chemotherapy; Pt-CHT-platinum-based chemotherapy
DCR-disease control rate; DR-duration of response; TTR-time to response; TTD-time to deterioration; HRQoL-Health Related Quality of Life; QTc-corrected QC; ph-pharmacodynamics; MTD-maximum tolerated dose; AE-adverse events; ND-not defined
AT13387 - Hsp90 inhibitor; PF-00299804 - panHER inhibitor; STA-9090 - Hsp90 inhibitor

showed promising antitumor activity of crizotinib in one patient with ROS1 rearrangement treated in clinical trial NCT00585195 (ref.[145]).

Mutations in PIK3CA, BRAF and AKT genes were reported in up to 3% of lung cancer cases. Large scale of BRAF, MEK, AKT and mTOR inhibitors are tested in ongoing clinical trials and are a promise for new personalized treatment opportunities in NSCLC patients[147,148].

## CONCLUSION

Personalized medicine requires molecular genetic testing prior to decision about which therapeutic regimen is appropriate for an individual patient. Several predictive markers have been identified in NSCLC patients but only the minority of them is clinically used for therapy individualization. Nevertheless, personalized therapeutic opportunities of NSCLC are expected to increase in the following years. Number of clinical trials is currently evaluating efficiency of inhibitors directed against various genetic markers and ongoing intensive research is focused on identification of new therapeutic targets as well as testing new therapeutics. In future, clear algorithm reflecting clinically importance of each marker will be required for the routine diagnostics in NSCLC because of limited sample material. New methodologies combining currently using methods able to evaluate several markers simultaneously will be needed for appropriate NSCLC patient care management.

In conclusion, EGFR mutations and EML4-ALK rearrangement are currently the strongest predictive markers and only clinically applicable markers for patient selection to targeted therapy in NSCLC.

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## CONFLICT OF INTEREST STATEMENT

**Author's conflict of interest disclosure**. The authors stated that there are no conflicts of interest regarding the publication of this article.

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