Charles University in Prague
Faculty of Medicine

Morphologic and molecular characterization of triple negative breast carcinoma for identification of clinically relevant subtypes

Morfologická a molekulární typizace triple-negativních karcinomů prsu – jako prostředek pro identifikaci klinicky relevantních subtypů

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This dissertation thesis was written during the *combined* Pathology doctoral study (PhD) programme at the Fingerland Department of Pathology, Faculty of Medicine in Hradec Králové, Charles University in Prague.

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Prof Aleš Ryška MD PhD
Chairperson of the Commission for Dissertation Defences
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SUMMARY (CZE) Morfologická a molekulární typizace triple-negativních karcinomů prsu – jako prostředek pro identifikaci klinicky relevantních subtypů

Úvod: Karcinom prsu je celosvětově nejčastějším maligním nádorem u žen a přední příčinou úmrtí žen na zhoubné novotvary. I přes nedávné pokroky v diagnostice a léčbě rakoviny prsu, představují přetrvávající problém relativně vzácné takzvané triple negativní karcinomy prsu (TNBC). TNBC neexprimují hormonální (estrogenový a progesteronový) receptory a HER2. Vzhledem k malé pravděpodobnosti pozitivní odpovědi na hormonální nebo anti-HER2 terapii mají tyto nádory zpravidla špatnou prognózu. TNBC je však poměrně heterogenní skupinou nádorů zahrnující celé spektrum diagnostických jednotek s odlišným biologickým potenciálem. Pro odlišení jednotlivých subtypů nádorů neexistuje doposud žádná obecně akceptovaná a zejména klinicky relevantní klasifikace. Rovněž na rozdíl od ostatních subtypů karcinomu prsu neexistuje pro TNBC doposud žádná ověřená cílená terapie. Jako jeden z možných cílů pro léčbu TNBC je zkoumán receptor pro epidermální růstový faktor (EGFR). Cílem předložené studie byla snaha identifikovat klinicky relevantní morfologické a/nebo imunofenotypické subtypy triple negativních karcinomů prsu. Naším cílem také bylo studovat vztah mezi změnami EGFR, fenotypem TNBC a klinickým chováním nádorů.
Metodika: Na souboru 52 archivních případů TNBC před zahájením léčby jsme provedli morfologickou, imunohistochemickou a in situ hybridizační studii. Hodnocena byla imunohistochemická exprese tzv. 'bazálních' markerů: p-cadherinu, p63, CD10, CK5/6, HMW a EGFR a dále exprese luminálního markeru CK18. Provedli jsme duální in situ hybridizaci za účelem detekce změn v počtu kopií EGFR genu v absolutních hodnotách i vzhledem k počtu kopií chromosomu 7. Byla korelována klinická data pacientů s morfologií nádoru a výsledky detekce molekulárních markerů.
Výsledky a diskuse: Zaznamenali jsme velké rozdíly v klinickém průběhu jednotlivých případů TNBC. Tato skutečnost by mohla znamenat, že paušální přiřazení špatné prognózy TNBC je zavádějící a mohlo by u některých nemocných vést ke zbytečně agresivní léčbě. Všechny naše případy TNBC exprimovaly alespoň jeden bazální marker a pouze dva nádory byly CK18 negativní. Exprese EGFR proteinu byla pozorována u 88,5% případů. Zatímco 8 (15,4%) nádorů mělo ≥ 4 kopií genu EGFR na buňku, amplifikace genu EGFR byla nalezena pouze v jednom případě. Výsledky detekce molekulárních markerů a morfologických znaků nebyly využitelné jako podklad pro klinicky relevantní klasifikaci TNBC. Průkaz "basal-like" fenotypu pomocí různých imunohistochemických kritérií neměl u naší skupiny TNBC žádný klinický význam. Na základě našich nálezů lze proto doporučit "kombinovaný" přístup ke stanovení prognózy u pacientek s TNBC pomocí jednoduchých nástrojů jako je Nottinghamský prognostický index (NPI). NPI prokázal statisticky významný vztah s biologickým chováním a prognózou onemocnění (p= 0.036). Protože se nám nepodařilo prokázat, že změny v EGFR jsou sdruženy s nepříznivým chováním nádoru, jeví se hypotéza o vhodnosti využití EGFR jako terapeutického cíle pro TNBC jako málo pravděpodobná. Vzhledem ke skutečnosti, že byl prokázán statisticky významný vztah mezi vysokým počtem kopii EGFR genu (≥ 4 kopií na buňku) a příznivou prognózou onemocnění (p=0.036) lze předpokládat, že nelze u blokády této signální dráhy předpokládat příznivý klinický efekt. Naše výsledky ale naznačují, že vysoký počet kopií EGFR by mohl sloužit jako potenciální marker příznivého biologického chování TNBC.

SUMMARY (ENG) Morphologic and molecular characterization of triple negative breast carcinoma for identification of clinically relevant subtypes

Introduction: Breast carcinoma is the most common malignancy in women worldwide, and the number one cause of cancer death in women. Despite the recent advances in breast cancer diagnosis and treatment, a problem is still posed by the so called 'triple negative breast carcinomas' (TNBCs). These tumours are associated with poor prognosis and are unlikely to respond to hormonal or anti-HER2 therapy. TNBCs are grouped together despite the clear heterogeneity within the subtype; there is no widely accepted evidence-based and clinically relevant way to sub-classify them. Neither is there any standard form of targeted therapy for the disease. Epidermal growth factor receptor (EGFR) is being investigated as a potential target for treatment of TNBC. The purpose of this study was to try to identify clinically significant morphologic and/or immunophenotypic subtypes within the triple negative group of breast carcinomas. We also aimed to study the associations between EGFR changes and TNBC phenotype and clinical behaviour.

Materials and methods: We performed an immunohistochemical (IHC), in situ hybridization and histo-morphological study on a set of 52 archive cases of pre-treatment TNBC. Immunohistochemical expression of the following 'basal' markers was assessed: p-cadherin, p63, CD10, CK5/6, HMW and EGFR; we also assessed immunohistochemical expression of luminal marker CK18. Dual in situ hybridization was performed to detect EGFR gene copy number changes. Clinical data obtained from patients' clinical charts were compared with morphology and molecular marker status for determining possible links between tumour phenotype and clinical behaviour.

Results and Discussion: We observed a wide variation in the clinical behaviour and outcome of different cases of TNBC showing that assigning a blanket 'poor' prognosis in all cases is misleading and could result in overtreatment of patients that may not need aggressive adjuvant therapy.

All of our TNBCs expressed at least one basal marker. Only two tumours did not express CK18. EGFR protein expression was observed in 88.5% of cases. While 8 (15.4%) tumours had ≥4 EGFR gene copies per cell, EGFR gene amplification was seen in only 1 case.

The molecular markers and morphological characteristics we investigated were not useful in providing a basis for clinically relevant classification of TNBC. Identification of the basal-like phenotype, using varied IHC definitions, had no clinical impact. Based on our findings, we recommend a combinatorial approach to prognostication in TNBC using simple tools such as the Nottingham Prognostic Index, as this showed a statistically significant association with outcome ($p=0.036$).

We were not able to demonstrate that EGFR changes are associated with poor outcome, making us question the suitability of EGFR as a therapeutic target for TNBC. The observation of a statistically significant link between high EGFR gene copy number (≥4 copies per cell) and good outcome ($p=0.036$) makes us further doubt that blocking the action of EGFR in TNBCs will produce favourable results. Instead, we propose that high EGFR copy number should be further investigated as a potential marker of good outcome in TNBC

INTRODUCTION

Triple negative breast cancer is a diagnosis that includes all primary epithelial breast malignancies that do not over-express HER-2 and are negative for hormone (oestrogen and progesterone) receptors (1). They account for 10-17% of all breast carcinomas with some authors reporting incidences of up to 24% (2-8).
TNBCs are thought to be more common in young women (<50 years of age) and in women of African, Hispanic and South East Asian origin (4). An American population based case-control study showed that women with triple negative (TN) tumours are also more likely to be obese, of lower socioeconomic status and likely to have experienced menarche at a younger age than women with hormone receptor positive tumours or controls (9).
This group of malignancies is morphologically, immunophentypically and biologically heterogeneous (1, 10). In general TNBCs are thought to be more aggressive with worse outcome than other immunophenotypic subtypes (10). They are considered to have bad prognosis with a high risk of death in the first 5 years following therapy but with reduced late recurrences/deaths (11). Relapse of TNBC usually occurs within the first three years of the breast cancer diagnosis (3, 12, 13). The liver, lungs and central nervous system are common metastatic sites (4). TNBCs are unlikely to respond to hormonal or anti-HER2 therapy thus the only standard systemic treatment option available is conventional chemotherapy (1, 10).
The morphological features associated with TN ductal NOS carcinoma are high nuclear grade, marked cellular pleomorphism, lack of tubule formation, scant stromal content, tumour necrosis, pushing border of invasion lymphoplasmacytic inflammatory infiltrate and central acellularity (14, 15). There are however, certain rare morphological subtypes of breast cancer with specific histological features and predictable clinical behaviour that are associated with triple negativity. Adenoid cystic carcinomas and medullary carcinomas for example are known to be associated with good prognosis while metaplastic breast carcinomas are reported to have worse outcome than any other subgroup of breast carcinoma (16-18).
Since the landmark Perou study in 2000 on breast cancer intrinsic subtypes (19), there has been great interest in the basal-like subtype; one of the four originally described intrinsic subtypes identified by cDNA microarray studies. The basal-like breast carcinomas (BLBCs) were strikingly similar to TNBCs; in terms of morphology, protein expression and clinical behaviour. Since then however, numerous authors have emphasized that TNBC and BLBC are not the same but that an overlap exists between the two groups of tumours (1, 20).
The significance of the basal-like subtype is controversial. While most studies have indicated that basal-like breast carcinomas have poor prognosis, not all of them have done so (21). Different investigators have used varied immunohistochemical definitions for identifying basal-like breast carcinoma (15, 22). The most widely used immunohistochemical definition for basal-like breast carcinoma is that proposed by Nielsen *et al*. They recommend defining basal-like breast carcinoma as ER and HER2 negative tumours that show any CK5/6 and/or EGFR positivity (23).
EGFR is a very attractive molecule for study in TNBC not only because it helps to identify the potentially prognostically significant basal phenotype but because it is a potential target for biological therapy. Clinical trials are currently underway to determine the efficacy of anti-EGFR agents in TNBC (6, 24, 25). So far, results are unconvincing. Much remains unknown about the role of EGFR in TNBC biology.

OBJECTIVES

The main objectives of this work were twofold:

- To identify clear prognostically significant immunophenotypic and morphological subtypes within the triple negative group of breast carcinomas using immunohistochemical detection of p-cadherin, p63, CD10, CK5/6, EGFR, HMW, CK18 for classification as well as assessment of characteristic morphological features

and

- To study EGFR in triple negative breast carcinoma using in situ hybridization for detection of gene or chromosomal numerical abnormalities that may be of predictive and/or prognostic significance.

MATERIALS AND METHODS

From the archives of the Fingerland Department of Pathology, we selected 52 consecutive cases of pre-treatment TNBC for which adequate material for further studies and sufficient clinical data were available. Triple negativity was defined as immunohistochemical immunoreactive score (IRS) = 0 for oestrogen and progesterone receptors (0% positive tumour cells) with HER score = 0, 1+ or 2+ non-amplified by fluorescence in situ hybridization (FISH) or dual in situ hybridization (HER2 gene:chromosome 17 ratio < 2). In each case one representative formalin-fixed paraffin-embedded tissue block was selected for further studies.
From the pathology reports, the following information was obtained in each case: patient age at diagnosis, tumour type and histological grade as well as immunohistochemically detected expression of Ki-67 and p53. Positive nuclear staining for Ki-67 in ≥20% of tumour cells was used to define high proliferative activity. P53 positivity was defined as nuclear staining in ≥ 50% tumour cells.
A clinical chart review was also performed to determine clinical course and outcome at the end of the follow-up period. Where available, the pathologic stage at time of diagnosis (pTNM) was also obtained. For the cases in which the patients received neoadjuvant chemotherapy, clinical stage (TNM) at the time of diagnosis was recorded instead. TNM stage IV tumours were excluded from our study.
In each case, Nottingham Prognostic Index (NPI) was calculated (NPI= lymph node stage + histological grade + tumour size x 0.2) and the following morphological features were assessed from the original haematoxylin and eosin slides used for diagnosis.

- Nuclear atypia (degree of atypia scored on a scale of 1-4)
- Tumour architecture (syncytial vs. non-syncytial)
- Tumour borders (infiltrating vs. pushing)
- Intra/peritumoural lymphoplasmacytic infiltrate
- Presence/absence of central acellular zone (scar or necrosis)
- Presence/absence of tumour necrosis
- Presence/absence of in situ component
- Stromal features
- Presence/absence of angioinvasion

Table 1 Prognostic groups of Nottingham Prognostic Index (27)

NPI	Prognosis
2,02-3,4	Good
3,41-5,4	Moderate
5,41-7,4	Poor

Indirect immunohistochemical staining for p-cadherin, p63, CD10, HMW, CK5/6, EGFR and CK18 was performed in all cases. Characteristics of the monoclonal antibodies used are shown in table 2. Manual staining was performed for p-cadherin. For the rest of the antibodies, staining was performed using the fully automated BenchMark ULTRA platform (Ventana, Arizona, USA). All specimens were assessed by light microscopy without knowledge of the case histories. A semi-quantitative method was used to assess the immunohistochemical stains.
Based on expression of the above mentioned markers, we classified the tumours as having basal, luminal or luminal progenitor differentiation.
Basal differentiation was defined as expression of any of the following markers – p-cadherin, p63, CD10, EGFR, CK5/6 and HMW, without expression of CK18.
Luminal differentiation was defined as expression of CK18 without expression of any of the following markers – p-cadherin, p63, CD10, EGFR, CK5/6 and HMW.
Luminal progenitor differentiation was defined as co-expression of CK18 with any of the following markers – p-cadherin, p63, CD10, EGFR, CK5/6 and HMW.
Dual in situ hybridization for detection of EGFR gene and chromosome 7 was performed in all cases. We used a dual colour staining technique for visualization of the EGFR gene and chromosome 7 centromere; with Ventana Silver *in situ* hybridization detection kit (Ventana, Arizona, USA) for the EGFR gene and Ventana Alk Phos Red ISH detection kit for chromosome 7 centromere (Ventana, Arizona, USA). Automated staining was performed using the BenchMark ULTRA platform (Ventana, Arizona, USA). For each case, the numbers of copies of EGFR gene and chromosome 7 were counted and recorded in 40 different tumour cell nuclei. The average number of copies of the gene and chromosome for each case was recorded and the gene-chromosome copy (G:C) ratio was calculated.
Clinical charts for all patients were reviewed. From these, we recorded type of treatment (neoadjuvant or adjuvant chemotherapy, radiotherapy, surgical therapy), duration of follow up (in months) and outcome at the end of the follow up period. Only those that were disease free at the end of the follow up period were considered to have good outcome. All the others (locoregional residual disease, metachronous breast cancer, distant metastases and death) were collectively viewed as having bad outcome.
Basis descriptive statistical analysis was performed using Microsoft Excel. Fisher's exact test was employed for determining associations between our selected prognostic markers, tumour characteristics and survival data. P-values <0.05 were considered to be statistically significant.

Table 2 Details of antibodies used

Antibody	Manufacturer	Clone	Dilution
P-cadherin	Vector laboratories	56C1	1:25
TP63	Ventana	4A4	original
CD10	Novocastra	56C6	1:10
CK5/6	DAKO	D5/16B4	1:100
HMW	DAKO	34βE12	1:25
CK18	DAKO	DC10	1:50
EGFR	DAKO	pharmDx™ kit	---

RESULTS

Table 3 Clinico-pathologic characteristics of cohort

	Number	Percentage
Patients (all female)	52	100
Age		
Range	28-84	
≤40	7	13.5
>40	45	86.5
Tumour type		
Ductal	46	88.5
Apocrine	3	5.8
Atypical medullary	2	3.8
Mucinous	1	1.9
Histological grade		
1	0	0
2	10	19.2
3	42	80.8
P53		
Positive	33	63.5
Negative	19	36.5
Ki-67		
Low	0	0
High	52	100

Table 4 Outcomes of all patients in cohort

	N=52*	%
Outcomes		
Disease free	39	75.0
Locoregional residual disease	2	3.8
Recurrence	2	3.8
Distant metastasis	8	15.4
Death		
TNBC related	4	7.7
Non-TNBC related	1	1.9

Abbreviation: TNBC= triple negative breast cancer
***There is an overlap in outcomes; one patient that developed distant metastases and another that had a recurrence were both disease-free at the end of the follow up period. Two patients that developed distant metastases died during the follow up period.**

Table 5 Traditional prognostic markers in metastasizing and non-metastasizing tumours

	Metastasis (%) N=8	No Metastasis (%) N=44	P-value
Lymph node stage			
0 (22)	1 (12.5)	21 (47.7)	**0.183**
1 (17)	4 (50.0)	13 (29.6)	
2 (10)	2 (25.5)	8 (18.2)	
3 (3)	1 (12.5)	2 (4.5)	
Tumour stage			
1 (21)	1 (12.5)	20 (45.5)	**0.242**
2 (20)	5 (62.5)	15 (34.1)	
3 (7)	1 (12.5)	6 (13.6)	
4 (4)	1 (12.5)	3 (6.8)	

TNM stage			
1 (15)	0 (0.0)	15 (34.1)	**0.124**
2 (25)	5 (62.5)	20 (45.5)	
3 (12)	3 (37.5)	9 (20.4)	
Histological grade			
2 (10)	2 (25.0)	8 (18.2)	**0.642**
3 (42)	6 (75.0)	36 (81.8)	
NPI (modified)			
Good prognosis (18)	0 (0.0)	18 (40.9)	**0.036**
Moderate prognosis (24)	5 (62.5)	19 (43.2)	
Poor prognosis (10)	3 (37.5)	7 (15.9)	
P53			
Negative (19)	5 (62.5)	14 (31.8)	**0.097**
Positive (33)	3 (37.5)	30 (68.2)	

Abbreviations: NPI= Nottingham Prognostic Index; TNM= tumour node metastasis

Table 6 Morphological features of metastasizing and non-metastasizing tumours

	Metastasizing N=8 (%)	Non-metastasizing N=44 (%)	P-value
Nuclear atypia			
1 (3)	0 (0.0)	3 (6.8)	**0.906**
2 (8)	1 (12.5)	7 (15.9)	
3 (33)	5 (62.5)	28 (63.6)	
4 (8)	2 (25.0)	6 (13.7)	
Borders			
Pushing (10)	1 (12.5)	9 (20.5)	**1.00**
Infiltrating (42)	7 (87.5)	35 (79.5)	
Architecture			
Non-syncytial (35)	6 (75.0)	29 (65.9)	**1.00**
Syncytial (17)	2 (25.0)	15 (34.1)	
DCIS			
Absent (33)	6 (75.0)	27 (61.4)	**0.694**
Present (19)	2 (25.0)	17 (38.6)	
Lymphoplasmacytic infiltrate			
Minimal (8)	2 (25.0)	6 (13.6)	
Moderate (37)	5 (62.5)	32 (72.7)	**0.827**
Prominent (7)	1 (12.5)	6 (13.6)	
Central acellular zone			
Absent (35)	5 (62.5)	30 (68.2)	**1.00**
Present (17)	3 (37.5)	14 (31.8)	
Necrosis			
Absent (26)	3 (37.5)	23 (52.3)	**0.674**
Focal (9)	2 (25.0)	7 (15.9)	
Geographic (17)	3 (37.5)	14 (31.8)	
Stroma			
Minimal (14)	5 (62.5)	9 (20.5)	**0.128**
Moderate (31)	3 (37.5)	28 (63.6)	
Abundant (6)	0 (0.0)	6 (13.6)	
Mucinous (1)	0 (0.0)	1 (2.3)	
Angioinvasion			
Absent (41)	5 (62.5)	36 (81.8)	**0.343**
Present (11)	3 (37.5)	8 (18.2)	

Abbreviation: DCIS= ductal carcinoma in situ

Table 7 P-cadherin expression and selected tumour characteristics/clinical behaviour

	Intensity of p-cadherin staining			
	Weak N=7	Moderate N=19	Strong N=26	**P-value**
Histological grade				
2 (10)	1	2	7	**0.441**
3 (42)	6	17	19	
Lymph nodes				
Positive (30)	3	11	16	**0.686**
Negative (22)	4	8	10	
Distant metastasis				
No (44)	5	15	24	**0.230**
Yes (8)	2	4	2	
Final outcome				
Good (39)	5	12	22	**0.245**
Bad (13)	2	7	4	

Table 8 p63 expression and selected tumour characteristics/clinical behaviour

	Percentage of p63 positive tumor cells				
	0% N=23	<10% N=23	≥10%, <50% N=4	≥50 N=2	**p-value**
Histological grade					
2 (10)	5	4	0	1	**0.535**
3 (42)	18	19	4	1	
Lymph node status					
Positive (30)	14	12	2	2	**0.701**
Negative (22)	9	11	2	0	
Distant metastasis					
No (44)	20	20	2	2	
Yes (8)	3	3	2	0	**0.295**
Final outcome					
Good (39)	19	18	1	1	**0.085**
Bad (13)	4	5	3	1	

Table 9 CD10 expression and selected tumour characteristics/clinical behaviour

	Percentage of CD10 positive tumor cells				
	0% N=36	**<10%** N=8	**≥10%, <50%** N=4	**≥50%** N=4	**p-value**
Histological grade					
2 (10)	7	2	1	0	**0.810**
3 (42)	29	6	3	4	
Lymph node status					
Positive (30)	22	3	3	2	
Negative (22)	14	5	1	2	**0.653**
Distant metastasis					
No (44)	32	5	3	4	
Yes (8)	4	3	1	0	**0.215**
Final outcome					
Good (39)	30	4	2	3	**0.084**
Bad (13)	6	4	2	1	

Table 10 CK5/6 expression and selected tumour characteristics/clinical behaviour

	Percentage of CK5/6 positive cells				
	0% N=11	**<10%** N=9	**≥10%, <50%** N=11	**≥50** N=21	**p-value**
Histological grade					
2 (10)	2	0	2	6	**0.308**
3 (42)	9	9	9	15	
Lymph node status					
Positive (30)	4	6	6	14	**0.425**
Negative (22)	7	3	5	7	
Distant metastasis					
No (44)	9	7	10	18	**0.759**
Yes (8)	2	2	1	3	
Final outcome					
Good (39)	9	5	9	16	**0.557**
Bad (13)	2	4	2	5	

11 HMW expression and selected tumour characteristics/clinical behaviour

	Intensity of HMW staining				
	0 N=3	**1** N=12	**2** N=11	**3** N=26	**p-value**
Histological grade					
2 (10)	0	3	1	6	**0.806**
3 (42)	3	9	10	20	
Lymph node status					
Positive (30)	1	6	6	17	
Negative (22)	2	6	5	9	**0.659**
Distant metastasis					
No (44)	3	9	9	23	**0.715**
Yes (8)	0	3	2	3	
Final outcome					
Good (39)	3	8	9	19	**0.762**
Bad (13)	0	4	2	7	

Table 12 EGFR score and selected tumour characteristics/clinical behaviour

	EGFR Score				
	0 N=6	**1-4** N=21	**5-8** N=15	**9-12** N=10	**p-value**
Histological grade					
2 (10)	0	3	5	2	**0.379**
3 (42)	6	18	10	8	
Lymph node status					
Positive (30)	2	14	8	6	
Negative (22)	4	7	7	4	**0.531**
Distant metastasis					
No (44)	5	18	12	9	**0.899**
Yes (8)	1	3	3	1	
Final outcome					
Good (39)	5	17	11	6	**0.680**
Bad (13)	1	4	4	4	

Table 13 EGFR gene copy number and selected tumour characteristics/clinical behaviour

	EGFR gene copy number per cell			
	2-2.99 N=26	**3-3.99** N=18	**≥4** N=8	**p-value**
Histological grade				
2 (10)	5	3	2	**0.898**
3 (42)	21	15	6	
Lymph node status				
Positive (30)	16	10	4	**0.863**
Negative (22)	10	8	4	
Distant metastasis				
No (44)	23	13	8	**0.211**
Yes (8)	3	5	0	
Final outcome				
Good (39)	21	10	8	**0.036**
Bad (13)	5	8	0	

Table 14 CK18 expression and selected tumour characteristics/clinical behaviour

	CK18 staining			
	Normal staining N=30	**Partial loss** N=20	**Complete loss** N=2	**p-value**
Histological grade				
2 (10)	8	2	0	**0.359**
3 (42)	22	18	2	
Lymph node status				
Positive (30)	16	13	1	**0.781**
Negative (22)	14	7	1	
Distant metastasis				
No (44)	25	17	2	**1.00**
Yes (8)	5	3	0	
Final outcome				
Good (39)	22	15	2	**1.00**
Bad (13)	8	5	0	

Table 15 Morphological and immunophenotypic subtypes

	Immunophenotypic subtype		
	Luminal N=0	Luminal progenitor N=50	'True' basal N=2
Ductal NOS	0	44	2
Apocrine	0	3	0
Atypical medullary	0	2	0
Mucinous	0	1	0

Table 16 Characteristics of metastasizing tumours

Age	Histologic subtype	Immuno subtype	TNM stage	NPI (modified)	EGFR score	EGFR gene copy number	Location of metastases
56	Ductal	Luminal progenitor	IIB	Moderate	3	2.75	Bone, subclavian lymph nodes
50	Ductal	Luminal progenitor	IIB	Moderate	4	3.90	Lung, brain
28	Ductal	Luminal progenitor	IIIA	Poor	0	2.20	Lung, liver, bone, meninges, cervical lymph nodes
70	Ductal	Luminal progenitor	IIIC	Poor	8	3.30	Brain
60	Ductal	Luminal progenitor	IIA	Moderate	4	3.60	Liver, bone, pleura
57	Ductal	Luminal progenitor	IIB	Moderate	6	2.20	Lung, brain
69	Atypical medullary	Luminal progenitor	IIA	Moderate	6	3.49	Pleura
67	Ductal	Luminal progenitor	IIIA	Poor	12	3.80	Subclavian, cervical and mediastinal lymph nodes

Abbreviations: EGFR= epidermal growth factor receptor; Immuno.= immunophenotypic; NPI= Nottingham Prognostic Index; TNM= tumour node metastasis

DISCUSSION

Results from studies on triple negative carcinomas, with emphasis on basal-like carcinomas, are difficult to compound and compare because of the lack of a standard definition of triple negativity. We excluded from our definition of triple negative all tumours that showed any staining for hormone receptors as studies show that even tumours with minimal hormone receptor expression could respond to hormonal therapy (26). By using such strict criteria in our definition, we ensured that only the tumours that are most likely to be truly biologically distinct from hormone receptor positive carcinomas were included in the study.

TNBC and NPI

NPI combines time dependent (tumour size and lymph node metastases) and tumour dependent (histological grade) characteristics (27, 28). According to the modified NPI, 34.6% (18/52) of our TNBCs had good prognosis, 46.2% (24/52) had moderate prognosis and 19.2% (10/52) had poor prognosis. None of the patients with good prognosis developed distant metastases, 20.8% of the patients with moderate prognosis developed metastases and 30.0% of those with poor prognosis developed metastasis. NPI was thus a useful tool in stratifying risk of metastases. The association between NPI and outcome was statistically significant (p=0.036). The finding suggests that tumour burden and histological grade may play a more important role in outcome of TNBC than other tumour characteristics.

Morphology of TNBC

It seems that TNBCs as a group of carcinomas are as morphologically heterogeneous as breast carcinomas in general. We observed a wide range of morphologic pictures even within the group of ductal NOS (not otherwise specified) carcinomas. One common morphologic feature of the ductal NOS carcinomas was the presence of a population of spindled cells.

None of the tumour characteristics we studied was significantly associated with development of distant metastasis. As all of our tumours expressed basal markers, we could not compare morphological characteristics in basal versus non-basal TNBCs. Half (4/8) of the tumours that showed high EGFR copy numbers were 'special' histological types of breast cancer (apocrine, mucinous and atypical medullary breast carcinomas). This suggests that morphology is an important manifestation of breast tumour biology and thus could play a significant role in patient selection for various types of targeted therapy. Reis-Filho *et al* also showed a link between breast tumour morphology and EGFR changes. They observed frequent overexpression of EGFR and EGFR gene amplification in metaplastic breast carcinomas and suggested that some patients with metaplastic breast carcinomas might benefit from EGFR targeted therapy (29).

TNBC, basal-like breast cancer and other immunophenotypic subtypes

We did not find identification of the basal-like phenotype useful in prognostic stratification of TNBC. This was because according to the 'gold standard' definition (TNBC with CK5/6 and/or EGFR+), 96.2% of our cases were basal-like, with only 2 tumours being non-basal. Even these two tumours showed expression of other markers of basal differentiation (i.e. p-cadherin/p63 and p-cadherin/HMW, respectively). Many authors have repeatedly emphasized that not all TNBCs are basal-like (1, 20); our findings rather suggest the opposite, possibly as a result of our strict immunohistochemical definition of triple negativity.

In our opinion the term basal-like is too ambiguous for use in daily medical practice and provides less clinical information than the triple negative designation. While both TNBC and BLBC are known to be heterogeneous groups generally associated with poor prognosis, unlike BLBC, the TNBC designation provides predictive information useful for directing patient management.

Not surprisingly, none of our tumours showed exclusive luminal marker expression. All of them fell into either the 'true' basal (2/52) category or the dual/luminal progenitor (50/52) category. Our finding of co-expression of luminal marker CK18 with basal markers (and minimal expression of p63 and CD10) in the majority of our cases is in support of the hypothesis that the so called basal-like tumours, which form the vast majority of TNBCs, may arise from luminal progenitor cells rather than stem cells or myoepithelial progenitor cells.

The clinical significance of subtypization in TNBC based on cell of origin is questionable. In our sample, identification of immunophenotypic subtypes with focus on identification of the 'basal' phenotype was not clinically useful.

TNBC and response to therapy

Nine of the 19 patients that received neoadjuvant chemotherapy achieved pCR. AC-T chemotherapeutic regimen was administered in all the 9 cases. All nine of them underwent partial mastectomy with axillary dissection and all but one had adjuvant radiotherapy. All of the patients that achieved pCR remained disease free at the end of

the follow up period (average: 83 months; range: 54-98 months). These patients showed varied clinico-pathologic characteristics and varied expression patterns of the molecular markers we investigated. We found no single unifying characteristic amongst all the patients that achieved pCR to distinguish them from the other patients that underwent neoadjuvant chemotherapy but did not have pCR. We note that 2 of the patients that underwent neoadjuvant chemotherapy and ended up not having surgical treatment were without signs of disease progression at the end of the follow up period (79 and 46 months respectively). Both patients had stage IIIA disease and underwent radiotherapy.
Choice of treatment did not appear to play a big role in development of distant metastasis or patient outcome.

Predicting outcome in TNBC
Rakha *et al* reported that time dependent pathological factors are the most useful in predicating outcome (5). Our findings are in accordance with theirs as in our sample, NPI was statistically significantly associated with development of distant metastasis. It is important to note that NPI, which also takes into account tumour histological grade, was a better predictor that the purely time dependent TNM stage. P53 expression was higher in patients that did not develop distant metastasis (though not statistically significant; p=0.097). This could be a reflection of better response of p53 expressing TNBCs to chemotherapy as suggested by Bidard *et al* (30).
EGFR gene copy number was the only molecular characteristic we observed that showed a statistically significant, however small, ability to predict outcome. All the TNBCs with 4 and higher EGFR gene copies per cell showed no sign of progression at the end of the follow up period. None of the other molecular markers we investigated were able to stratify TNBCs in a clinically significant way, neither were the morphological features we assessed.

Anti-EGFR therapy for TNBC
Though 88.5% of our sample showed EGFR expression, amplification of the gene was seen in only one case. Our finding of rare EGFR gene amplification in TNBC was also reported by Nakajima *et al* in their study of 84 TNBCs (31). None of their tumours showed EGFR amplification. In addition to IHC and in dual in situ hybridization, they also performed EGFR gene mutation analysis. No EGFR gene activating mutations were found. Jacot *et al* also found no EGFR-activating mutations in their group of 229 TNBCs (32); neither did Uramoto *et al* in their PCR-based study on 84 breast carcinomas, 45% of which were ER negative. They concluded that EGFR tyrosine kinase inhibitors are unlikely to provide any benefit for Japanese breast cancer patients (33).
Lv *et al* on the other hand observed a higher incidence of EGFR gene amplification in their set of 139 unselected breast carcinomas; positivity in 33.1% of all cases (34). They did, however, also report a low rate of EGFR mutations (in 1.4% of all cases) and concluded that EGFR mutations should not be used in trials testing anti-EGFR therapy in breast cancer.
Based on a study comparing 40 patients with TNBC to 158 patients with non-TNBC, Tang *et al* proposed that EGFR overexpression predicts better response to neoadjuvant chemotherapy in TNBC as EGFR overexpression was significantly associated with pCR in these patients (35).

We were not able to demonstrate that EGFR changes are associated with poor outcome, neither are we certain that EGFR abnormalities are critical drivers of TNBC. This makes us question the suitability of EGFR as a therapeutic target for TNBC, certainly not for all subtypes of TNBC. The observation of a statistically significant link between high EGFR copy number and good outcome further makes us doubt that blocking the action of EGFR in TNBCs will produce favourable results.
After a phase II trial testing cetuximab in patients with metastatic TNBC Carey *et al* concluded that '*therapy targeting growth factor pathways in this subtype* (TNBC) *may require a far better understanding of the pathways maintaining EGFR activity'* (36). Our findings put us in agreement with this statement. The role of EGFR in breast cancer appears to be highly complex and using anti-EGFR agents for treatment of TNBC at this point seems premature.

CONCLUSIONS

- Axillary lymph node involvement is a relatively common feature of TNBC
- Time dependent factors are significant predictors of prognosis in TNBC
- All TNBCs express at least 1 basal marker and could thus be considered 'basal-like'
- Complete loss of CK18 is a rare feature of TNBC
- EGFR gene amplification is a rare event in TNBC
- High EGFR gene copy numbers (≥4 copies per cell) may be associated with favourable outcome in TNBC.

Our results show that assigning a blanket 'poor' prognosis in TNBC is misleading and could lead to overtreatment in patients that may not need aggressive adjuvant therapy. Based on our findings, we recommend a combinatorial approach to prognostication in TNBC using simple tools such as NPI, which proved to be useful in the stratification of patients into prognostic groups.
Identification of the basal-like phenotype, using varied IHC definitions, in TNBC had no clinical significance. Also, as the vast majority of our cohort showed co-expression of luminal marker CK18 and markers of basal differentiation, we are in favour of adopting the term luminal progenitor-like to better describe this group of TNBCs to differentiate them from carcinomas with (basally located) myoepithelial differentiation. The clinical significance of molecular classification based on cell of origin is doubtful and limited, partially because of the significant overlap in protein expression amongst the cell types comprising the TDLU.
The unexpected finding of good outcome in patients with carcinomas showing high EGFR copy number calls to question the suitability of anti-EGFR treatment in patients with TNBC. Though our study was limited by its size and its retrospective nature, we found nothing to indicate that EGFR was a driver of aggressive behaviour. We were thus unable to provide evidence supporting the use of anti-EGFR therapy in unselected cases of TNBC. We did, however, discover that EGFR gene copy number may be of use in determining prognosis in TNBC. High EGFR gene copy number could be an independent marker of good outcome in TNBC.
The molecular markers and morphological characteristics we investigated were not useful in providing a basis for clinically relevant classification of TNBC. Instead, we

confirmed the importance of simple combinatorial prognostic tools like the Nottingham Prognostic Index.
We propose that high EGFR copy number should be further investigated as a potential marker of good outcome in TNBC.

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1. SOBANDE F. and RYSKA A. Breast cancer in young women – lessons from a clinico-pathological institutional review. *The Breast Journal.* (Accepted March 2013) ISSN 1075-122X. **(IF=1.831)**

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