Research Paper

Subtyping Of Triple Negative Breast Carcinoma On The Basis Of RTK Expression

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Abstract

Background: "Triple-negative breast cancers" (TNBC) comprise a heterogeneous group of about 15% of invasive BCs lacking the expression of estrogen and progesterone receptors (ER, PR) and the expression of HER2 (ERBB2) and are therefore no established candidates for targeted treatment options in BC, i.e., endocrine and anti-HER2 therapy. The aim of the present study was to use gene expression profiling and immunohistochemical (IHC) characterization to identify receptor tyrosine kinase (RTK) profiles that would allow patient stratification for the purposes of target-oriented personalized tumor therapy in TNBC.

Methods: Twenty-nine cases of TNBC selected according to routine diagnostic IHC/cytogenetic criteria were examined by reverse transcription polymerase chain reaction (RT-PCR). RTK mRNA expression profiles were generated for a total of 31 tumor-relevant biomarkers, mainly belonging to the IGF- and EGF-receptor families but also including biomarkers related to downstream signaling. Protein expression of selected biomarkers was investigated by IHC.

Results: Hierarchical cluster analysis revealed a dichotomous differentiation pattern amongst TNBCs. A significant difference in gene expression was observed for 16 of the 31 RTK-associated tumor relevant biomarkers between the two newly identified TNBC subgroups. The findings were verified at the posttranslational level by the IHC data. The RTKs HER4, IGF-1R and IGF-2R and the hormone receptors ER and PR below the IHC detection limit play a central role in the differentiation of the two TNBC subgroups. Observed survival was reported as Kaplan-Meier estimates and point towards an improved survival of patients with RTK-high with superior three-year survival rate of 100% compared to RTK-low gene signatures with superior three-year survival rate of 60% (log-rank test, p-value = 0.022).

Conclusion: Gene-expression and IHC analysis of the EGF and IGF receptor families and biomarkers associated with downstream signaling point to the existence of two distinct TNBC subtypes. The RTKs HER4, IGF-1R, IGF-2R and the hormone receptors ER and PR appear to be of particular importance here. Based on survival analysis the differentiation of TNBC with RTK-high and RTK-low gene signatures seems to be of prognostic relevance. Additionally, correlation analysis of the relationship between RTKs and ER suggests co-regulatory mechanisms that may have potential significance in new therapeutic approaches.

Key words: triple-negative breast cancer, subtyping, personalized tumor therapy, Real-Time RT-PCR, receptor tyrosine kinase (RTK), RTK-high gene signature, RTK-low gene signature

Introduction

About one in eight women will be diagnosed with breast cancer during their lifetime, making this the most frequent malignant diseases in women [1].

Figures published by the WHO indicate that, worldwide, about 1,000,000 women develop breast cancer every year. Breast cancer comprises a

heterogeneous group of malignant diseases that can be differentiated primarily on the basis of histopathological criteria into several distinct sub-groups of varying incidences [2].

Intense research efforts in recent years have provided insight into a large number of the complex molecular mechanisms involved in the pathogenesis of breast cancer [3]. Current attempts to develop individualized tumor therapies are aimed at identifying and treating further distinct subtypes on the basis of therapeutically relevant surrogate markers [4, 5, 6, 7, 8]. In addition to the classical histopathological parameters, the IHC characterization of hormone receptor status and HER2 as well as when indicated the cytogenetic detection of HER2 gene amplification represent the first important parameters upon which individualized tumor-specific therapy for breast carcinoma can be based. About 85% of all breast cancer patients currently receive such individualized therapy, which is aimed at the inhibition of HER2 (Trastuzumab, Lapatinib) and estrogen receptors (Tamoxifen, Letrozol) and is now considered standard treatment [9].

Thus, breast carcinoma can be roughly divided into tumors that are positive for estrogen/progesterone receptors and/or HER2 (triple-positive) which are suited to established, personalized therapy and those that are negative for these markers (triple-negative) without such benefit.

Triple-negative tumors are primarily defined, therefore, by an absence of surrogate markers of relevance for individualized therapy. As has been shown in numerous studies, triple-negative tumors vary in tumor progression, the development of therapeutic resistance, and the clinical course [10, 11, 12, 13, 14, 15], which indicates that they comprise a heterogeneous group. Further characterization and subtyping of triple-negative tumors on the basis of therapeutically relevant biomarkers is therefore strongly necessary.

To this end, a number of international microarray and NGS studies have produced data on the characterization of triple-negative tumors at the molecular level [13, 16, 17, 18]. In addition, further attempts to subtype these tumors have differentiated between basal-like and myoepithelial tumors [19, 20, 21] the findings being primarily of prognostic relevance. However, little therapeutic strategies targeting basal-like tumor cell specific surrogate markers have yet been developed.

The search for new approaches to individualized therapy during recent years has revealed biomarkers with predictive relevance for a large number of malignant diseases, which have been used in the development of individualized tumor therapy modalities in clinical trials. Various RTK families are of particular importance here. The treatment success achieved with trastuzumab (Herceptin) in HER2+ breast cancer has stimulated a search for other therapeutic targets including other members of elements of the epidermal growth factor receptor (EGFR) family.

The concept of growth factor receptor based anti-tumor therapy has been investigated and developed in recent years, especially in relation to EGFR. This has produced a series of EGFR inhibitors that have been approved for the treatment of epithelial tumors. In addition to the therapeutic monoclonal antibodies cetuximab and panitumumab, which are of great importance in the treatment of colorectal carcinoma, antineoplastic effects have been demonstrated for the receptor tyrosine kinase inhibitors (RTKI) gefitinib (Iressa) and erlotinib (Rarceva) in non-small cell lung cancer and for lapatinib (Tykerb) in metastatic breast cancer [22, 23, 24].

In addition to the representatives of the EGF receptor family, insulin-like growth factor receptor 1 (IGF-1R) is a further promising candidate for the development of individual therapeutic approaches in oncology. Various specific therapeutic monoclonal antibodies and RTKI against IGF-1R, growth factor antagonists and IGF-1R are currently undergoing clinical trials [25, 26, 27].

The aim of the present study was, by means of gene expression analysis and IHC investigations, to identify various RTK of the EGFR and IGFR families that would enable patient stratification for the purposes of target-oriented individualized tumor therapy in TNBC. In addition, correlation analysis was undertaken to shed light on possible interactions amongst the various RTK and provide a basis for innovative approaches to individualized tumor therapy.

Materials and Methods

Patients and tumors

A total of 29 triple-negative invasive ductal breast carcinomas, which had been diagnosed in the course of routine histopathological investigation at the Institute of Pathology, Ludwig-Maximilians-Universität Munich during the years 2003 to 2005, were selected for investigation. All tumors were re-determined for negative hormone receptor and negative HER2 status according to up-to-date histopathological guidelines and recommendations [28, 29]. ER and PR negativity was defined as positive staining in <1% of tumor cells. HER2 negativity was

defined as an IHC score of 0 or 1+; those with an immunoreactive score of 2+ underwent HER2-specific FISH analysis to exclude HER2 gene amplification. Further selection criteria included the high-risk parameters of high tumor grade (grade 3) and low age (cut-off of ≤ 55 years) at primary manifestation of disease (average age 41 years; x = 41.3 years; s = 7.8 years) at the time of diagnosis. Tumor size was documented in relation to the patient specific chemotherapeutic treatment regimen before adjuvant respectively before neoadjuvant chemotherapeutic treatment. The mean tumor size was 3.3 cm (x = 3.3 cm, s = 3.5 cm). Nineteen patients received adjuvant chemotherapy and 9 patients neoadjuvant chemotherapy. Within the observation time of this study recurrence rate was 24% for patients treated with adjuvant chemotherapy and 63% for patients with neoadjuvant treatment. None of the patiens with neoadjuvant chemotherapy showed a pathological complete response (pCR). Tumors in 6 patients showed an infiltration of lymph or blood vessels (lymphangiosis-, hemangiosis carcinomatosa) at the time of diagnosis and one patient had distant metastases. Details of the diagnostically relevant parameters in the study group are given in Table 1.

Material investigated

The investigations were performed on formalin-fixed, paraffin-embedded tissue (FFPET) processed for routine histopathological diagnostic investigation. All analyses were performed at resection specimens.

Deparaffinisation and RNA extraction

For each case, nine 4-μm FFPET whole sections comprising more than 70% tumor were cut and deparaffinized according to standard protocols. Total RNA was isolated by means of a commercial RNA isolation kit (High-Pure RNA Isolation Kit, Roche). RNA samples were analyzed photometric for their qualitative and quantitative content (NanoDrop, Thermo Scientific, USA). The minimum RNA content required for gene expression analysis was 300 ng/μl (total volume: 45 μl). An adequate amount of total RNA could be isolated from a total of 29 of the 31 triple-negative tumors that were initially selected for investigation.

cDNA synthesis

cDNA synthesis was performed by primer-specific reverse transcription of total RNA according to a standard protocol (Transkriptor First Strand cDNA Synthesis Kit, Roche). In brief, 3 μl RNA (50 ng/μl), 1 μl sequence-specific primer (1 μM), 9 μl distilled water, 4 μl 5X buffer, 2 μl nucleotide mix (each 10 mM), 0.5 μl protector RNase inhibitor (40 U/μl) and 0.5 μl transcriptor reverse transcriptase (20 U/μl) per reaction were mixed, preincubated for 10 minutes at 95°C and incubated for one hour at 50°C.

Table 1. Diagnostically relevant parameters in the study group

Case	Age	Grade	Estrogen	Progesterone	HER2/neu	Lymph nodes	Tumor size (cm)	Lymphangiosis carcinomatosa / Angiosis carcinomatosa	Neoadjuvant chemo-therapy	Adjuvant chemo-therapy	Distant metastases
1	42	3	0	0	0	0	2.3			x	
2	31	3	0	0	1+	1	1.6	x	x		
3	43	3	0	0	0	1	1.5			x	
4	42	3	0	0	0	0	1.1			x	
5	53	3	0	0	0	0	1.3			x	
6	38	3	0	0	1+	0	1.6			x	
7	45	3	0	0	0	1	15	x		x	
8	48	3	0	0	1+	0	2.5	x	x		
9	28	3	0	0	1+	0	1.9			x	
10	42	3	0	0	0	0	nd	x	x		
11	34	3	0	0	0	nd	3.3		x		
12	31	3	0	0	0	1	3.6		x		
13	47	3	0	0	0	nd	nd		nd	nd	
14	53	3	0	0	0	0	4.3			x	
15	34	3	0	0	0	1	2.2			x	
16	51	3	0	0	0	0	0.7			x	
17	40	3	0	0	0	0	2.2			x	
18	49	3	0	0	0	nd	0.9			x	
19	53	3	0	0	0	1	2.5		x		x
20	51	3	0	0	2+	0	5		x		
21	35	3	0	0	2+	1	3	x	x		
22	49	3	0	0	1+	0	2.1			x	
23	43	3	0	0	1+	1	2	x		x	
24	35	3	0	0	0	0	2		x		
25	33	3	0	0	2+	0	2.6			x	
26	41	3	0	0	1+	0	10			x	
27	32	3	0	0	0	0	2.5			x	
28	30	3	0	0	0	1	2			x	
29	46	3	0	0	0	0	5			x	

Per 96-well plate a standard dilution series (dilution factor 1:5), calibrator, negative control and 29 tumor samples were processed. A total of 34 biomarkers, including representatives of the EGFR and IGF-1R families and biomarkers related to downstream signaling, and five housekeeping genes (HKG) were analyzed in separate runs.

PCR Amplification

Gene expression data were obtained using sequence-specific detection probes (hydrolysis probes). With the help of the Universal ProbeLibrary Software [30], primer-specific sequences and target-specific hydrolysis probes for 34 biomarkers and 5 HKG were determined on the basis of freely accessible data banks (Table 2).

Table 2. Biomarkers / Universal ProbeLibrary Assays

Biomarker	Primersequence (5' -> 3'-direction)	Probe No.	Reverse Primer RT
EGFR Fwd	cat gtc gat gga ctt cca ga	44	ttc tcc tct cct gca cc
EGFR Rev1	ggg aca gct tgg atc aca ct		
HER2 Fwd	tgg ctc agt gac ctg ttt tg	75	tca ggt ttc aca ccg ct
HER2 Rev1	ggt cct tat agt ggg cac agg		
Her3 Fwd	cac aat gcc gac ctc tcc	86	tgg gca atg gta gag tag ag
Her3 Rev1	cac gag gac ata gcc tgt ca		
Her4 Fwd	ttc cac ttt acc aca aca tgc ta	78	aca gca aat gtc aga ccc
Her4 Rev1	cag aat gaa gag ccc acc a		
TGF-alpha Fwd	tgc tgc cac tca gaa aca gt	63	agt gct gtc ctg aag aag c
TGF-alpha Rev1	atc tgc cac ag tcc acc tg		
EGF Fwd	tgg ttg tgg ttc atc cat tg	68	tca cag cct ccg ttt tga ta
EGF Rev1	tca cag cct ccg ttt tga ta		
beta-cellulin Fwd	act gca tca aag gga gat gc	49	acc ttg ctc caa tgt agc
beta-cellulin Rev1	tcc aat gta gcc ttc atc aca		
Amphiregulin Fwd	cgg aga atg caa ata tat aga gca c	38	tgt caa tca tgc tgt gag ttt
Amphiregulin Rev1	cac cga aat att ctt gct gac a		
HB-EGF Fwd	tgg ggc ttc tca tgt tta gg	55	ccg att cct tga gca ca
HB-EGF Rev1	tgc cca act tca ctt tct ctt c		
NRG-Common Fwd	gat cag caa att agg aaa tga cag	53	tct gaa gac aca tat gct cct
NRG-Common Rev1	ggc ata cca gtg atg atc tcg		
NRG-GGF2 Fwd	gct gcc act act gct gct g	70	cgg gga cga gta gca cac
NRG-GGF2 Rev1	cgg gga cga gta gca cac		
NRG-SMDFsecSet Fwd	aga acg ccc aag tca gca	5	atg gct tgt ccc agt g
NRG-SMDF secSetRev1	ttg tcc cag tgg tgg atg ta		
NRG2 Fwd	ttc gca tca aat atg gca ac	39	gac ggt gtc ctt ccc c
NRG2 Rev1	ggc ctc gca gac ata ctc c		
NRG3 Fwd	gcc agt ctg tca aac acc ac	26	cca cct agc cta ctt cgg
NRG3 Rev1	atg gag cat gcc act tct tt		
NRG4 Fwd	ggg gct ttg tta tgt gat acc t	23	ctg gag cct ggg aga a
NRG4 Rev1	cct gta tag ttt tca acg cac ct		
BCL2 Fwd	agg tgc atc tgg tga tgt ga	26	cac tcc aac ccc cga tct
BCL2 Rev1	cac tcc aac ccc cga tct		
G6PDH secSet Fwd	aga gac cgt gga tgc tga ag	65	ctc cgc act gct gac a
G6PDH secSet Rev1	tga gga cct ccg tca gat g		
beta-Aktin Fwd	att ggc aat gag cgg ttc	11	atg tcc acg tca cac ttc at
beta-Atkin Rev	gga tgc cac agg act cca t		
ER-Fwd	aac cag tgc acc att gat aaa a	69	att ctc cct cct ctt cgg
ER-Rev	tcc tct tcg gtc ttt tcg tat c		
PGR-Fwd	ttt aag agg gca atg gaa gg	11	ttt ttc tgc gga ttt tat caa
PGR-Rev	cgg att tta tca acg atg cag		
PTEN-Fwd	gca caa gag gcc cta gat ttc	60	aat aca cat agc gcc tct ga
PTEN-Rev	cgc ctc tga ctg gga ata gt		
PIK3CA-Fwd	cga gat cct ctc tct gaa atc ac	2	gaa ttt cgg gga tag tta cac aa
PIK3CA-Rev	gaa ttt cgg gga tag tta cac aa		
PIK3R1 Iso 1-Fwd	aat gaa cga cag cct gca c	16	cag tac cat tca gca tct tgt aa
PIK3R1 Iso 1-Rev	ccg ttg ttg gct aca gta gta gg		
AKT common-Fwd	ggc tca ccc agt gac aac tc	41	agc agc ttc agg tac tca aa
AKT common-Rev	act caa act cgt tca tgg tca c		
SRCcom-Fwd	cga gaa agt gag acc acg aa	34	gtt gag gcc ctt ggc
SRCcom-Rev	ttg gcg ttg tcg aag tca		
SCUBE2-Fwd	gct gcc atc cac agt aca ag	17	gac agt gtc ctc tcg ctc caa
SCUBE2-Rev	gac agt gtc ctc tcg ctc caa		
Ki-67-Fwd	tga att cca aga aaa at acg tga	36	agc ttt ctc atc agg gtc a
Ki-67-Rev	tca ggg tca gaa gag aag cta ga		
Cyclin B1-Fwd	cgc ctg agc cta ttt tgg t	34	aga aag cct gac aca ggt c
Cyclin B1-Rev	gca cat cca gat gtt tcc att		
HNF3α-Fwd	agg gct gga tgg ttg tat tg	1	atg ttg ctg acc ggg a
HNF3α-Rev	acc ggg acg gag gag tag		

Biomarker	Primersequence (5' -> 3'-direction)	Probe No.	Reverse Primer RT
Survivin com-Fwd	ctt gaa agt ggc acc aga gg	29	cct cac ttc tca cct ggt aa
Survivin com-Rev	caa aaa tga gcc ccc aaa a		
TRIP13-Fwd	ctc atg cgc tgt atg tcc a	65	gca agc ttc ttt ctc tct tca
TRIP13-Rev	gtc cac tgc cag aga cag g		
HPRT-Fwd	tga cct tga ttt att ttg cat acc	73	aat gtg atg gcc tcc ca
HPRT-Rev	cga gca aga cgt tca gtc ct		
PBGD common-Fwd	agc tat gaa gga tgg gca ac	25	gcc tgc atg gtc tct tgt a
PBGD common-Rev	ttg tat gct atc tga gcc gtc ta		
PPP1CA common-Fwd	cct ata aga tca agt acc ccg aga	15	cag ttg aag cag tca gtg aa
PPP1CA common-Rev	gat gtt gta gcg tct ctt gca c		
IGF1 Fwd	tgt gga gac agg ggc ttt ta	67	cac tca tcc acg atg cct
IGF1 Rev	atc cac gat gcc tgt ctg a		
IGF2 Fwd	gct ggc aga gga gtg tcc	10	cat tgg gat tcc cat tgg
IGF2 Rev	gat tcc cat tgg tgt ctg ga		
IGF-1R Fwd	ttc agc gct gct gat gtg	10	caa gtt ccc ggc tca tg
IGF-1R Rev	ggc tca tgg tga tct tct cc		
IGF-2R Fwd	cag acc aca tat acc acg agg a	1	agt tga gag aaa aga tgg ggt
IGF-2R Rev	aga aaa gat ggg gtg gct gt		

Table 3. Antibodies employed in the study

Antigen	Source	Order No.	Dilution	Method / System	Pretreatment	Incubation
EGFR	Ventana/Roche	790-2988	ready to use	Ventana /3,3`-diamino-benzidine kit[1]	20 min, Protease	32 min
HER2	Ventana/Roche	800-2996	ready to use	Ventana /3,3`-diamino-benzidine kit[1]	30min, CC1	16 min
IGF-1R	Ventana/Roche	790-4346	ready to use	Ventana /3,3`-diamino-benzidine kit[1]	60 min, CC1	28 min
ER	Ventana/Roche	790-4325	ready to use	Ventana /3,3`-diamino-benzidine kit[1]	60 min, CC1	28 min
PR	Ventana/Roche	790-4296	ready to use	Ventana /3,3`-diamino-benzidine kit[1]	60 min, CC1	28 min
CK 5/6	Ventana/Roche	760-4253	ready to use	Ventana /3,3`-diamino-benzidine kit[1]	60 min, CC1	32 min

[1] (XT Ultra View DAB kit, Ventana Medical Systems, Tucson, Arizona)

For quantitative PCR, 3.5 µl cDNA (50ng/µl) were added to 16.5 µl PCR Master Mix according to the manufacturer´s instructions (LightCycler® 480 Probes Master, Roche). For all targets, uniform ThermoCycler running conditions were used. In brief, initial preincubation at 95°C for 5 minutes was followed by 45 cycles of 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 1 second. Gene expression profiles were generated on the LightCycler® 480 Real-Time PCR System (Roche).

A standard curve, consisting of diluted human reference RNA (Universal Human Reference RNA, Stratagene), was included for every RT reaction and subsequent RT-PCR. For a run to be accepted there had to be a valid negative control and an amplification efficiency of between 1.9 and 2.1. Expression data were normalized to five averaged HKG. All experiments were performed in duplicate and the results averaged. Calibrator based calculations were used to determine relative gene expression profiles [31].

Immunohistochemical analysis

IHC investigation for various surrogate markers was performed to substantiate the results of gene expression analysis and to identify basal-like breast carcinomas. Markers investigated included the RTK EGFR, HER2 and IGF-1R, the hormone receptors ER and PR, and the basal-like marker CK5/6 [32]. Whole FFPET sections cut at 3 µm were stained with a Ventana Benchmark XT autostainer (Ventana Medical

Systems). Details of the antibodies and methods employed are given in Table 3.

Slides were counterstained with haematoxylin (Ventana). System and isotype controls were included. IHC investigation for ER and PR and for HER2 was performed according to up-to-date histopathological guidelines and recommendations [28, 29].

Immunostaining for EGFR and IGF-1R was evaluated semiquantitatively with a scoring system similar to that established for HER2 (0: no membrane-specific staining, 1+: weak, incomplete cell membrane staining in <10% of cells, 2+: weak or moderate staining of the complete cell membrane in > 10% of cells, 3+: strong, complete membrane staining in > 10% of cells. Immunostaining for CK 5/6 was evaluated according to the criteria of Dabbs et al. [33] (0: no staining, R: single cells stained, 1+: 5-30% of cells stained, 2+: >30-60% of cells stained, 3+: > 60% of cells stained). The scores 0 and R were considered negative, 1+ to 3+ positive.

Statistical analysis

Statistical evaluation was performed with the help of the statistics software SPSS (Chicago, USA) and R [34]. The Kolmogorov Smirnov test was used to test for normal distribution. The Mann-Whitney U test and Student's t-test were used to test for significant differences in gene expression between the postulated subtypes RTK-high and RTK-low. T-test for equality of means and Levene´s test for equality of variances

was applied to demonstrate the statistic independence of tumor samples with adjuvant and neoadjuvant chemotherapeutic treatment in context to the gene-expression analyses. The influence of clinical parameters on the gene expression profile was investigated by multivariate analysis on the basis of the Chi-square test and the Mann-Whitney U test. Correlation between variables was observed with Pearson respectively Spearman rank correlation analyses. Hierarchical cluster analysis was performed to identify subtypes within the study cohort. The 3-year survival rate was analyzed with the Kaplan-Meier curve.

Ethics approval and consent to participate

Specimens and data were anonymized, and the need for consent was waived by the institutional ethics committee of the Medical Faculty of the Ludwig Maximilians University of Munich.

Results

Biomarker detection efficiencies within RT-PCR analyses

In the present study 34 tumor associated biomarkers relevant for EGFR and IGF-1R families and related biomarkers of the downstream signaling as well as 5 HKG were analyzed. Expression profiles for all tumor samples could be obtained for 31 of the biomarkers and all five HKG.

Differentiation of triple-negative tumors into two distinct subtypes on the basis of gene expression profiling of RTK associated biomarkers

Hierarchical cluster analysis revealed a dichotomous differentiation pattern amongst the triple-negative tumors (Figure 1).

The two subtypes were characterized by low (subtype RTK-low; N=15) and high (subtype RTK-high; N=14) gene expression of the biomarkers investigated. The corresponding relative gene expression values of each biomarker are shown in Table 4.

Further statistical analysis confirmed significant differences in gene expression between the subgroups RTK-low and RTK-high for 16 of the 31 biomarkers evaluated (Table 4). Of particular interest were significant differences for 3 of the 6 analyzed RTK (HER4, p≤ 0.001; IGF-1R, p < 0,001; IGF-2R, p = 0.015; Figure 2).
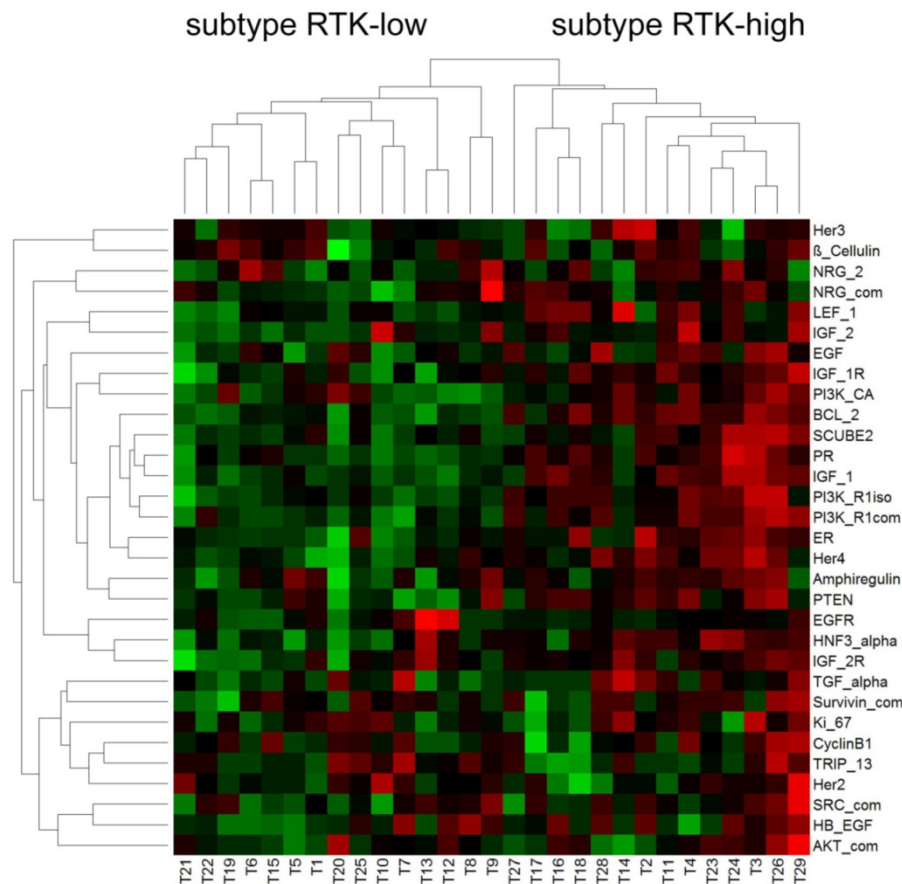

Figure 1. Pearson correlation – triple-negative breast cancer

Table 4. Differential biomarker expression in triple-negative tumors of the subtypes RTK-low and RTK-high

Biomarker	p	TNBC subtype	Mean	95 % CI	Biomarker	p	TNBC subtype	Mean	95 % CI
PR	0.000	RTK-low	2.789	1.802 - 3.776	Amphiregulin	0.24	RTK-low	2.880	0.840 - 4.920
		RTK-high	17.582	5.256 - 29.908			RTK-high	4.610	2.122 - 7.278
HER4	0.000	RTK-low	1.510	0.827 - 2.373	NRG-com	0.408	RTK-low	16.995	-16.498 - 50.488
		RTK-high	10.550	3.887 - 17.213			RTK-high	3.390	1.669 -5.111
EGF	0.043	RTK-low	3.728	1.153 - 6.303	Ki-67	0.407	RTK-low	1.390	1.114 - 1.665
		RTK-high	18.825	4.383 - 33.267			RTK-high	1.628	1.069 - 2.186
PI3K-R1iso	0.000	RTK-low	4.494	3.659 - 5.329	SRC-com	0.254	RTK-low	17.375	13.012 - 21.738
		RTK-high	12.392	8.023 - 16.762			RTK-high	22.269	14.142 - 30.397
SCUBE2	0.003	RTK-low	5.696	3.953 - 7.439	Survivin-com	0.119	RTK-low	0.262	0.185 - 0.339
		RTK-high	22.497	9.788 - 35.206			RTK-high	0.3981	0.228 - 0.568
BCL2	0.000	RTK-low	10.077	7.813 - 12.340	TRIP13	0.382	RTK-low	1.214	0.948 - 1.479
		RTK-high	34.702	26.705 - 42.699			RTK-high	1.035	0.690 - 1.381
IGF-1R	0.001	RTK-low	1.424	0.876 - 1.971	Cyclin B1	0.425	RTK-low	0.92	0.738 - 1.102
		RTK-high	4.332	2.159 - 6.504			RTK-high	1.082	0.696 - 1.467
IGF-2R	0.015	RTK-low	2.545	1.827 - 3.263	HER2	0.794	RTK-low	6.353	5.173 - 7.533
		RTK-high	3.493	2.980 - 4.007			RTK-high	6.093	4.299 - 7.888
IGF2	0.004	RTK-low	0.376	0.224 - 0.976	HER3	0.798	RTK-low	6.324	5.258 - 7.389
		RTK-high	0.473	0.069-1.015			RTK-high	8.650	5.070 - 12.231
HNF3-α	0.001	RTK-low	0.142	0.025-0.308	β-Cellulin	0.889	RTK-low	63.354	37.120 - 89.588
		RTK-high	0.306	0.135 - 0.477			RTK-high	60.973	35.728 - 86.218
IGF1	0.000	RTK-low	31.943	25.742 - 38.143	HBEGF	0.446	RTK-low	3.408	1.849 - 4.968
		RTK-high	113.493	73.942 - 153.044			RTK-high	4.189	2.689 - 5.688
ER	0.000	RTK-low	0.833	0.520 - 1.146	NRG-2	0.727	RTK-low	5.416	1.366 - 9.467
		RTK-high	2.554	1.452 - 3.656			RTK-high	4.651	2.572 - 6.730
PTEN	0.000	RTK-low	3.704	2.278 - 5.129	AKT-com	0.311	RTK-low	4.606	3.578 - 5.633
		RTK-high	6.786	4.883 - 8.689			RTK-high	5.675	3.640 - 7.710
Pi3K-CA	0.043	RTK-low	5.415	3.521 - 7.310	EGFR	0.257	RTK-low	23.781	-12.782 - 60.344
		RTK-high	8.481	6.383 - 10.579			RTK-high	3.645	2.668 - 4.621
LEF1	0.003	RTK-low	0.871	0.684 - 0.106	TGF-α	0.219	RTK-low	3.587	1.112 - 6.062
		RTK-high	0.2561	0.131 - 0.381			RTK-high	5.865	2.817 - 8.913
PI3K R1 iso	0.000	RTK-low	4.494	3.659 - 5.329					
		RTK-high	12.392	8.023 - 16.762					
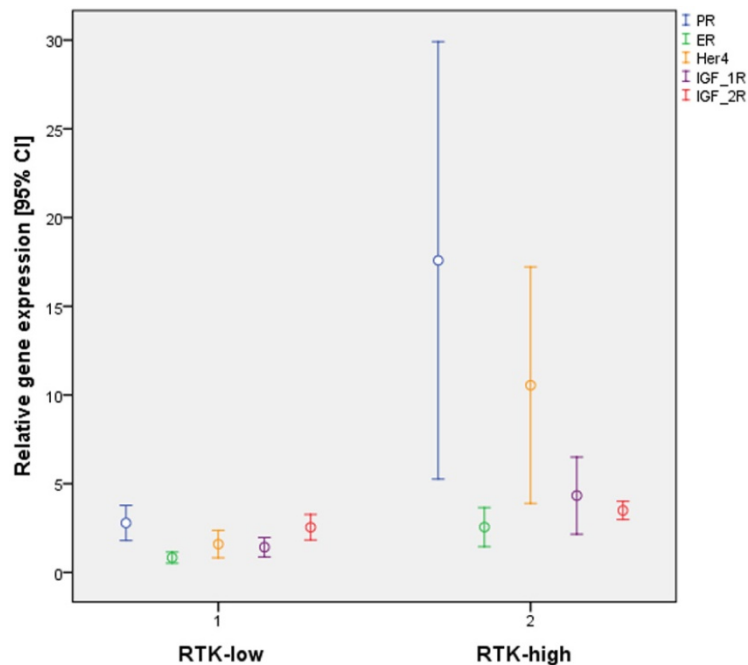
Figure 2. Differential gene expression by the triple-negative subtypes RTK-low und RTK-high in relation to PR, ER, HER4, IGF-1R and IGF-2R

Interestingly, significant differences in gene expression patterns for ER (p = 0.001) and PR (p < 0.001) were also found between the two subtypes (Figure 2). By contrast, all cases of triple-negative tumors, by definition lacked IHC staining for ER and PR.

Correlation analysis was performed to determine whether the RTK and hormone receptors investigated are subject to functional interaction (Table 5).

Table 5. Correlation coefficients for RTK, ER and PR gene expression

	ER	PR	EGFR	egfr	HER3	HER4	IGF-1R	IGF-2R
ER	1							
PR	0.654**	1						
EGFR	0.034	0.001	1					
HER2	0.057	-0.081	0.099	1				
HER3	-0.109	0.078	0.023	0.000	1			
HER4	0.675**	0.623**	-0.021	-0.105	0.222	1		
IGF-1R	0.419*	0.555**	-0.056	0.112	0.206	0.394*	1	
IGF-2R	0.293	0.284	0.519**	-0.034	0.354	0.369*	0.264	1

** $p<0.005$; * $p<0.05$

Increased correlation, in particular for HER4 with ER (r = 0.675; p≤0.001), HER4 with PR (r=0.623, p≤0.001), as well as IGF-1R with ER (r = 0.419; p=0.024) and IGF-1R with PR (r=0.555; p=0.002) was found (Figure 3).

Additionally, we compared the patient survival based on the RTK-high and RTK-low gene signature. Kaplan-Meier analysis demonstrated superior three-year survival in the RTK-high group as opposed to the patients in the RTK-low group (three-year survival rate of 100% v. 60%; log-rank test, p-value = 0.022; Figure 4).

In summary, two subtypes of TNBC could be identified on the basis of gene expression profiles of 16 biomarkers of the EGF and IGF receptor families and biomarkers of their downstream signaling as well as the hormone receptors ER and PR (Table 4). Of special interest are the RTK HER4 and IGF-1R, as well as the hormone receptors ER and PR, for which the findings of correlation analysis indicate functional interaction in cell signaling.

Comparison of IHC and gene expression findings

In order to determine to what extent the differences in gene expression between the subtypes RTK-high and RTK-low as detected by RT-PCR are also reflected at the protein level, IHC investigations were carried out for certain key proteins from the biomarker panel (IGF-1R and EGFR: study findings, HER2, ER and PR: findings of routine diagnostic
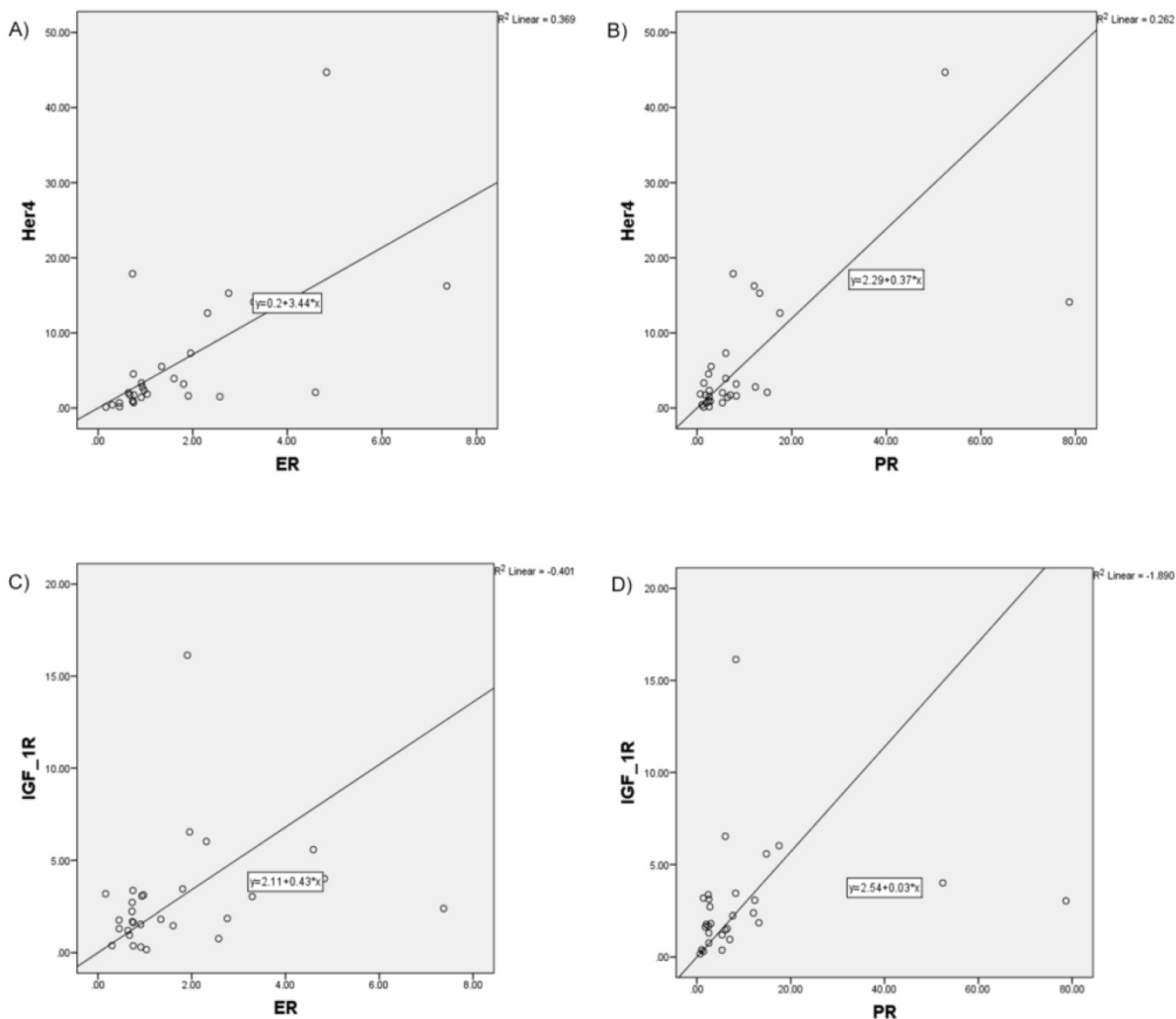
Figure 3. Increased correlation between analyzed RTK and hormone receptors pictured in scatter plot charts A) HER4 and ER, B) HER4 and PR, C) IGF-1R and ER, D) IGF-1R and PR
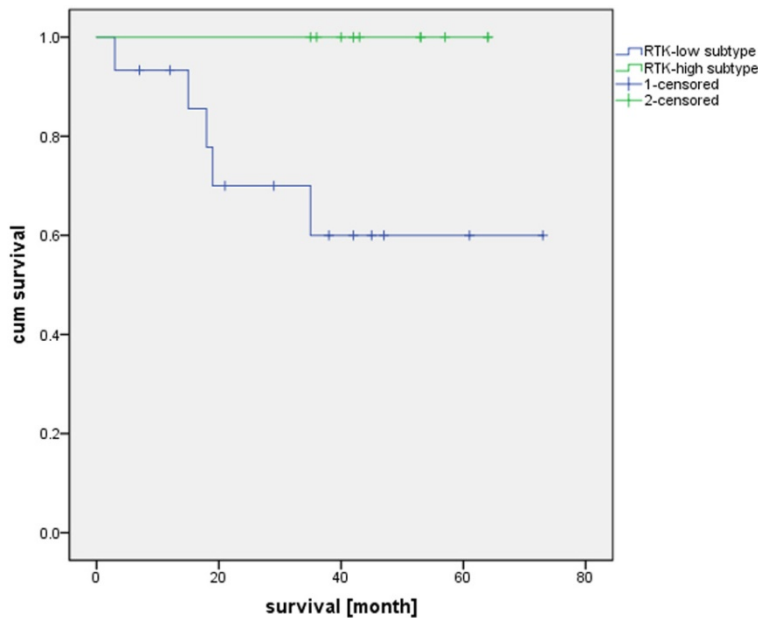
Figure 4. Overall survival of patients with RTK-high and RTK-low gene signature

work-up). For IGF-1R and EGFR the IHC findings were consistent with the gene expression data. Accordingly, differences in IGF-1R expression at both transcriptional and translational levels were found between the subtypes RTK-high and RTK-low (IHC: p Mann-Whitney U test = 0.005, RT-PCR: p Mann-Whitney U test < 0.001; r Spearman-Rho = 0.706, $p \leq 0.001$). The expression profile of EGFR in both subtypes of triple-negative tumors could also be confirmed at the IHC level (IHC: p Mann-Whitney U test = 0.377, RT-PCR: p t-test = 0.257; r Spearman-Rho = 0.311, $p = 0.100$).

As shown in Table 1, each case in the study group exhibited a triple negative phenotype according to established histopathological criteria. However, there were highly significant differences in the gene expression profiles for both ER (p Mann-Whitney U test = 0.001) and PR (p Mann-Whitney U test $\leq$ 0.001) amongst the triple-negative tumors in relation to the postulated subtypes RTK-high and RTK-low. As with the IHC findings, no significant difference was found for HER2 at the transcriptional level between the postulated subtypes RTK-high and RTK-low (p t-test = 0.794; r Spearman-Rho = -0.087, $p = 0.655$).

Immunohistochemical characterization of basal-like subtypes

In relation to the much debated question as to the extent to which triple-negative tumors correspond to the basal-like breast cancer subtype, and to determine to what extent one of the two triple-negative subtypes defined in the study correlates with the basal-like subtype, IHC staining for the basal-like tumor associated biomarker CK5/6 and EGFR was performed. The IHC analyses revealed

a basal-like phenotype within 79% of the samples. Statistical analysis did show no significant difference between the triple-negative subtypes defined in this study on the basis of gene expression analyses and the basal-like and non-basal-like TNBC subtypes (p Pearson Chi-Square test = 0.080).

Comparison of clinical parameters and genetic expression of tumor-related biomarkers

By means of multivariate analysis the variables nodal status, age, tumor size and angiosis carcinomatosa were tested for a possible relationship to biomarker expression and subtype (RTK-high and RTK-low). The Chi-square test and Mann-Whitney U test failed to show a significant relationship between any of the clinicopathological parameters and the subtypes.

T-test for equality of means and Levene's test for equality of variances demonstrated the statistic independence of tumor samples with adjuvant and neoadjuvant chemotherapeutic treatment in context to the RTK gene-expression analyses (Table 6). None of the changes was significant after Bonferroni correction.

Discussion

Characterization and subtyping of triple-negative tumors based on therapeutically relevant RTK

The search for individualized treatment strategies in recent years has revealed biomarkers with predictive relevance for a large number of malignancies. This information has been applied to the development of individualized tumor therapies. The various RTK are of special importance here, particularly representatives of the EGF and IGF receptor families which, in the case of overexpression, represent target proteins for individualized tumor therapy for an increasing number of tumor entities.

In the case of breast cancer, too, gene expression data and IHC findings have characterized patients for whom target-specific therapy is suitable. A number of clinical trials are concerned with the question as to what extent inhibition of EGFR or IGF-1R by specific monoclonal antibodies or RTKI can benefit patients with advanced breast cancer [35, 36, 37, 38, 39]. The combination of chemotherapy with EGFR inhibition appears so far to have mixed results. Adequate results of an individual therapeutic IGF-1R inhibition are not yet available.

Table 6. Statistic independence of tumor samples with adjuvant and neoadjuvant chemotherapeutic treatment in context to the RTK gene-expression analyses

Biomarker	Fold Change (neoadjuvant versus adjuvant)	p-value (Welch)	p-value (Levene)	Biomarker	Fold Change (neoadjuvant versus adjuvant)	p-value (Welch)	p-value (Levene)
PR	-1.847	0.269	0.212	PI3K_R1iso	-1.589	0.078	0.607
ER	-1.145	0.755	0.254	AKT_com	1.176	0.325	0.478
EGFR	-1.022	0.960	0.158	SRC_com	-0.114	0.511	0.780
HER2	1.135	0.393	0.834	SCUBE2	-0.135	0.515	0.222
Her3	-1.009	0.970	0.777	BCL2	-1.712	0.081	0.915
Her4	-1.170	0.804	0.376	IGF-1R	-1.454	0.390	0.690
TGF_alpha	-1.079	0.817	0.943	IGF-2R	-1.403	0.090	0.250
EGF	-2.634	0.150	0.604	IGF1	-1.557	0.234	0.477
ß_Cellulin	-1.125	0.826	0.279	IGF2	-1.297	0.729	0.605
Amphiregulin	-1.945	0.259	0.482	HNF3-alpha	-1.842	0.318	0.046
HB_EGF	1.183	0.573	0.924	Survivin com	-1.600	0.117	0.765
NRG_com	-1.506	0.531	0.962	TRIP 13	1.044	0.794	0.256
NRG_2	0.140	0.348	0.153	CyclinB1	-1.159	0.386	0.151
PTEN	-1.546	0.102	0.881	Ki-67	1.037	0.840	0.161
PI3K_CA	-1.242	0.385	0.274	LEF1	-1.855	0.041	0.973
PI3K_R1com	-1.558	0.082	0.233				

At the same time, it has been shown in recent years that despite the importance of single tumor-relevant biomarkers, the neoplastic behaviour of tumors is influenced by numerous interactions in downstream signaling. The crosstalk of various overexpressed RTK, hormonal transcriptional regulation (ER) or mutational, constitutive activation in downstream signaling (EGFR, KRAS) represent multifactorial mechanisms involved in tumor growth. Because of the complexity of the situation, more recent clinical trials have focused increasingly on combination treatment strategies in narrowly defined patient groups. The characterization of breast tumors using a panel of tumor-specific surrogate markers not only contributes to a better understanding of functional mechanisms involved in tumor differentiation, growth and invasion, but also represents a basis for new combination treatment strategies. In addition, exact subtyping of breast cancer cases such as luminal A, luminal B, normal breast-like, ERBB2-overexpressing and basal-like molecular subtypes of breast cancer is still used for optimal patient selection in clinical trials.

In the study presented, we examined gene expression levels of various RTK and related biomarkers in TNBC without individualized treatment options with the aim of defining heterogeneous expression patterns and possible TNBC subtypes. Hierarchical cluster analysis was able to identify a panel of biomarkers that allows two distinct subtypes of TNBC to be distinguished. Further analysis of the genetic cluster emphasizes in particular the significance of the two RTK IGF-1R and HER4, as well as the hormone receptors ER and PR. Cases with overexpression of these markers described above (RTK-high) represent a potentially interesting group for clinical investigations. In addition, the possibility to identify those breast cancer patients who are potential responders for personalized medicine targeting IGF-1R or ER is of particular significance.

Differentiation of triple-negative tumors into basal-like positive and negative subtypes

Several microarray-based studies have demonstrated a differentiation of breast cancer into at least 5 different subtypes [40, 41, 42, 43]. Selected gene sets for around 500 biomarkers enable subtyping of breast carcinoma into luminal A, luminal B, normal breast-like, ERBB2-overexpressing and basal-like tumors. Tumors with the basal-like phenotype which are characterized by, among other features, by the absence of IHC ER and HER2 expression, are of particular clinical interest. Scientific studies employing IHC techniques and gene expression analysis have shown that up to 15% of all breast cancers exhibit the basal-like subtype [32, 33]. Numerous studies have attempted to equate triple-negative tumors with basal-like breast cancer, and indeed an accumulation of the basal-like phenotype amongst triple-negative tumors has been documented. Nevertheless, it has been shown that the two are not identical with each other [44]. Comparative analysis has shown that, although triple-negative tumors (immunohistochemically defined) often show a basal-like phenotype, and tumors diagnosed as basal-like breast cancer (on the basis of gene expression) are predominantly triple-negative, the two categories are up to 30% discordant [19, 45].

Methods for the identification of the basal-like phenotype rely mainly on the detection of expression of proliferation-specific biomarkers by gene expression analysis and immuno-histochemical techniques. Our study utilized IHC staining for CK 5/6. Eighty-one percent of the tumor samples were found to express a basal-like phenotype, a prevalence consistent with figures quoted in the literature.

In our study, comparison of the subtypes RTK-high and RTK-low and the group classed as CK 5/6 positive revealed no significant correlation. Therefore, on the basis of our findings it can be assumed that this is a newly defined and independent gene expression signature that permits further subtyping of TNBC into RTK-high and RTK-low genotypes.

Gene expression of ER in triple-negative breast cancer

IHC analysis of the hormone receptor status, is one of the standardized clinical investigations used in the diagnostic work-up of patients with breast cancer for treatment prediction. Selection of the cases for our study was based upon the findings of routine histopathological investigations. By definition, all exhibited a negative (<1%) IHC score for ER and PR. By contrast, our gene expression analysis revealed over-expression of ER and PR in some cases, there being a highly significant difference between the subtypes RTK-high and RTK-low. The discrepancy may be due to the increased sensitivity of RT-PCR. Differences in regulation of protein expression at the translational level are also conceivable.

The results of correlation analysis pertaining to IGF-1R, HER4 and ER in tumors with the RTK-high genotype suggest that these receptors interact with each other through regulatory mechanisms. Hence, further in-vivo investigations, especially into the potential of new immunotherapeutic approaches using combined immunotherapeutic approaches in TNBC patients with the RTK-high genotype, would be of great interest [46, 47, 48].

Crosstalk between IGF-1R and ER signaling

Recent studies have shown that there is crosstalk in cell signaling between RTK and ER. Various different trans-activating mechanisms with stimulating effects on the downstream signaling of adjacent RTK and ER were generally found [49, 50].

IGF-1R is one of the most important representatives of the IGF receptor family and, like ER, is associated with neoplastic properties when overexpressed [51, 52, 53].

In our study we found increased expression of IGF-1R and ER in TNBC of the RTK-high subtype. In addition, strong correlation between IGF-1R and ER was seen, which suggests functional interaction between the two receptors. As shown in recent studies, IGF-1R and ER are able to trans-activate each other and lead to activation or enhancement of downstream signaling [52]. In addition, in vivo experiments have demonstrated their combined significance in tumorigenesis [54].

In studies of IGF-1R-mediated ER activation [55, 56, 57] it has been shown that tyrosine kinases involved in IGF-1R downstream signaling, such as MAPK, RSK and AKT, are able to phosphorylate and activate ER at the AF-1 domain (Serin118, Serin167). Furthermore, in vivo experiments showed that IGF-1 is able to increase the expression of PR, an estrogen response element.

In addition to IGF-1R-mediated ER phosphorylation and activation, effects of ER on IGF-1R signaling have also been demonstrated [47]. ER is able, by means of various different regulatory mechanisms, to impact on IGF-1R downstream signaling. Studies have shown that anti-estrogens like tamoxifen can inhibit IGF-mediated growth [58]. Thus, it has been demonstrated that estrogens are able to regulate IGF-1R at the transcriptional level and to transactivate IGF-1R by phosphorylation. ER belongs to the steroid hormone receptor family and functions primarily as a transcription factor. IGF-1R is one of the most important estrogen response elements [58].

Besides the ability of ER to inactivate inhibitory elements of IGFR signaling, such as IGFBP-3, binding of ER to Shc with subsequent phosphorylation of MAPK have also been described. In addition to the singular importance of hormone receptors as a therapeutic target, several studies have demonstrated the existence of complex regulatory mechanisms and interactions in downstream signaling between ER/PR and growth factors [59, 60]. Several clinical trials concerning the combined use of IGF-1R and ER-inhibitors are under investigation [61, 62].

Crosstalk between HER4 and ER signaling

The c-erbB-4 gene (HER4) belongs to the EGFR family. Little is known about the functional significance of HER4 in the pathogenesis of breast cancer. In normal breast tissue, its functions include the differentiation of myocardial cells and mammary epithelial cells and the development of the central nervous system [63, 64]. Compared to the most important representatives of the EGFR family, EGFR and HER2, the prognostic impact of HER4 expression in breast cancer are basically unclear and controversially discussed [65, 66]. Unlike the remaining members of the EGF receptor family, which act primarily as mitogenic effectors in breast cancer cells, HER4 appears to have a large number of different functions in normal and neoplastic breast tissue. With regard to the overexpression of HER4, experimental studies have shown both oncogenic and tumor suppressive functions [67, 68]. Other investigations have also shown that HER4 is generally associated with differentiated and less aggressive tumors, and that it correlates with a better prognosis

and a longer disease-free interval [66, 69, 70]. In addition, it has been shown that breast cancer patients who exhibit coexpression of HER4 and ER have a lower recurrence rate [71] and a higher survival rate [72] than those who express ER alone.

Interestingly, several in vivo and in vitro studies have shown a strong correlation and crosstalk between HER4 and ER expression in breast cancer [68, 73, 74]. Intense efforts to elucidate mechanism of crosstalk between HER4 and ER could identify an autocrine HER4/ER signaling pathway where the factor HER4 intracellular domain (4ICD), a cleavage product of HER4, acts as transcriptional coregulator of ER [73, 75, 76, 77, 78].

Our results point to an increased rate of coexpression of HER4 and ER in triple-negative tumors with the RTK-high phenotype. The coexpression of HER4 and ER suggests the existence of crosstalk in downstream-signaling within defined subpopulations of breast cancer patients.

ER / RTK crosstalk– development of intrinsic and acquired resistance

Antihormonal therapy (tamoxifen, anastrozole) and HER2 inhibition (trastuzumab, lapatinib) represent important cornerstones in personalized therapy for the treatment of breast cancer in women with overexpression of the corresponding receptors. Further promising targets for individualized therapy the RTK EGFR and IGF-1R and targeting the PI3K-Akt pathway are currently subject of several studies and clinical trials [79, 80, 81, 82]. However, follow-up studies did show the development of resistance in some patients receiving antihormonal therapy and/or RTK inhibitors.

Studies have demonstrated intrinsic or acquired resistance to treatment with tamoxifen in 20-30% of hormone-receptor-positive breast cancer patients [83]. Although the mechanisms involved have not yet been clarified in detail, it appears that, in addition to coregulatory and epigenetic mechanisms, transactivating processes between RTK and ER could be responsible [80, 84]. Further investigations have revealed complex co-operation of genomic and non-genomic/fast ER signaling and their crosstalk with growth factor receptors, such as EGFR, HER2, HER4 or IGF-1R, in the development of endocrine resistance [85, 86, 87]. In brief, activated cytoplasmic and membrane-bound ER show the ability to stimulate RTK, either directly via adaptor proteins like Shc, or indirectly by nuclear ER through increased release of RTK-specific ligands, for example HB-EGF. Following subsequent activation of the downstream signaling kinases MAPK and AKT, phosphorylation and thus activation of nuclear ER occurs, which leads to increased gene expression, including the expression of RTK-related genes. Thus, innovative treatment strategies aim at inhibition of both RTK and ER.

Current indications for hormone receptor therapy are based on standardized IHC findings of routine diagnostic investigations. According to the histopathological guidelines and recommendations [28], antihormonal treatment is indicated when more than 1% of the tumor cells are ER and/or PR positive. Large overviews of randomized clinical trials have confirmed the therapeutic value of antihormonal treatment only in immunreactive ER-positive breast cancers.

Our investigations demonstrated a significant increase in ER gene expression level in a newly characterized subtype (RTK-high) of TNBC. In addition, correlation analysis suggests functional interaction between IGF-1R, HER4 and ER. It is not yet clear to what extent antihormonal therapy would be effective in tumors in which hormone receptor status is negative by immunohistochemistry but gene expression is elevated. However, on the basis of crosstalk between ER and RTK, it is conceivable that a combination of inhibition of both ER and corresponding RTK, like IGF-1R or HER4, could be of benefit.

Conclusions

RTK-associated gene expression profiles generated in this study revealed dichotomous differentiation within the triple-negative study group. IHC analysis verified this at the posttranslational level. Correlation analysis of the biomarkers investigated suggests a functional connection between IGF-1R, IGF-2R, HER4, PR and ER. Based on survival analysis the differentiation of triple-negative tumors with RTK-high and RTK-low gene signature seems to be from prognostic relevance.

Abbreviations

TNBC: Triple-negative breast cancers; ER: estrogen receptor; PR: progesterone receptors; IHC: immunohistochemical; RTK: receptor tyrosine kinase; RT-PCR: reverse transcription polymerase chain reaction; EGFR: epidermal growth factor receptor; RTKI: receptor tyrosine kinase inhibitors; IFF-1R: insulin-like growth factor receptor 1; pCR: pathological complete response; FFPET: formalin-fixed, paraffin-embedded tissue; HKG: housekeeping genes; 4ICD: HER4 intracellular domain.

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Competing Interests

The authors have declared that no competing interest exists.

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