Triple-negative breast cancer: New therapeutic options via signalling transduction cascades

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Abstract. Triple-negative breast cancer is a highly aggressive type of mammalian carcinoma. It is defined by a rather weak expression of estrogen-, progesterone- and Her2-receptor, and is thus difficult to treat, resulting in low disease-free and overall survival rates of the affected patients. Hence it is important to find new therapeutic options. To this aim we analysed the incidence of some molecules from different signal transduction cascades by immunohistochemistry, which are known to correlate with triple-negative breast cancer, and correlated the expression of these molecules to different tumour traits, such as size, grading, menopausal stage, histology, lymph node affection, remote metastasis formation, and to the incidence of local and lymph node recurrence and metastasis by statistical analysis. Statistically significant correlations were found for a number of tumour characteristics and signalling molecules: HIF1α is correlated to tumour grading, β-catenin to the menopausal state of the patient, and for Notch1 a relation to lymph node affection is seen. In terms of different recurrences, a correlation of β-catenin to metastasis formation and lymph node affection could be shown, as well as coherences between XBP1 and lymph node recurrence, Notch1 and metastasis formation and FOXP3 and the occurrence of local recurrence. The presented results are in accordance with formerly published studies and therefore might comprise opportunities to develop new therapeutical strategies, which could help to handle this aggressive form of breast cancer in a manner, by which side effects would be reduced and therapeutical efficiency is increased.

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Abbreviations: CSCs, cancer stem cells; DFS, disease-free survival; ECM, extracellular matrix; EGFR, endothelial growth factor receptor; EMT, epithelial to mesenchymal transition; ER, estrogen receptor; FOXP3, forkhead box protein 3; Her2, human epidermal growth factor receptor 2; HIF1α, hypoxia inducible factor 1α; IRS, immunreactive score; LRP6, low-density lipoprotein receptor-related protein 6; MCL1, myeloid leukemia cell differentiation protein 1; MMP13, matrix metalloprotease 13; OAS, overall survival; PARP, poly(ADP-ribose) polymerase; PR, progesterone receptor; TNBC, triple-negative breast cancer; VEGF, vascular endothelial growth factor; XBP1, X-box binding protein 1

Key words: triple-negative breast cancer, signal transduction cascades, cancer treatment, immunohistochemistry, statistical analysis, tumour traits, recurrence

Introduction

Breast Cancer is still the most frequent malignant disease worldwide, and the most frequent cause of death in women (1). One out of eight women is diagnosed with breast cancer during her lifetime. Although lethality has declined in the last 40 years, still 30% of the affected patients die from the consequences of breast cancer (2).

Approximately 10-20% of all diagnosed breast cancers are characterized by a lack of expression or a only very weak expression of the hormone receptors, estrogen- and progesterone-receptor, and of the human epidermal growth factor receptor 2 (Her2) (3). These quite aggressive tumours, which are termed 'triple-negative breast cancer' [TNBC (4)], occur frequently in younger women, many of them show up with BRCA-1 mutations (5). Patients suffering from TNBC may develop visceral metastases, have a high risk of recurrence and a reduced overall survival (6), independent of tumour size, staging and lymph node affection (7).

Furthermore, the possibilities for treatment are sparse, as endocrine therapy with Tamoxifen or aromatase-inhibitors as well as anti-Her2 therapy with Trastuzumab are ineffective. Actually, TNBCs are treated postoperatively with a dose-dense or metronome chemotherapy using anthracyclins or taxans and radiation (8). Only in the neoadjuvant setting TNBC shows a better follow-up as non-TNBC (9). Recent therapeutic concepts introduced for example inhibitors of poly(ADP-ribose) polymerase (PARP) in addition to chemotherapy, such as Iniparib or Olaparib, which are normally responsible for the repair of single- and double-strand DNA breaks. The aim is, to accumulate severe DNA-damage within the tumour cells, that the cells stop entering mitosis, thereby stopping cell division. Thus disease-free survival can be prolonged (10). The risk of recurrence can be reduced by the additional application

of VEGF inhibitors such as bevacizumab, which decelerates tumour growth by reduction of neoangiogenesis (11). However, the disadvantage of this treatment is, that bevacizumab has rather strong side effects (12,13).

From all those facts the importance of new treatment options can be explained. New therapeutic strategies could target for example inter- and intracellular signal transduction pathways, regulation of cell adhesion and proliferation. Clinical studies are carried out using cetuximab, an inhibitor of epithelial growth factor receptor (EGFR) (14,15) which seems to improve survival rates without further tumour progression (16), or the mTOR-inhibitor everolimus (17). Another treatment strategy could target the Notch1 pathway. Notch1 plays an important role in normal breast development and cell fate determination. In breast cancer tissue, especially TNBC, this pathway is activated in an aberrant manner. Additionally it has been shown, that an inhibition of Notch signalling by gamma-secretase inhibitors (GSIs) results in antitumour activity by cell cycle arrest, apoptosis and disruption of angiogenesis (18,19).

Notch-pathway members are moreover regulated by HIF1α, which thereby constitutes another possibility for TNBC treatment. Overexpression of the transcription factor HIF1α is associated with a poor prognosis for the affected patients, and in a mouse model it was shown, that it is important for promoting carcinoma onset and formation of lung metastasis. The deletion of HIF1α resulted in reduced primary tumour growth, suppression of lung metastasis and prolonged survival (20). HIF1α and XBP1 in turn form a transcriptional complex, which is known to drive tumorigenicity. XBP1 thereby regulates the expression of HIF1α targets via recruitment of polymerase II. Hence, XBP1 also plays a role in tumour progression, especially in TNBC, contributing to a poor prognosis. In a cell culture model it was shown, that an inhibition of XBP1 results in a retardation of tumour growth, giving hints to another therapeutic target (21).

Another transcription factor, which is highly expressed in tumour cells is FOXP3, a potent repressor of several oncogenes. Its involves in TNBC susceptibility and prognosis (22), but the prognosis is dependent on the cellular localisation of FOXP3: If it is located in the cytoplasm, it is a marker for poor OAS, but if it remains in the nucleus, OAS is markedly improved (23). Furthermore the Wnt/β-catenin signalling pathway could be used for therapeutical intervention, as this pathway regulates the cell cycle, cell growth and tumour progression and is responsible for poor clinical outcomes.

The Wnt/β-catenin pathway was shown to be activated in TNBC, the inhibition of Wnt-receptors by, e.g. salinomycin, is already known as therapeutic target, as it induces LRP6 degradation (24). LRP6 in turn, is frequently produced in response to obesity stimuli and induces cell proliferation by interaction with Ki67 and reduces efficiency of treatment. LRP6 is of great use in TNBC-therapy, as it has no relation with ER, PR, and Her2. Its use as drug target receptor significantly prolonged survival time in a mouse model (25). Another signalling molecule within the Wnt-signal transduction pathway, which might be of therapeutic significance, is MCL1, which modulates mitochondrial physiology, and is associated with enhanced metastasis formation and decreased DFS (26).

Table I. Patient/tumour characteristics.

Characteristics	No. of patients
Tumour size	
pT1	19
pT2	8
pT3	1
pT4	2
pTx	1
Lymph node affection	
pN0	17
pN1	11
pN2	2
pNx	1
Metastatic stage	
pM0	20
pM1	1
pMx	10
Grading	
G1	1
G2	7
G3	14
Gx	9
Histology	
Ductal	18
Lobular	4
Medullar	7
Other	2

Materials and methods

Patient samples. Tumour tissue of breast cancer patients, who underwent breast cancer surgery between 2001 and 2002 were collected by the Department of Obstetrics and Gynaecology of Ludwig-Maximilians University of Munich, and subsequently embedded in paraffin. Ethics approval compliant to the Declaration of Helsinki for the collection of these samples was available (LMU 048-08 and 148-12). Hormone receptor and Her2 status were determined pathologically. Thirty-one patients had a triple-negative receptor state and were studied by immunhistochemical analysis. At the time of surgery, patients had an average age of 62 years. Further tumour characteristics are listed in Table I.

Immunohistochemical staining. The paraffin-embedded tissues samples were cut in thin sections by a sliding microtome and transferred onto specially covered microscope slides (SuperFrost Plus, Menzel GmbH, J1800AMNZ/ground 90˚). The slides were air-dried overnight at 56-58˚C. For the staining procedure paraffin was removed by incubation of the slides in xylol (Merck, 81500) for 20 min, followed by washes in different dilutions of ethanol (100, 90, 75%). Slides were then incubated in 3% H_2O_2 (VWR International,

Table II. Primary antibodies used for staining.

Antibody	Clonality	Working dilution	Distributor	Order no.
Anti-HIF1α	Monoclonal Rabbit-IgG	1:2000	Sigma Aldrich	HPA001275
Anti-β-catenin	Polyclonal Rabbit-IgG	1:300	Diagnostic Biosystems	RP080
Anti-XBP1	Monoclonal Rabbit-IgG	1:400	Sigma Aldrich	HPA044305
Anti-FOXP3	Monoclonal Mouse IgG1	1:300	Abcam	ab20034
Anti-NOTCH1	Monoclonal Mouse-IgG	1:100	Sigma Aldrich	N5163
Anti-MCL1	Monoclonal Rabbit-IgG	1:1000	Abcam	ab53709
Anti-LRP6	Rabbit-IgG	1:80	Millipore	06-017

ACRO42600100) to reduce activity of endogenous peroxidase and thereby to prevent unspecific staining of tissue samples. Following slides were again washed in ethanol and water and boiled in Na-Citrate (Merck, 106448) Buffer (pH 6.00) for 5 min to reconstitute the antigens. After cooling down, samples were again washed in water and PBS (Biochrom, order no. L1835). To prevent unspecific binding of the primary antibody, samples are blocked in 10% normal goat serum (Vector Laboratories, S-1012) for 20 min, then the blocking solution was removed and primary antibodies were applied in the appropriate concentrations (see Table II).

Incubation of primary antibodies was carried out at 4˚C for 18 h, following slides were washed twice with PBS and incubated with the biotinylated secondary antibody for 30 min at room temperature. When the secondary antibody was removed, the samples were treated with ABC-reagent (Vector Laboratories, order no. AK-5200) for 30 min, then DAB-reagent (Dako, K-3468), diluted in H_2O_2 was added to the slides for 1 min. Enzyme reaction was stopped by washing the slides in water. Nuclei were then counterstained by Hemalaun (Applichem, A0884) for 5 min before slides are again dehydrated with ethanol and xylol and embedded in Eukitt (Sigma, 03989).

To be sure of the function of the primary antibody, and also to determine its optimal working dilution, a tissue sample, which was confirmed to express the complimentary antigen was stained (tissues used for the antibodies and used concentrations are also given in Table II). Furthermore an isotype control was carried out, staining the same tissue as for the positive control, replacing the primary antibody by a control serum. Thereby the unspecific background of each antibody could be determined.

Microscopy. Staining of the samples was observed and evaluated by two independent persons by a Leitz Diaplan light microscope (Ernst Leitz GmbH, Wetzlar, Germany), equipped with four objectives for different magnifications (x6.3, x10, x25, x40). Evaluation was carried out following the immunoreactive score [IRS (27)]. In brief, staining intensity is rated in groups from 0 (no staining) to 3 (strong colour reaction), and number of stained cells is also classified in groups from 0 (no stained cells) to 4 (81-100% of cells stained). A multiplication of both values results in the IRS score, ranging from 0 to 12. The IRS is then set into reference with different tumour characteristics.

Statistical evaluation. Statistical analysis was performed by SPSS (SPSS Inc., Chicago, IL, USA) version 22.0. Correlations were calculated by the non-parametric Kruskal-Wallis test. A p-value of ≤0.05 was regarded to be statistically significant.

Results

Correlation of staining with different tumour characteristics. The TNBC-tissue sections were prepared and stained immunohistochemically (Fig. 1) as described above and an IR-Score was calculated. The IRS was then set into correlation with different tumour characteristics using SPSS software for calculation (Table III). The non-parametric Kruskal-Wallis test revealed a statistically significant correlation of cytoplasmic HIF1α-staining and tumour grading (p=0.030). A borderline significance was seen for the cytoplasmic staining of β-catenin and menopausal state (p=0.068). Furthermore, an association of Notch1 staining, cytoplasmic and nuclear, and lymph node affect on tumours were detected (p=0.049 and p=0.063, respectively). For the other tumour parameters such as tumour size, metastatic affection and histological classification no significant correlations could be found. In addition, the other signal molecules investigated in our study, such as nuclear HIF1α, and nuclear β-catenin, XBP1, MCL1, LRP6 and FOXP3 did not seem to have an influence on tumour characteristics.

Correlation of staining with recurrence. In the following we investigated, if the signal transduction molecules could be correlated to different types of recurrence, such as local recurrence, lymph node recurrence or metastatic recurrence (Table IV). Two statistically strong correlations were seen for β-catenin: the cytoplasmic staining correlated with the occurrence of remote metastases (p=0.007), whereas the nuclear staining was associated with a lymph node recurrence (p=0.018). The nuclear β-catenin staining was also correlated in a borderline manner to the appearance of remote metastasis (p=0.100). Significance of the three more borderline values were found: XBP-1 could be linked to a lymph node recurrence (p=0.059), nuclear Notch1-staining was connected to remote metastasis formation (p=0.082) and nuclear FOXP3 seemed to be related to the appearance of local recurrence (p=0.083). No statistically significant correlations could be found for HIF1α, cytoplasmic Notch1 and FOXP3, Mcl1 and LRP6.

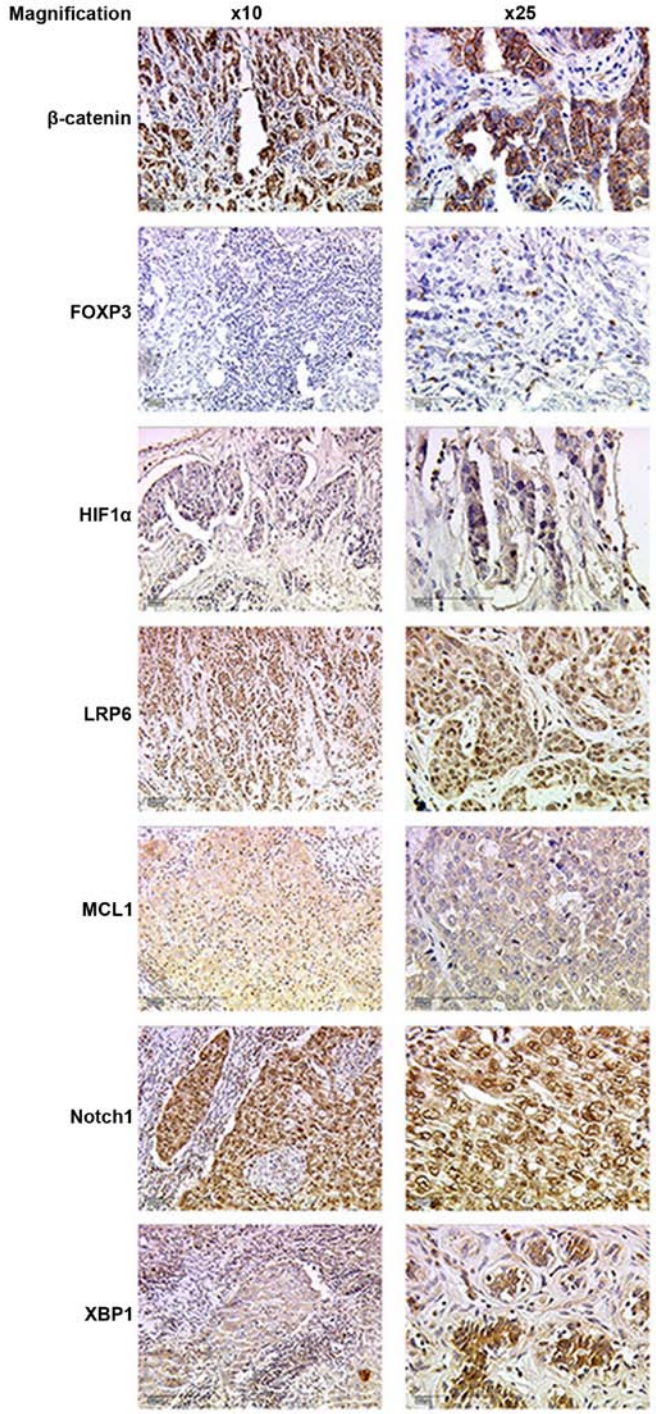

Figure 1. Staining of triple-negative breast cancer tissue with antibodies against the different signal transduction molecules. Images were taken with different objectives (x10, x25) resulting in different magnifications of tissue structures. Brown, DAB-staining; blue, Hemalaun counterstaining of the nuclei.

Discussion

The results clearly indicate that some of the analysed signal transduction molecules have a correlation to the incidence of patient and tumour traits, and are furthermore correlated to different recurrences. The novelty of this study lies in the combination of marker molecules of breast cancer tissue samples and their correlation to other tumour and patient characteristics, especially of the rather aggressive and hard to treat triple-negative breast cancer subtype.

A drawback of the presented study is of course the small number of patient samples analysed, so that the results have to be considered as preliminary. However, the data already show up a certain trend, which should be clarified by the analysis of a larger patient collective. A higher number of samples analysed would also improve the statistical significance.

Nevertheless, some of these findings are in accordance to recently published data, either in breast cancer or in other tumour entities. The correlation of HIF1α to the histological grading was already described for ovarian cancer (28), where this factor might become important for prognostic evaluation and clinical treatment. Moreover, the linkage between β-catenin and menopausal stage might be of clinical relevance, as Wang *et al* found, that the Wnt-pathway influenced the pathogenesis of postmenopausal osteoporosis (29). The Wnt/β-catenin pathway also contributes to radioresistence of TNBC cells. Niclosamide, a potent inhibitor of the Wnt/β-catenin pathway might be able to abolish the radioresistence of these cells, improving the TNBC-therapy (30).

Wnt/β-catenin and Notch1 are normally responsible for mammary gland morphogenesis during embryonal development and are frequently found to be upregulated during tumorigenesis, creating another treatment option (31). The role of Notch1 in lymph node affected infiltrating ductal carcinomas was already described in the literature, claiming the involvement of Notch1 in epithelial-mesenchymal transition (32) which is a rather important process in metastasis formation. Furthermore patients with high expression of Notch1 had a worse OAS and DFS (33). Recently, the microRNA, miR9, was identified to regulate Notch1 in a manner to suppress its tumorigenic capacity (34). Furthermore, the ATPase, a2V-ATPase, was identified, which is necessary for the processing of Notch1-receptor. A deficiency of this ATPase was shown to disrupt Notch signalling and mammary gland development, which might also represent a new way in breast cancer treatment (35).

However, we did not find correlations of tumour characteristics with XBP1, MCL1, LRP6 and FOXP3, and no relation could be shown between tumour size, remote metastasis formation and tumour histology and one of the investigated signalling molecules.

Additionally we investigated the coherence between the different signalling molecules and the formation of recurrence such as lymph node recurrence, local recurrence or remote metastasis formation. We found a correlation between the expression of β-catenin and the incidence of remote metastasis. It was published earlier, that β-catenin plays a role in metastasis formation by interactions with ECM (extracellular matrix)1 protein, what increased the progress on EMT and CSC (cancer stem cell) phenotype maintenance in the cancer cells (36). Furthermore, β-catenin is known to support the action of matrix-metalloproteinases (MMPs) and uPA (urokinase plasminogen activator), its inhibition thereby inhibits EMT (37). The inhibition of β-catenin seems to correlate with the inhibition of metastasis formation (38,39). However, β-catenin also plays a role in lymph node recurrence formation (40), as we also demonstrated with our experiments. An association of Notch1 and remote metastasis formation, which we found in our analysis, was also already described (41) and seems to work via angiogenesis.

Table III. Statistical correlation of stainings with tumour characteristics (Kruskal-Wallis test).

Tumour trait	HIF1α		β-catenin		XBPI	Notch1		Mcl1		LRP6	FoxP3	
	Cytoplasm	Nucleus	Cytoplasm	Nucleus		Cytoplasm	Nucleus	Cytoplasm	Nucleus		Cytoplasm	Nucleus
Grading	**0.030**	0.269	0.980	0.516	0.225	0.530	0.537	0.302	0.279	0.443	0.711	0.445
Size	0.849	0.331	0.384	0.701	0.154	0.112	0.369	0.672	0.705	0.355	0.282	0.369
Lymph node affection	0.208	0.751	0.377	0.656	0.615	**0.049**	*0.063*	0.605	0.233	0.154	0.189	0.707
Metastases	0.704	0.982	0.189	0.659	0.921	0.294	0.706	0.769	0.938	0.458	0.667	0.627
Histology	0.594	0.317	0.192	0.426	0.984	0.350	0.315	0.426	0.556	0.315	0.755	0.392
Menopausal state	0.750	0.591	*0.068*	0.609	0.767	0.476	0.298	0.557	0.151	0.802	0.663	0.542

Bold, statistically significant p-values (p≤0.05); italics, borderline significant values (≥0.1-p≤0.05).

Table IV. Statistical correlation of stainings with tumour recurrence (Kruskal-Wallis test).

Type of recurrence	HIF1α		β-catenin		XBPI	Notch1		Mcl1		LRP6	FoxP3	
	Cytoplasm	Nucleus	Cytoplasm	Nucleus		Cytoplasm	Nucleus	Cytoplasm	Nucleus		Cytoplasm	Nucleus
Local	0.188	0.787	0.946	0.209	0.909	0.188	0.163	0.621	0.112	0.270	0.448	*0.083*
Lymph node	0.312	0.953	0.239	**0.018**	*0.059*	0.312	0.418	0.638	0.241	0.470	0.508	0.906
Metastatic	0.896	0.965	**0.007**	*0.100*	0.225	0.370	*0.082*	0.387	0.560	0.426	0.661	0.761

Bold, statistical significant p-values (p≤0.05); italics, borderline significant values (≥0.1-p≤0.05)

Rather new are the coherence of XBP1 and lymph node recurrences and FOXP3 and local recurrences, showing ultimately, that all these signal transduction pathways are rather important in tumorigenicity and recurrence formation.

As a conclusion of the experiments, the correlation of staining of different signal transduction molecules to tumour traits, indicated that signal transduction pathways influence tumour progression and recurrence or metastasis formation. As the number of samples used in the study is rather small, the results have to be considered as preliminary and further research has to be done to verify these data. Carrying out such experiments could help to refine prognosis and find ways to inhibit tumour progression and metastasis formation and thereby might help to find new therapeutic strategies.

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