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PRECLINICAL STUDY

Rare oncogenic mutations of predictive markers for targeted therapy in triple-negative breast cancer

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Abstract Women with triple-negative breast cancer (TNBC) do not benefit from endocrine therapy or trastuzumab. Chemotherapy is the only systemic therapy currently available. To reduce the elevated risk of disease progression in these patients, better treatment options are needed, which are less toxic and more targeted to this patient population. We performed a comprehensive analysis of potential targetable genetic aberrations affecting the receptor tyrosine kinase/RAS/MAPK pathway, which are observed at higher frequencies in adenocarcinomas of other organs. Sixty-five individual TNBCs were studied by sequence analysis for *HER2* (exon 18–23), *EGFR* (exon 18–21), *KRAS* (exon 2), and *BRAF* (exon 15) mutations. In addition, a tissue microarray was constructed to screen for *EGFR* gene copy gain and *EML4-ALK* fusion by FISH. Triple-negative status was confirmed by immunohistochemistry and FISH on tissue microarray sections. EGFR and CK5/6 immunohistochemical analyses were performed for identification of the basal-like phenotype. In addition, mutation analysis of *TP53* (exon 5–8) was included. Sequence analysis revealed *HER2* gene mutation in only one patient (heterozygous missense mutation in exon 19: p.L755S). No mutations were found in *EGFR*, *KRAS,* and *BRAF*. High polysomy of *EGFR* was detected in 5 of the 62 informative cases by FISH. True *EGFR* gene amplification accompanied by strong membranous EGFR protein expression was observed in only one case. No rearrangement of the *ALK* gene was detected. Basal-like phenotype was identified in 38 of the 65 TNBCs (58.5 %). *TP53* gene mutation was found in 36/63 (57.1 %) tumors. We conclude that targetable genetic aberrations in the receptor tyrosine kinase/RAS/MAPK pathway occur rarely in TNBC.

Keywords Triple-negative breast cancer · *HER2* mutation · *EGFR* mutation · *EGFR* gene copy gain · *EML4-ALK* fusion · *KRAS* mutation · *BRAF* mutation

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Introduction

Triple-negative breast cancer (TNBC), defined by the lack of estrogen receptor (ER) and progesterone receptor (PR) expression and the absence of *HER2/neu* (*ERBB2*) gene amplification, accounts for approximately 15 % of all breast carcinomas [1]. Although TNBC is common in patients with *BRCA1* mutations, it is not restricted to this group [2]. TNBCs represent a heterogeneous group of tumors in terms of presentation, morphology, and molecular aberrations. These tumors may present as invasive

ductal (not otherwise specified, NOS), metaplastic, medullary, apocrine, or other histological type. However, most of them share distinct phenotypic and genotypic features like high tumor grade and accelerated tumor cell proliferation [1]. Molecular profiling has shown a close association to the basal-like subtype [3]. However, there are TNBCs that belong to other intrinsic subtypes, i.e., the claudin-low and molecular apocrine type [4]. TNBC has attracted the attention of both pathologists and oncologists as an "easily recognizable" prognostic group. Triple-negative tumors are associated with shorter time to recurrence and higher mortality, and the affected women are younger compared to the total group of breast cancer patients [5, 6]. Currently, TNBCs lack the benefit of available targeted therapy. Hormone therapies and HER2-targeted therapies are ineffective in their treatment, and thus, searching for new drug targets selective for this subtype of tumors is a major challenge in modern oncology [7].

Activation of distinct growth factor receptor pathways by mutation and/or amplification of the corresponding genes are a major mechanism in the development and progression of cancer and a main target for newly developed drugs. Up to 20 % of breast cancers show amplification of *HER2* which is a strong predictive marker for therapy with monoclonal antibodies directed against HER2 (trastuzumab) [8]. *HER2* belongs to the family of receptor tyrosine kinases. This family includes additional targetable proto-oncogenes such as the epidermal growth factor (EGFR) and the anaplastic lymphoma kinase (ALK). In adenocarcinomas of the lung, *EGFR* mutation/amplification and *EML4-ALK* fusion is frequently found, but *HER2* amplification is less common [9]. The introduction of tyrosine kinase inhibitors against EGFR and ALK expanded the therapy options for lung cancer. In addition, activating mutations of *HER2* and *BRAF* have been described at low frequency in adenocarcinoma of the lung and might be targeted by inhibitory drugs in the future [9]. As mutations of all these proto-oncogenes, along with *KRAS*, are involved in the activation of the same transforming MAP-kinase pathway, their occurrence is mutually exclusive [10].

In breast cancer, genetic aberrations of these biomarkers are described with low frequencies. *EGFR* mutations are reported in 0–11 % of breast cancer [11, 12], while *EGFR* amplifications are found in 1–6 % [13–16]. Two studies on *EML4-ALK* fusion report a frequency of 0 and 2 % [17, 18]. Mutations of the *HER2* gene in breast cancer were found in 4/94 (4.3 %) tumors in one study [11]. *KRAS* mutations are found in 5 % of breast cancer [19], while *BRAF* mutations have been identified only in breast cancer cell lines and three primary tumors so far [20–22]. However, breast cancer is a heterogeneous group of diseases with divergent molecular mechanisms of pathogenesis. We hypothesized that mutations of these targetable oncogenic kinases, which are observed at higher frequencies in adenocarcinomas of other organs that are also not endocrine responsive, might accumulate in the group of hormone receptor-negative breast cancers without activation of the MAP-kinase pathway by *HER2* amplification. We therefore performed a comprehensive analysis of potential targetable mutations and amplifications affecting the receptor tyrosine kinase/RAS/MAPK pathway, including *EGFR* mutation, *EGFR* amplification, *HER2* mutation, *EML4-ALK* fusion, *KRAS* mutation, and *BRAF* mutation, in 65 triple-negative primary breast cancers. In addition, we performed *TP53* mutation analysis in all cases.

Materials and methods

Specimen collection and TMA construction

All TNBCs diagnosed at the Institute of Pathology, University Medical Center Hamburg-Eppendorf, between January 2008 and January 2011 were reviewed for this study. All 65 cases with available surgical specimens of the primary tumor and a tumor size larger than 5 mm were included (one patient with a tumor size of 4 mm and one patient with complete remission after neoadjuvant chemotherapy were excluded). Negativities for hormone receptors (ER and PR) and HER2 were assessed during initial diagnostic procedures by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), respectively, on core biopsy or surgical specimen following the same procedures as described later. Five of the patients had received neoadjuvant chemotherapy, including taxanes, anthracyclines, cyclophosphamide, and 5-fluorouracil, before surgery. All cases were reviewed for histological type and grade. The pathologic stage was obtained from the original pathology report.

A tissue microarray (TMA) of the 65 TNBC specimens was constructed as previously described [23]. Each case is represented in duplicate on the TMA. Consecutive sections of the TMA were used for H&E-stained reference, immunohistochemical analysis, and FISH.

Immunohistochemistry

Immunohistochemical analyses were performed on 4-µm-thick TMA sections. Staining for ER alpha (ER; Clone 6F11, Novocastra, dilution scale 1:80), progesterone receptor (PR; Clone 16, Novocastra, dilution scale 1:200), and cytokeratin 5/6 (Clone D5/16B4, Dako, dilution scale 1:100) was carried out after heat pretreatment in Bond Epitope Retrieval Solution 2 (pH9; Leica Microsystems) in a Bond automated system (Leica Microsystems). HER2 (HercepTest, Dako) and EGFR (EGFR pharmDx, Dako) IHC were performed in an Autostainer (DAKO). The test

kits were applied exactly according to the manufacturer's instructions. ER and PR stains were considered negative if immunostaining was seen in <1 % of tumor nuclei. EGFR and CK5/6 stains were considered positive if any (weak, moderate, or strong) cytoplasmic and/or membranous stainings were observed [24]. For HER2 status, tumors were considered negative if scored 0 or 1+ according to the HercepTest criteria. FISH ratio was used to segregate immunohistochemical equivocal (2+) results (see below).

DNA extraction and targeted sequence analysis

DNA was extracted from formalin-fixed and paraffin-embedded tissues. A representative area containing at least 60 % tumor cells was marked on H&E sections of the original tumor blocks and sampled by punching two tissue cores with a diameter of 0.6 mm. DNA was extracted as per standard protocols (QIAmp DNA Mini Kit, Qiagen). Quantity and quality of DNA were evaluated using a Nanodrop spectrophotometer ND-1000 (Thermo-Scientific). 100 ng of DNA was subjected to PCR using the AmpliTaq Gold PCR master mix (Applied Biosystems) under the conditions recommended by the manufacturer. Primer sequences for *HER2* (Exons 18–23), *KRAS* (Exon 2), and *BRAF* (Exon 15) are given in supplementary Table 1. PCR and primer sequences for *EGFR* (Exons 18–21) and *TP53* (Exons 5–8) have been previously described [25, 26]. After verification of the amplicons on QIAxcel system (Qiagen), PCR products were purified using an enzymatic method (ExoSAP-IT; USB Products) and subjected to sequencing reactions using BigDye Terminator Cycle v1.1 Sequencing Kit (Applied Biosystems). Sequence reactions were analyzed on ABI PRISM 3100 genetic analyzer (Applied Biosystems).

FISH

FISH was performed on 4-μm-thick TMA sections using a commercially available kit for proteolytic slide pretreatment (paraffin pretreatment reagent kit, Abbott Molecular). *HER2* FISH was carried out using the PathVysion kit (Abbott Molecular). *EGFR* copy gain was assessed using the Vysis EGFR/CEP7 FISH Probe Kit (Abbott Molecular). Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe (Abbott Molecular) was used to detect any rearrangement involving the *ALK* gene. FISH preparation was carried out exactly according to the manufacturer's guidelines. HER2 status was considered negative (not amplified) if the ratio of gene signal to centromere signal number was below 2. *EGFR* FISH was evaluated according to the suggested guidelines for non-small cell lung cancer by the Colorado group [27, 28]. Tumors were classified as *EGFR* FISH-positive if *EGFR* amplification or high polysomy was detected. Diagnosis of *ALK* rearrangement required rearranged FISH signals in more than 15 % of tumor cells as suggested by the manufacturer for the evaluation of non-small cell lung cancer.

Results

Study group

Triple-negative primary breast cancers of 65 patients were included in the study. All patients were women with a mean age of 56.1 years (range 32–86) at the time of surgery. Triple-negative status of all included tumors was confirmed by ER, PR, and HER2 IHC, as well as *HER2* FISH on TMA sections representing two samples of each tumor. Basal-like phenotype was identified in 38 of the 65 analyzed TNBCs (58.5 %) using the five biomarker surrogate that includes cytokeratin 5/6 and EGFR IHC [24]. An overview of the studied cases is given in Table 1.

Mutation analysis of *EGFR*, *HER2*, *KRAS*, *BRAF*, and *TP53*

Sequence analysis of all exons was successful in 63 tumors. In two samples, severe DNA degradation after formalin

Table 1 Clinical and pathological overview of the studied triple-negative breast cancer cohort

Total cases	65 (%)
Histologic type [40]	
Ductal	60 (92.3)
Apocrine	3 (4.6)
Adenoid cystic	1 (1.5)
Micropapillary	1 (1.5)
Histological grade [41]	
I	1 (1.5)
II	9 (13.8)
III	55 (84.6)
Stage[a] [42]	
pT1	25 (41.7)
pT2	29 (48.3)
pT3	5 (8.3)
pT4	1 (1.7)
Lymphnode metastasis[b] [42]	
pN0	35 (57.4)
pN1-3	25 (41.)
pNX	1 (1.6)
Basal-like phenotype acc. to [24]	38 (58.5)

[a] Five neoadjuvant treated cases not included

[b] Four cases with axillary lymph node dissection after neoadjuvant treatment not included

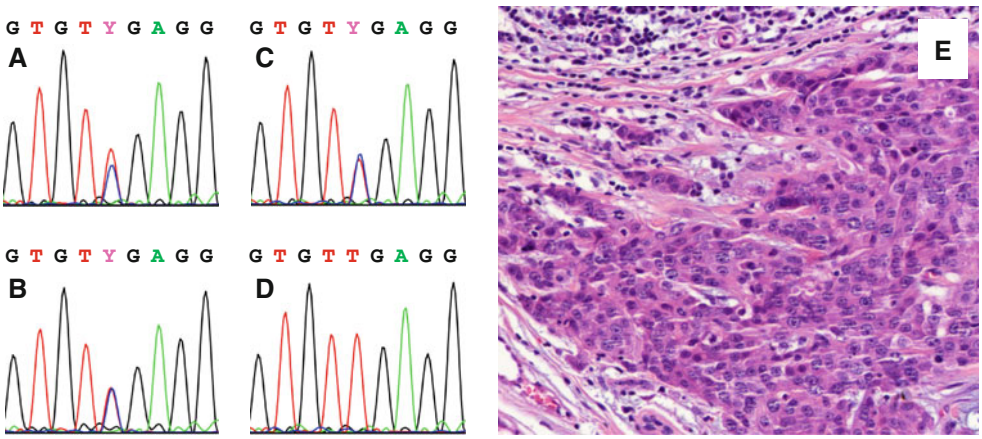

Fig. 1 *HER2* mutation in case 61. Sequence analysis of primary invasive carcinoma (**a**), lymph-node metastasis (**b**), and carcinoma in situ (**c**) indicates substitution c.2264T>C (p.L755S) of the *HER2* gene. Non-neoplastic tissue shows wild-type sequence (**d**). H&E stained detail of the primary tumor is shown in (**e**)

fixation impeded successful amplification in some reactions. No mutations were found in the tyrosine kinase domain (exons 18–21) of *EGFR* (0/64). *HER2* sequence analysis of exons 18–23 revealed mutation in one case (1/64). This tumor of a premenopausal patient showed high-grade morphology of ductal type (G3, pT1c, pN1). A heterozygous missense mutation in exon 19 of the *HER2* gene, p.L755S (c.2264T>C), was detected (Fig. 1). The mutation was confirmed to be of somatic nature, and the tumor of an axillary lymph-node metastasis also showed this mutation. Using laser-capture microdissection, the mutation was also confirmed in the concomitant intraductal tumor component (DCIS). HER2 expression as assessed by IHC was negative (1+), and an additional mutation of the *TP53* gene (p.A138S) was detected in this tumor. No mutations were found in *KRAS*, codon G12 and G13 (0/63), and *BRAF*, codon V600 (0/63). We found *TP53* mutations within exons 5–8 in 36/63 (57.1 %) tumors. These mutations comprised 21 missense mutations, five nonsense mutations, nine frameshift mutations, and one in-frame deletion. This *TP53* mutation frequency is in line with previously published results in TNBC [29] and provides evidence that the applied method of mutation analysis by Sanger sequencing of enriched tumor cells is of sufficient sensitivity.

FISH analysis of *EGFR* gene copy gain and *ALK* rearrangement

FISH analysis on the TMA was successful in 62/65 cases for *EGFR* and 57/65 cases for *ALK*. The two cases, which failed to be amplified due to low DNA quality, also failed in both FISH assays. *EGFR* FISH showed high polysomy in five cases, i.e., ≥4 *EGFR* copies in ≥40 % of cells [28]. Two of these cases showed moderate expression of the EGFR protein, while in the other three cases, no or weak expression was detectable by IHC. True *EGFR* amplification with 30–50 gene copies in clusters accompanied by strong membranous EGFR protein expression of the tumor cells was found in one case (Fig. 2). This premenopausal patient was treated with neoadjuvant chemotherapy (CAT regime) before surgery, which did not result in complete remission. *EGFR* amplification was also detected in axillary and supraclavicular lymph-node metastases removed before neoadjuvant treatment indicating that the therapy regimen had no influence on the development of the *EGFR* amplification. The tumor showed high-grade morphology of ductal type (G3, ypT1b, pN3), and a concomitant *TP53* mutation (p.R273C) was detected. No rearrangement of the *ALK* gene indicating *EML4-ALK* fusion was detected in any tumor.

Discussion

Triple-negative breast cancer represents a major clinical challenge due to its aggressive behavior that comes along with a lack of available therapy targets. Until now, it is unknown what genetic aberrations induce self-sufficiency in growth signals in these tumors that are endocrine non-responsive and do not exhibit *HER2* gene amplification. HER2 is a receptor tyrosine kinase and gene amplification results in an activation of the RAS/MAPK pathway. Studies of hormone receptor-negative adenocarcinomas of other organs have shown the importance of the receptor tyrosine kinase/RAS/MAPK pathway for tumor growth even in the absence of HER2 gene amplification. Driver mutations affecting this pathway have been well documented in non-small cell lung cancer. These genetic aberrations with potential clinical relevance for targeted therapy include *EGFR* mutation and amplification, *EML4-ALK* fusion, *HER2* mutation, *KRAS* mutation, and *BRAF* mutation. In breast cancer, these mutations have been only punctually studied and are observed at low frequencies. Since these studies included hormone receptor positive and HER2 amplified breast cancers, we hypothesized that these infrequent driver mutations might accumulate in the group of TNBCs.

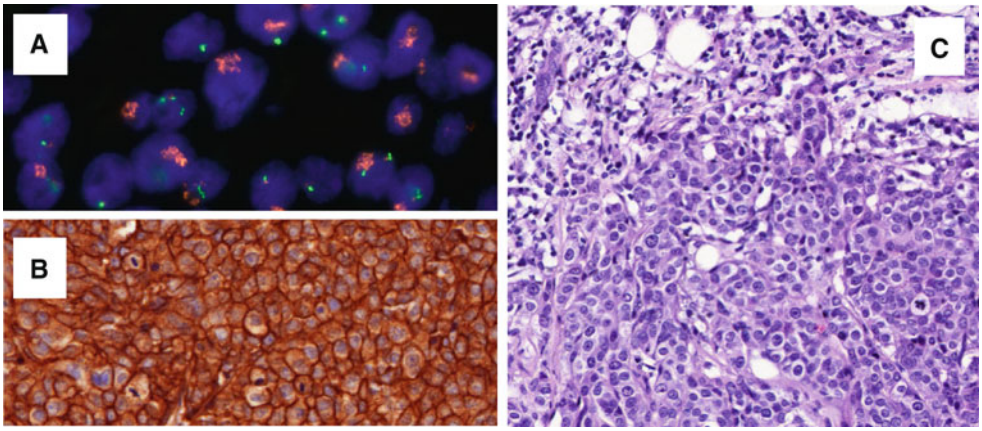

Fig. 2 *EGFR* amplification in case 64. EGFR FISH analysis (**a**) shows 30–50 EGFR gene copies per tumor cell nucleus in clusters (*red signals*) and 2 CEP7 signals (*green*). EGFR IHC; (**b**) demonstrates strong membranous EGFR expression. H&E stained detail of the primary tumor is shown in (**c**)

To the best of our knowledge, this study represents the first comprehensive and concurrent analysis of these potentially targetable driver mutations in a cohort of triple-negative breast cancers. We analyzed these tumors for activating mutations of *EGFR*, *HER2*, *BRAF,* and *KRAS*. In addition, a screening for *ALK* rearrangement and *EGFR* gene copy gain was performed by FISH. We found genetic aberrations in only two of the examined cases. One tumor harbors a *HER2* missense mutation p.L755S. The mutation is also detectable in the in situ component (DCIS) of the tumor, suggesting an early event in the carcinogenesis of this tumor. This type of mutation affects the kinase domain of HER2 and has been previously described in four tumors including two breast cancers [11]. As shown in lung cancer, HER2 inhibitors such as lapatinib or afatinib might well be effective in tumors with activating *HER2* mutations [30, 31]. The second case shows high level *EGFR* gene amplification which might render the tumor amenable to either EGFR tyrosine kinase inhibitors or monoclonal antibodies directed against EGFR as described for *EGFR*-amplified lung cancers [32–34].

Only few data are available on these predictive markers in TNBC. Gumuskaya et al. [15] reported *EGFR* gene amplification in one and high polysomy in 9 of 62 TNBCs, which is consistent with our data. In contrast to our results, Teng et al. have reported *EGFR* mutations in 8/70 TNBCs in their study performed in Singapore. We did not detect any *EGFR* mutations. Remarkably, *EGFR* mutations in lung cancer are much more frequent in Asian than in the Caucasian populations [35]. One might speculate that the frequency of *EGFR* mutations in breast cancer might also be higher in Asian populations. However, two studies from Japan and Korea did not find any *EGFR* mutations in a total of 177 unselected breast cancers [36, 37]. Therefore, it is currently unclear if the frequency of EGFR mutations in TNBC depends on the analyzed population. In accordance with our results, a recently published study on *KRAS* mutations in TNBCs found no mutations in 35 tumors [38]. Because of the small sizes of cohorts published so far, a systematic review of these studies is needed to accurately analyze the frequency of the different mutations and association with distinct populations.

Molecular profiling has shown that TNBC and basal-like subtype are closely associated but not congruent. To ensure the representativeness of our study collective, we used a five-biomarker surrogate immunopanel to identify the rate of basal-like phenotype in our collective. Consistent with the literature, 58.5 % of the analyzed TNBC were basal using the five-marker method [24, 39].

In summary, we found that the analyzed targetable genetic aberrations in the receptor tyrosine kinase/RAS/MAPK pathway are rare in TNBC. Therefore, these potential predictive markers might be helpful only in individual patients with TNBC. Our data support the view that TNBC is a biologically peculiar group of breast cancers. As these tumors only rarely harbor activating aberrations of tyrosine kinase receptors, their mechanism of independency on growth factors is still unclear and differs from the already known pathways in breast cancer.

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Conflict of interest The authors declare that they have no conflict of interest.

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