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Prognostic Evaluation of Epidermal Growth Factor Receptor (EGFR) Genotype and Phenotype Parameters in Triple-negative Breast Cancers

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Abstract. *Background: Epidermal growth factor receptor (EGFR) aberrations have been implicated in the pathogenesis of triple-negative breast cancer (TNBC) but their impact on prognosis and, therefore, druggability, remain controversial. Herein, we studied EGFR aberrations at different molecular levels and assessed their prognostic impact in patients with operable TNBC treated with adjuvant anthracycline-based chemotherapy. Materials and Methods: We evaluated the prognostic impact of EGFR gene status by fluorescent in situ hybridization (FISH), EGFR coding mutations by Sanger and next-generation sequencing, relative EGFR messenger RNA (mRNA) levels by qPCR (upper quartile) and EGFR and p53 protein expression by immunohistochemistry (IHC), in 352 centrally-assessed tumors from an equal number of TNBC patients. Results: Approximately 53.5% of the tumors expressed EGFR, 59.3% p53 and 35.9% both EGFR and p53 proteins; 4.1% showed EGFR gene amplification and 4.4% carried EGFR mutations. The latter were located outside the druggable kinase domain region and presented at low frequencies. Amplification and mutations overlapped only in*

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one case of glycogen-rich carcinoma. EGFR and CEN7 copies were higher in tumors from older patients (p=0.002 and p=0.003, respectively). Patients with amplified tumors (n=11) had excellent prognosis (0 relapses and deaths). Upon multivariate analysis, high EGFR copies conferred significantly favorable disease-free survival (HR=0.57, 95% CI 0.36-0.90, Wald's p=0.017) and high CEN7 copies favorable overall survival (HR=0.49, 95% CI=0.29-0.83, Wald's p=0.008). Patients with EGFR–/p53+ and EGFR+/p53– tumors had significantly higher risk for relapse than those with EGFR–/p53– and EGFR+/p53+ tumors (HR=1.73, 95% CI=1.12-2.67, Wald's p=0.013). Conclusion: EGFR gene amplification and mutations are rare in TNBC, the latter of no apparent clinical relevance. Surrogate markers of EGFR-related chromosomal aberrations and combined EGFR/p53 IHC phenotypes appear to be associated with favorable prognosis in patients with operable TNBC receiving conventional adjuvant chemotherapy.

TNBC lacks expression of estrogen receptor (ER), progesterone receptor (PgR) and over-expression of human epidermal growth factor receptor 2 (HER2), diminishing the response from targeted therapies other than chemotherapy and presenting the worst prognosis among all breast cancer subtypes (1). It is now clear that TNBC is a clinically and molecularly heterogeneous disease (2, 3). No established markers exist significantly associated with prognosis. Gene expression analysis has categorized TNBC into 6 subtypes with different prognosis, namely basal-like 1 and 2 (BL1 & BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and luminal androgen receptor (LAR) or apocrine (4). Possible differential sensitivity to chemotherapy and targeted agents has been tested in several clinical trials (5, 6). More recently, another subtype has been described, called claudin-low, mostly composed of "M" and "MSL" subtypes (7, 8). "BL1", "BL2", "IM" and "M" subtypes are mainly associated with carcinomas with basal features, while "LAR" consists of luminal and HER2 subtypes (8). However, TNBC subtyping is difficult to apply in routine clinical practice due to cost and infrastructure availability.

Basal-like or basal-type tumors are tumors expressing high levels of myoepithelial-cell type cytokeratins (CK5/6, CK 14, CK 17, CK8/18), displaying high proliferative activity and adverse pathological characteristics *e.g.* high histological grade, high mitotic index, central scar, tumor necrosis, squamous metaplasia and stromal lymphocytic infiltration as well as enhanced expression of p53, vimentin and EGFR (9-14). EGFR expression has been well studied in breast cancer and occurs more frequently in TNBC, where it has been associated with poor prognosis (9, 15). At least three lines of experimental evidence support the stimulating effect of EGFR in oncogenesis; its up-regulation and aberrant activation in many human epithelial cancers, its co-expression with its natural ligands in the same tumor tissues and finally, the inhibitory effect in tumor growth of a number of anti-EGFR agents, including small-molecule inhibitors and EGFR-neutralizing monoclonal antibodies (16-22) (23).

The prognostic value of EGFR protein expression, *EGFR* gene copy number and *EGFR* hotspot mutations in breast cancer have been investigated in several studies (15, 24-26). However, a study comparing *EGFR* alterations at various molecular levels has not been reported for TNBC. Herein, we examined the prognostic role of EGFR protein expression by immunohistochemistry, *EGFR* gene amplification by fluorescence in situ hybridization (FISH), *EGFR* mRNA expression by quantitative PCR (qPCR) and *EGFR* gene mutations. In addition, because we have previously shown that TNBC frequently express p53 protein (27), we also assessed this marker along with EGFR protein expression.

Materials and Methods

Using the Hellenic Cooperative Oncology Group registry data, we identified 352 women with early high-risk triple-negative breast cancer treated with adjuvant chemotherapy with anthracyclines and/or taxanes between 1997 and 2012. Clinicopathological parameters, treatment information, as well as retrospectively collected follow-up data were recorded. All patients had signed informed consent, which allowed the use of their biological material for future research purposes. The study was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine and was conducted in accordance with ethical principles stated in the latest version of the Declaration of Helsinki. The study was conducted at the Laboratory of Molecular Oncology of the Hellenic Foundation of Cancer Research, Aristotle University of Thessaloniki School of Medicine and complied with the REMARK recommendations for tumor marker prognostic studies using biological material (28).

Central tumor assessment and tissue microarray (TMA) construction. Formalin-fixed and paraffin-embedded (FFPE) tissues from each patient were obtained. Hematoxylin-eosin (H&E) stained sections from the tissue blocks were reviewed by two experienced breast cancer pathologists who recorded histologic parameters in detail (histopathological type, grade, presence of lymphocytic infiltrations, necrosis) and marked the most tumor dense areas for subsequent TMA construction. Histological grade was evaluated according to the Scarff, Bloom and Richardson system. Tumors were centrally assessed for the triple negative phenotype (ER, PgR and HER2) by IHC and FISH. HER2 status was considered to be positive if HER2 was amplified (ratio >2.2 or copy number >6) by FISH and/or HER2 score of 3+ was obtained by IHC (29). Tumors with inadequate tissue material for further assessment were excluded from the study.

A total of 352 tumors were assembled into low-density TMAs with the use of a manual arrayer (Model I, Beecher Instruments, San Prairie, WI, USA). For the TMA blocks construction, two cores (1.5 mm in diameter) were obtained from representative regions of each tumor and transferred to the recipient block. Each TMA block contained approximately 40 tumor tissue cores and 8-10 neoplastic

and non-neoplastic tissue cores as controls for slide-based assays. TMAs were stored at 4˚C. A total of eighteen tumor TMAs and nine additional duplicate TMAs were constructed in order to account for cores that were exhausted.

Tumor-infiltrating lymphocytes (TILs) were assessed morphologically on H&E sections. Both stromal TILs and TILs within the tumor nests were evaluated and interpretation of the measurements was defined according to the following semi-quantitative modification of the scoring system proposed by Lee *et al*.; "0" for none or few scattered lymphocytes (<5% of stroma), "1" for discontinuous peripheral distribution of small lymphocytic aggregates clearly discernible at 100x (6-25%), "2" for moderately organized continuous peripheral distribution of lymphocytes (26-75%), and "3" for marked infiltrations of lymphocytes mimicking a lymphoid organ, nearly always penetrating tumor mass (highly organized) (≥76%) (30, 31). Assessment of TILs was performed both on TMA cores and on whole sections (WS) and the agreement of the results was assessed by Cohen's kappa coefficient. The cases scored 0, 1 or 2 were considered to have low presence of TILs, whereas cases scored 3 were considered to be high. Tertiary lymphoid structures, areas with in situ carcinoma or lobular cancerization that often exhibited regional high TILs, and necrotic areas were not considered.

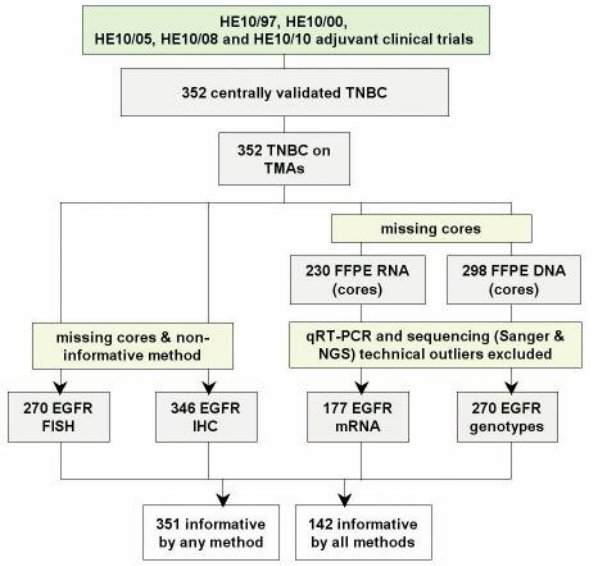

Figure 1. *REMARK diagram of study materials and methods*.

EGFR protein expression. IHC was applied on serial 2.5 μm thick TMA sections, using Bond Max (Leica Microsystems, Wezlar, Germany) autostainer. Samples with tumor in less than 5% of core surface were considered not interpretable and were excluded from further analysis. IHC staining for ER (ER clone 6F11, Novocastra™, Leica Biosystems), PgR (PgR clone 1A6, Novocastra™), Ki67 (clone MIB-1, Dako™, DK), HER2 (HER2/neu, A0485 polyclonal antibody, Dako™), EGFR (EGFR, clone 31G7, Invitrogen, Camarillo, CA), cytokeratin 5 (clone XM26, Novocastra™) and tumor protein 53 (p53, clone DO-7, Dako™) on each slide was performed as previously described (32, 33). The antigen-antibody complex was visualized using the Bond Polymer Refine Detection kit (DS 9800, Leica Biosystems) and DAB as a chromogen. ER and PgR were considered positive for nuclear positivity in at least 1% of cancer cells (34). Any CK5 specific staining in tumor cells was considered as positive (35). For EGFR, any membrane staining above the background in >1% of tumor cells was interpreted as positive (9). For p53, nuclear staining in ≥10% of tumor cells was considered as positive (33). Based on core availability and method performance, 346 tumors were considered for EGFR protein expression (Figure 1).

EGFR gene amplification. FISH assays were carried out on 4-μm TMA sections. After being deparaffinized, the sections were dehydrated and incubated in 2x SSC for 15 min and digested in pepsin solution for 10 min at 37˚C and then rinsed in 2x SSC at room temperature for 5 min. After denaturation at 75˚C for 10 min on a hot plate (Dako, DK-2600 Glostrup, Denmark), the ZytoLight® SPEC HER2/TOP2A/CEN17 triple color probe and ZytoLight® SPEC *EGFR*/CEN7 dual color probe (both from ZytoVision, Bremerhaven, Germany) were then applied on the sections and hybridized overnight in a hybridization oven at 37˚C. After post-hybridization washing, the slides were counterstained with DAPI for visualizing the nuclei. For all probes, sequential digital images (5 planes at 1.0 μm) were captured using the Plan Apo VC 100x/1.40 oil objective (Nikon, Japan) with specific filters for each probe. The resulting images were reconstructed using specifically developed software for cytogenetics (XCyto-Gen, ALPHELYS, Plaisir, France) (36).

EGFR gene status was assessed in 60 non-overlapping nuclei from the invasive part of the tumor, according to the classification proposed by Hirsch *et al*. (37). This classification employs *EGFR* gene and CEN7 copies, as well as the *EGFR*/CEN7 ratio. Thus, FISH results were classified as: (a) negative, for ≤6 copies of the gene in >40% of cells; (b) positive, for >6 copies of the gene in ≥40% of cells; (c) amplified, for *EGFR*/CEN7 ratio per cell ≥2. *EGFR* status was not amplified for (a), and amplified for (b) and/or (c). Based on core availability and informative FISH measurements, 298 tumors were analyzed for *EGFR* gene status (Figure 1).

EGFR mRNA expression. RNA was extracted from TMA cores (5x8um thick sections, total depth per block=0,04 mm) with magnetic beads (VERSANT® Tissue Prep Kit, Siemens Healthcare, Erlangen, Germany). DNase I was added to each extract to remove DNA and ensure the presence of pure RNA for gene expression analysis. cDNA synthesis was performed with random hexamers and SuperScript® III Reverse Transcriptase (Invitrogen™, cat. no. 48190011 and 18080044, respectively). Finally, cDNAs were assessed in duplicate 10 ul reactions in 384-well plates with quantitative Polymerase Chain Reaction (qPCR) in an ABI7900HT system for 45 cycles of amplification (default conditions). For the analysis of *EGFR* mRNA expression an exon-spanning (exons 20-21) premade Taqman-MGB assay (Hs00193306_m1/NM_005228.3/Applied Biosystems/Life Technologies) was selected. A Taqman-MGB expression assay (Hs00939627_m1/NM_000181.3) targeting β-glucuronidase (GUSB) mRNA was used for the assessment of relative quantification. GUSB was selected as the endogenous reference since it does not seem to be represented in pseudogenes. In addition, GUSB has been independently identified as one of the best preserved mRNA targets in FFPE tissues (38, 39). A commercially available reference RNA

derived from multiple transformed cell lines (TaqMan® Control Total RNA, cat. no 4307281, Applied Biosystems) was applied in multiple plate positions in each run as a positive control and for inter-run evaluation of PCR assay efficiency. No-template controls were also included. Samples were run in duplicates. To obtain linear Relative Quantification (RQ) values, relative expression was assessed as (40-dCT), whereby dCT (or delta Cycle Threshold, equivalent to Cq in MIQE guidelines) was calculated as (average target CT – (average GUSB CT) from all eligible measurements. Samples were considered eligible for analysis for GUSB CT <36 and delta RQ for each duplicate pair (intra-run variation) of <1. Inter-run RQ values for the reference RNA were <1 for all assays.

Out of 230 originally prepared RNA samples, 177 were considered eligible for relative *EGFR* mRNA expression (Figure 1). RQ values greater than 39.3 were classified as high expression, which accounted for the upper 25% of the samples in this study.

EGFR genotyping. DNA was extracted from TMA cores magnetic beads as described for RNA above, at a different time point. In the present FFPE series TCC was ≥50% on TMA cores. In total, 298 DNA samples were prepared. These were initially interrogated for classic *EGFR* mutations in the kinase domain with dd-sequencing. Nested PCR products, M13 coupled with intron spanning primers for exons 18 (GRCh37 coordinates on chr7: 55241512-55241795); exon 19 (55242380-55242570); exon 20 (55248954-55249194); and, exon 21 (55259354-55259591) were cycle sequenced and analyzed in an ABI3130XL system (Applied Biosystems / Life Technologies). Informative results for all targets were obtained in 286 samples. In a separate experimental series, 280 out of these 286 DNA samples had DNA quantity ≥2 ng/μl with the Qubit fluorometer (Life Technologies, Paisley, UK) and were submitted for massively parallel sequencing (NGS) with a previously described panel (40) targeting additional areas in the *EGFR* coding region (exon 1: 55086912-55087073; 55087034-55087200); exon 7 (55221684-55221843; 55221800-55221948); exon 17 (55240623-55240764); exon 27 (55270177-55270340; 55270285-55270452). Upon library construction and clonal amplification, samples were massively processed on Ion Proton™ Sequencer PI chips (96 samples/chip). For data retrieval, base calling was performed on the Torrent Server using Torrent Suite v3.6.2 and v4.0. Variants were called, annotated (Ion Reporter 1.6), and accepted for analysis at highly stringent setup with read quality filtering at $p<0.0001$, excluding 63% of all detected variants. Amplicons were checked for sequence specificity and reading quality (IGV). Informative results were obtained for 269 tumors (75.5%). Variants were accepted for position coverage >100 and variant coverage >40 for worst cases. *EGFR* coding mutations (amino acid changing; minor allele frequency <0.1% in dbSNP) were examined. Informative results for all amplicons were obtained for 270 tumors, which were further processed for *EGFR* genotype comparison (Figure 1).

Statistical analysis. Categorical data are presented as frequencies and corresponding percentages, while continuous data are presented as mean, standard deviation, median and range values. The Fisher's exact or Pearson Chi-square tests were used for group comparison of categorical data, while for continuous data the non-parametric Mann-Whitney or Kruskal-Wallis tests were used where appropriate. Available follow-up data for 351 patients were recorded and the follow up period ranged from 2 to 190 months. DFS was defined as the interval from the date of diagnosis to the first locoregional recurrence or distant metastasis and overall survival (OS) was defined as the interval from diagnosis until death from any cause or last contact. Locoregional recurrence corresponds to local relapse at the tumor bed and/or to the regional lymph nodes. Patients who survived without relapse were censored at the date of their last contact. Survival curves were estimated using the Kaplan-Meier method and compared across groups with the log-rank test. The associations between factors of interest and relapse/mortality rate were evaluated with hazard ratios estimated with univariate and multivariate Cox proportional hazards models. The optimal cut-off values for Ki67, *EGFR* and CEN7 copy number variables were selected using ROC (receiver operating characteristic) curve analysis with 3-year DFS as the outcome variable. We chose 3-year DFS rate because the risk of recurrence among TNBCs peaks at 1-3 years (41).

Model choice was performed using backward selection criteria with a removal criterion $p=0.15$, including in the initial step the following clinical parameters: age (<median *vs.* ≥median), menopausal status (postmenopausal *vs.* premenopausal), tumor size (≤2 *vs.* 2-5 *vs.* >5 cm), histological grade (I-II *vs.* III), number of positive nodes (0 *vs.* ≥4, 1-3 *vs.* ≥4), adjuvant hormonotherapy (yes *vs.* no), adjuvant radiotherapy (yes *vs.* no), Ki67 percent (as a continuous variable) and each of the three EGFR related parameters: *EGFR* copy numbers, CEN7 copy numbers, combined p53/EGFR variable. The three EGFR related parameters were also entered in a combined model.

The statistical analyses were performed using the SAS software (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA). Statistical significance was set at 2-sided $p=0.05$. The statistical analysis complied with the reporting recommendations for tumor marker prognostic studies (42).

Results

Clinicopathological characteristics and immunohistochemical markers. Results for at least one parameter were available in 351 cases (Figure 1). Table I summarizes the patient clinicopathological characteristics and follow-up data of this TNBC cohort. Hormonotherapy had been administered to patients with tumors positive for hormone receptors when assessed locally (approximately 20%). Survival status was updated in June 2014. Survival data were available for 349 cases. After a median follow-up time of 75.7 months (range=2.3-190.4), overall 108 women (30.9%) had relapsed and 88 (25.2%) had died. The median DFS was 166.9 months (95% CI=144.3–not estimable) and the median OS 171.2 months (95% CI=152.1-not estimable).

TILs were evaluable in 306 tumors on TMA cores and in 335 tumors on whole sections. High TILs rates were found in 9.2% and 12.2% of the cases when assessed on TMA cores and whole sections respectively. TILs assessment on cores showed only moderate agreement with assessment on whole sections (Cohen's kappa=46.52%, 95% CI 39.56-53.49). For this reason, we used only the whole section measurements for associations in this study.

EGFR and CK5 protein expression were positive in 53.5% and 71.6% of the cases, respectively. Positivity for these two markers defined the basal phenotype according to the most

Table I. *Patient and tumor characteristics*.

Variable	Measurements	Variable	Measurements
All patients; N=351		Histological grade	
		I-II	65 (18.6%)
Age (continuous)		III	283 (80.6%)
Mean (SD)	52.9 (12.1)	Not reported	3 (0.8%)
Median	53.3	Lymphocytic infiltrates* (4 scale)	
Min-Max	21-83	Informative	335 (95.4%)
Tumor size (in cm)		0 (≤1%)	148(44.2%)
Mean (SD)	2.9 (1.7)	1 (>1-25%)	87 (26.0%)
Median	2.5	2 (>25-75%)	59 (17.6%)
Min-Max	0-11	3 (>75%)	41 (12.2%)
Number of positive nodes		Lymphocytic infiltrates* (binary)	
Mean (SD)	4.2 (6.9)	Informative	335 (95.4%)
Median	1.0	High (>75%)	41 (12.2%)
Min-Max	0-54	Low	294 (87.8%)
Adjuvant hormonotherapy		Menopausal status	
Yes	70 (20.0%)	Postmenopausal	195 (55.6%)
No	281 (80.0%)	Premenopausal	149 (42.4%)
Adjuvant radiotherapy		Not reported	7 (2.0%)
Yes	258 (73.6%)	Multifocal	
No	93 (26.4%)	Yes	22 (6.2%)
Age (median cut off)		No	329 (93.8%)
<53.2	174 (49.6%)	Number of positive nodes	
≥53.2	177 (50.4%)	0	115 (32.8%)
ER/PgR (IHC local pathology)		1-3	120 (34.2%)
Positive	46 (13.2%)	≥4	106 (30.2%)
Negative	273 (77.8%)	Not reported	10 (2.8%)
Not reported	32 (9.2%)	Tumor size	
Histological type		≤2 cm	127 (36.2%)
Apocrine	12 (3.4%)	2-5 cm	188 (53.6%)
Lobular	16 (4.6%)	>5 cm	30 (8.6%)
Medullar	22 (6.2%)	Not reported	6 (1.8%)
Metaplastic	17 (4.8%)	Follow-up (months)	
NOS	273 (77.8%)	Median	75.7
Other	11 (3.2%)	Range	2.3-190.4

NOS, Non-specific. *Evaluated on whole sections.

frequently quoted definition (9), which was found in 283 cases (80.6%), whereas 62.3% of these cases also presented immunopositivity for p53 protein. Detailed information about the immunohistochemical marker measurements with respect to basal phenotype is shown in Supplementary Table I.

EGFR gene, genotype and mRNA expression analysis. For the entire cohort, mean±standard deviation for *EGFR* gene copies was 3.2±2.4 (median=2.8, range=1.1-24.6); for CEN7 copies 3.0±1.4 (median=2.8, range=1.2-19.9); and, for *EGFR*/CEN7 ratio 1.1±0.6 (median=1.0, range=0.3-6.2). Eleven out of 270 evaluable tumors (4.1%) had EGFR gene amplification, 7 presented an *EGFR*/CEN7 ratio ≥2.0 and 9 *EGFR* copies >6.0. *EGFR* mRNA expression values ranged from 34.6 to 45.5 (mean±SD=38.5±1.6). Forty-four patients (25% of evaluable tumors) showed high *EGFR* mRNA expression (≥39.3).

No mutation was identified in the kinase domain of the 270 tumors with informative results for all interrogated *EGFR* coding areas, but 17 mutations outside the kinase domain were detected in 12 patients, *i.e*., 4.4% of evaluable tumors carried a mutation in the *EGFR* gene. Mutation characteristics are shown in Supplementary Table II. Most had a SNP ID although with 0 or unknown minor allele frequency in the general population; all but two were missense mutations; and, all but one had variant allele frequency <20%, *i.e*. they were represented in small fractions of the cells in the samples. In one tumor four different mutations in exons 17 and 27 were identified, one of which was silent. The patient was a premenopausal woman with a ductal NOS type grade III adenocarcinoma, pT2N1, Ki67 labeling 93%, event free at 114 months of follow-up. Another patient, a postmenopausal woman with ductal NOS

Table II. *EGFR genotypes and phenotypic characteristics*.

Case	Histology	*EGFR* FISH	*EGFR* mRNA	EGFR IHC	CK5 IHC	p53	Ki67	*EGFR* mutation
1	Ductal NST	Amplified	N/A	Negative	Negative	Negative	Low	N/A
2	Ductal NST	Amplified	Low	Positive	Positive	Positive	Low	Wild type
3	Ductal NST	Amplified	High	Positive	Positive	Positive	high	Wild type
4	Ductal NST	Amplified	High	Positive	Positive	N/A	N/A	Wild type
5	Ductal NST	Amplified	N/A	Positive	Negative	Positive	Low	Wild type
6	Ductal NST	Amplified	High	Positive	Positive	Negative	Low	Wild type
7	Ductal NST	Amplified	High	N/A	N/A	N/A	N/A	Wild type
8	Ductal NST	Amplified	High	Positive	Negative	Positive	Low	Wild type
9	Ductal NST	Amplified	N/A	Positive	Positive	Negative	Low	Wild type
10	Glycogen rich^	Amplified	High	Positive	Negative	Positive	Low	Mutant
11	Ductal NST	Amplified	High	Positive	Negative	Negative	High	Wild type
Case	Histology	*EGFR* mutation	*EGFR* mRNA	EGFR IHC	CK5 IHC	p53	Ki67	*EGFR* FISH
1	Ductal NST	p.Ala289Val	N/A	Positive	Positive	Positive	High	Not-amplified
2	Ductal NST	p.Pro641Leu p.Leu655Phe p.Ala661Thr p.Ser1081Asn	Low	Positive	Positive	Positive	High	Not-amplified
3	Ductal NST	p.Ala661Val	N/A	Negative	Positive	Positive	Low	Not-amplified
4	Ductal NST	p.Ala7Val	Low	Negative	N/A	N/A	N/A	Not-amplified
5	Ductal NST	p.Arg1068* p.Ser1070Asn p.Gly1075Ser	N/A	Negative	Negative	Positive	Low	Not-amplified
6	Lobular	p.Arg1068Gln	N/A	Negative	Negative	Negative	Low	Not-amplified
7	Glycogen rich^	p.Asp1084Asn	High	Positive	Negative	Positive	Low	Amplified
8	Ductal with apocrine features	p.Cys1058Tyr	N/A	Positive	Negative	Negative	Low	Not-amplified
9	Metaplastic (sarcomatoid)	p.Ile1082Leu	N/A	Positive	Positive	Positive	Low	Not-amplified
10	Ductal NST	p.Leu269Pro	Low	Negative	Negative	Positive	Low	Not-amplified
11	Ductal NST	p.Leu655Phe	N/A	Negative	N/A	N/A	Low	Not-amplified
12	Medullary	p.Pro1059Ser	Low	Negative	Positive	Negative	Low	Not-amplified

^Same case.

type grade III adenocarcinoma, pT2N2, Ki67 56%, carried three different *EGFR* mutations in exon 27, one of which was nonsense, resulting in a stop codon and thus in protein truncation. She was lost to follow-up six months after diagnosis.

Table II shows the phenotypic characteristics of amplified and mutated tumors. *EGFR* gene amplification and mutations did not overlap except for one case. This tumor carried a low frequency mutation in exon 27 and had *EGFR* amplification, high *EGFR* mRNA expression, EGFR and p53 protein expression positive (Figure 2A-C). The patient was a postmenopausal woman with a grade II glycogen-rich carcinoma, pT2N1, Ki67 labeling 5%, disease-free at 112 months of follow-up.

The agreement of EGFR status with the applied methods, *i.e.* IHC, mRNA expression, FISH and mutation analysis is shown in Supplementary Table III. Relative *EGFR* mRNA expression and IHC positivity were strongly correlated. Tumors with amplification were also IHC positive in 9 out of 10 comparable cases and expressed high *EGFR* mRNA in 7 out of 8 comparable cases (Table II); however, no statistical significance was reached for these comparisons, evidently because of the very low numbers of tumors with gene amplification and mutations in the entire cohort.

Associations of EGFR parameters with clinicopathological characteristics. All examined associations and their detailed description are presented in supplementary Table IV. Higher *EGFR* and CEN7 copies were found in tumors from older patients (Figure 2D) and similarly in tumors from postmenopausal women (Mann-Whitney's, p=0.037 for *EGFR*; p=0.006 for CEN7). In the same line, 9/11 *EGFR* amplified tumors were found in older patients (Fisher's exact, p=0.031). The rate of amplified tumors did not differ with respect to the basal and non-basal phenotype; basal tumors had a relatively higher rate of *EGFR* mutations, expressed relatively higher *EGFR* mRNA and were more frequently p53 protein positive as compared to non-basal tumors (Figure 2E). Tumors of larger

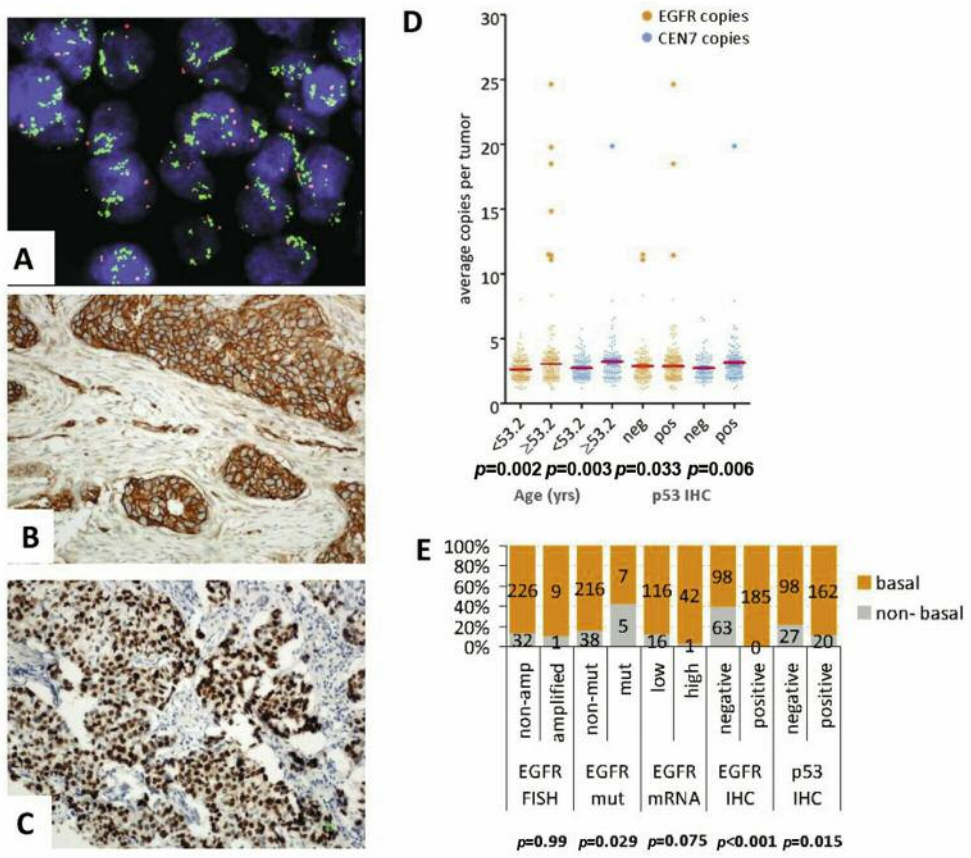

Figure 2. *EGFR parameters assessed with different methods. A-C, Example case, a glycogen-rich carcinoma: this tumor was EGFR amplified (A), and was positive for EGFR and p53 proteins with IHC (B and C); furthermore, the same tumor carried an EGFR mutation at 8% frequency and expressed relatively high EGFR mRNA (not shown). D: Significant associations between EGFR and CEN7 copies assessed by FISH with patient age and tumor p53 IHC. E: Associations between EGFR parameters and p53 IHC with the basal phenotype, which was classified based on EGFR and/or CK5 IHC positivity.*

size (>5 cm) had more frequently relatively high *EGFR* mRNA expression (11/19 tumors, p=0.005). EGFR protein expression was significantly associated with histological type (p=0.002). More specifically, EGFR IHC positivity was predominant in apocrine and metaplastic but it was less frequent in lobular carcinomas. EGFR and p53 IHC positive tumors had higher Ki67 index as compared to negative tumors (median 52.3 *vs*. 35.0 and 52.5 *vs*. 38, respectively; Mann-Whitney's, p-values=0.001). As compared to p53 IHC negative, p53 IHC positive tumors were more frequent in premenopausal patients (48.6% *vs*. 36.4%, Fisher's exact, p=0.044).

In comparison to patients with non-basal tumors, patients with basal tumors more often had low nodal burden (32/62 *vs*. 200/275, respectively; p=0.006) but significantly higher proliferation index (median Ki67 11.0 *vs*. 52.3, respectively, Mann-Whitney's p<0.001). Additionally, histological type was associated with basal phenotype (Fisher's exact p<0.001); apocrine (10/12), medullary (20/22) and metaplastic (16/17) histological types were frequently of the basal phenotype but lobular carcinomas were not (4/16).

Because EGFR and p53 protein expression were both associated with the basal phenotype, the first by definition and the second as revealed in the present analysis, we next created a combined variable for these two proteins (Supplementary Table IV), which, except for the associations described for EGFR and p53 proteins was not further associated with any parameter under study.

We then looked for associations between at least one positive result in EGFR parameters (IHC positive and/or mutant and/or high mRNA expression and/or amplified) and the clinicopathological parameters. Histological grade and Ki67 were significantly associated with positivity to at least one EGFR parameter (Supplementary Table V). More specifically, grade III carcinomas were more frequent in tumors positive to at least one EGFR parameter, while those tumors also had higher median Ki67.

Table III. *Multivariate analysis for EGFR related parameters (backwards selection models) in 243 TNBC patients.*

Parameter/Categories	N patients	N events	HR	95% CI	*p*-Value
Disease-free survival					
Age (median cutoff)					
≥53.2 *vs.* <53.2	119 *vs.* 124	34 *vs.* 44	0.42	0.19-0.94	0.033
Menopausal status					
Postmenopausal *vs.* premenopausal	133 *vs.* 110	41 *vs.* 37	1.88	0.85-4.16	0.12
Number of positive nodes					
0 *vs.* ≥4	83 *vs.* 73	16 *vs.* 41	0.26	0.14-0.49	<0.001
1-3 *vs.* ≥4	87 *vs.* 73	21 *vs.* 41	0.34	0.19-0.58	<0.001
Histological grade					
III *vs.* I-II	205 *vs.* 38	69 *vs.* 9	2.24	1.08-4.65	0.031
Adjuvant hormonotherapy					
Yes *vs.* No	45 *vs.* 198	23 *vs.* 55	2.02	1.21-3.39	0.007
Adjuvant radiotherapy					
Yes *vs.* No	185 *vs.* 58	55 *vs.* 23	0.44	0.26-0.75	0.003
p53/EGFR IHC (binary)					
One negative and one positive *vs.* Both negative or both positive	104 *vs.* 139	40 *vs.* 38	1.62	1.03-2.57	0.039
EGFR copies; cut off at 2.6*					
High *vs.* Low	136 *vs.* 107	37 *vs.* 41	0.57	0.36-0.90	0.017
Overall survival					
Age (median cutoff)					
≥53.2 *vs.* <53.2	119 *vs.* 124	28 *vs.* 36	0.35	0.14-0.85	0.020
Menopausal status					
Postmenopausal *vs.* premenopausal	133 *vs.* 110	34 *vs.* 30	2.57	1.03-6.37	0.042
Number of positive nodes					
0 *vs.* ≥4	83 *vs.* 73	12 *vs.* 36	0.26	0.13-0.52	<0.001
1-3 *vs.* ≥4	87 *vs.* 73	16 *vs.* 36	0.31	0.17-0.57	<0.001
Histological grade					
III *vs.* I-II	205 *vs.* 38	55 *vs.* 9	1.72	0.83-3.58	0.15
Adjuvant hormonotherapy					
Yes *vs.* No	45 *vs.* 198	21 vs.43	2.14	1.22-3.78	0.008
Adjuvant radiotherapy					
Yes *vs.* No	185 *vs.* 58	45 *vs.* 19	0.37	0.20-0.66	<0.001
CEN7 copies; cut off at 2.6*					
High *vs.* Low	138 *vs.* 105	30 *vs.* 34	0.49	0.29-0.83	0.008

*3-year ROC cut-off coinciding with median value; HR: hazard ratio.

EGFR parameters associated with patient prognosis. Univariate Cox regression analysis results for DFS and OS are shown in Supplementary Table VI and VII, respectively. Patients with small tumor size (T1), low nodal burden (≤3 positive lymph nodes) and high TILs rates presented better prognosis.

With log-rank testing, *EGFR* amplification was significantly associated with favorable outcome, with no relapses or deaths in the amplified group (Figure 3A and B); however, evidently because this group with only 11 patients corresponded to <5% of the cohort, statistical significance could not be reached for this parameter with Cox analysis (Supplementary Table VI and VII). In the same line, patients with tumors bearing higher *EGFR* (Figure 3C and D) and CEN7 (Figure 3E and F) copies showed a trend for better DFS and OS. *EGFR* and CEN7 cutoffs were defined by ROC curve analysis for 3-year DFS and coincided with the median values for these markers. Patients with *EGFR* mutated tumors had similar DFS and OS as patients without *EGFR* mutations. Lastly, *EGFR* mRNA expression did not affect patient outcome.

Next, we compared the combined p53/EGFR variable with survival data. Patients with tumors negative for both proteins fared better than those with tumors expressing either p53 or EGFR; interestingly though, patients with double negative tumors had similar outcomes to those with double positive tumors (Figure 4A and B). One hundred and seventy-four TNBC patients (49.4%) were classified as either double-positive or double-negative for EGFR and p53 protein and showed significantly increased 3-year DFS than patients classified otherwise (Supplementary Table VI).

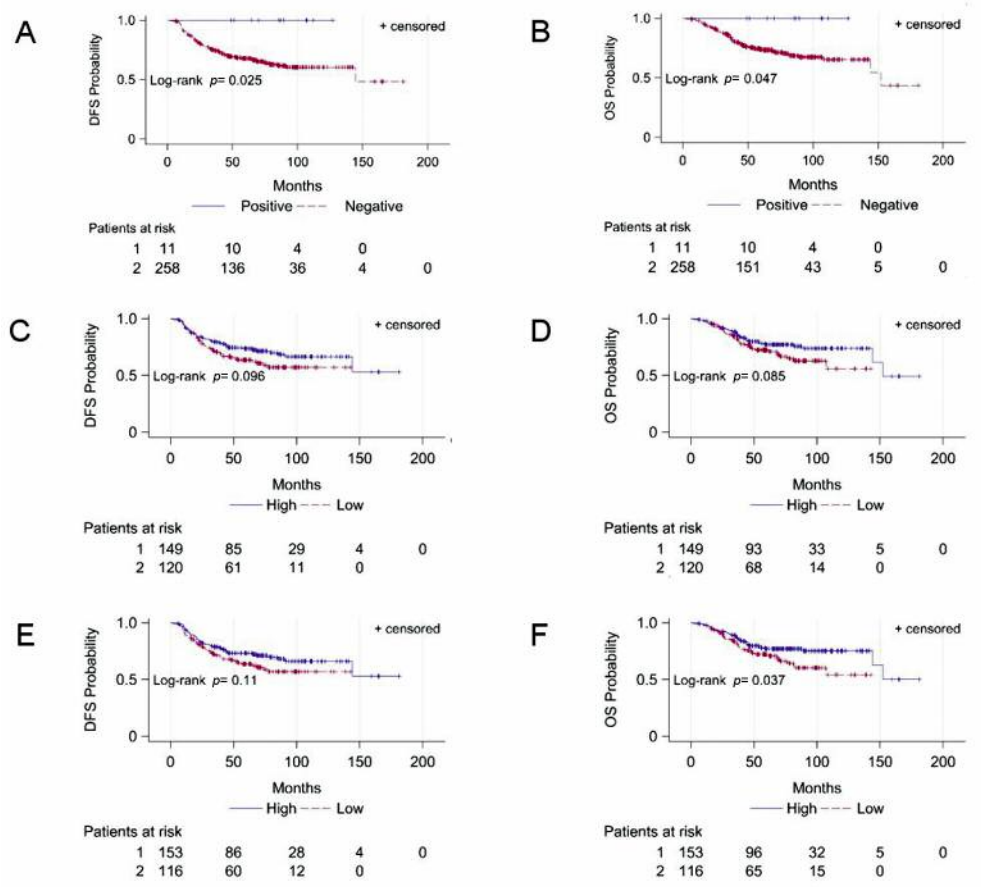

Figure 3. *Impact of EGFR-related chromosomal aberrations on patient outcome. A and B: EGFR gene amplification, no relapses or deaths for the 11 patients in this group; C and D: EGFR copies classified into high/low with 2.62 as a cut-off; E and F: CEN7 copies classified into high/low with 2.65 as a cut-off. Disease-free (DFS) and overall survival (OS) are demonstrated, as indicated.*

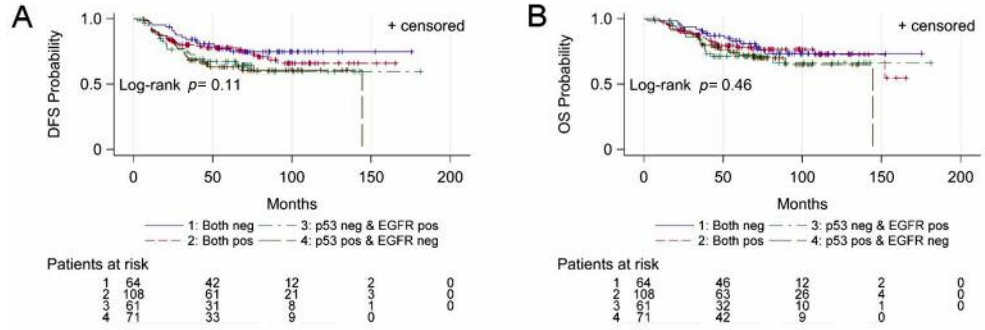

Figure 4. *Impact of combined EGFR/p53 phenotypes on patient outcome. A and B: DFS and OS, are indicated. No difference was observed for either positive phenotypes (p53-negative and EGFR-positive, or, p53-positive and EGFR-negative), while double-positive or double-negative tumors were associated with more favorable DFS but not OS.*

Finally, the effects of the three most clinically-relevant EGFR parameters, *i.e.*, *EGFR* and CEN7 copy numbers, and the binary combined p53/EGFR variable on DFS and OS were estimated, adjusting for standard clinicopathological characteristics and treatment and using backwards selection procedure, in order to examine their significance in the multivariate setting. The three parameters were entered in three separate (Supplementary Table VIII) and one combined

(Table III) models. Double p53/EGFR negative or positive tumors and high *EGFR* gene copies conferred favorable DFS to TNBC patients; these parameters did not retain their significance when analyzed in the same model for OS, while high CEN7 copies were significantly associated with fewer deaths (Table III). As expected, older age, higher nodal burden, larger tumor size and histological grade predicted for favorable DFS and, except for grade, also for OS; TNBC patients treated with adjuvant hormonotherapy based on local ER-positive classification had higher risk for relapse and death, while those treated with adjuvant radiotherapy had significantly more favorable outcome (Table III and Supplementary Table VIII).

All Supplementary Tables have been made publicly available at: http://hecog-images.gr/index.php?dir=/home /gkatak/public_html/TNBC_EGFR_SUPPLEMENTAL_TABLES/

Discussion

The heterogeneity of triple-negative breast cancer has made finding actionable targets and the development of targeted therapies particularly difficult. Several clinical trials tried to target a particular receptor or pathway, including EGFR, in patients with metastatic TNBC but failed to demonstrate a significant benefit (43-47). The result of abnormal EGFR mediation in molecular dysfunction remains unresolved and could be responsible for the failure of these trials together with inadequate patient selection (44). To date, no reliable and standardized methods for the measurement of constitutive activation of EGFR, appropriate for clinical and pathological practices, exist to properly classify patients with TNBC (48). Secq *et al*. first correlated different procedures, including IHC, silver in situ hybridization (SISH) and qPCR, to evaluate EGFR overexpression and *EGFR* amplification in TNBC and searched for *EGFR* mutations within exons 18-21 in TNBC (49). In the present study, we evaluated EGFR status at the chromosomal, genomic, mRNA and protein status.

With respect to EGFR IHC expression, our results are in accordance with the literature, ranging from 42-76% for immunopositivity in TNBC and basal-like tumors (9) (15) (9, 15, 50-54). However, there is no conclusive evidence concerning the impact of EGFR expression on patient outcome in the adjuvant setting (55). Notwithstanding the fact that EGFR expression and basal phenotype were associated with more aggressive pathological characteristics in our study, neither EGFR expression nor basal-like phenotype correlated with DFS or OS probability.

The Cancer Genome Atlas (TCGA) Research Group analysis demonstrated that among the most frequent loss-of-function alterations in TNBC are genes associated with DNA damage repair signaling pathway such as RB1 and BRCA1 and loss of TP53 function (56). Gain-of-function mutations in TP53, mostly missense mutations, are found in more than 60% of basal-like and TNBC tumors (3, 27, 56-58). Immunohistochemical evaluation of p53 protein expression has been widely used instead of *TP53* gene mutation analysis. Normal p53 protein and p53 protein resulting from nonsense mutations are unstable, due to their short half-life time and their abnormal length or morphology respectively; thus they cannot be detected immunohistochemically. On the contrary, missense mutations of *TP53* gene often result in high stability of the protein and its accumulation to the nucleus, thus they can be detected immunohistochemically (27, 59, 60). Interestingly, we found that patients with tumors double-positive or double-negative for EGFR and p53 expression had lower risk for relapse and death. Multiple reasons may integrate for this apparently contrasting finding. For example, the double-negatives and double-positives were highly represented among tumors with high TILs that are favorable prognosticators in TNBC (61, 62), double negatives may represent genomically stable TNBC that carry a better prognosis (63), while our finding on the either-positive phenotype may be related to the observed worse outcome observed in patients with tumors that are p53 protein positive but do not express CK5/6 and the androgen receptor (64). Plausibly, beyond these speculations, the obtained EGFR/p53 IHC profiles should be addressed as surrogate markers integrating different genomic/molecular alterations in TNBC that need further evaluation for their prognostic value in the clinical setting.

EGFR gene amplification broadly varies in breast cancer, ranging from 0.8% to 37% (24, 25, 51, 65-71). The optimal cut off in breast cancer has not yet been established, whereas several methods have been used to assess *EGFR* gene copy number alterations (FISH, CISH, DISH, RT-PCR). Nakajima *et al*., found no *EGFR* amplification among 84 patients with TNBC, while there was significant correlation between EGFR expression and high polysomy of chromosome 7 (48). Metaplastic breast carcinomas, mainly classified as basal, demonstrate high frequency of *EGFR* amplification among breast carcinomas (25, 69, 70). However, only one out of eleven amplified tumors was a metaplastic carcinoma (one out of seventeen such tumors) in our series. *EGFR* gene amplification has been described to result in increased protein expression in breast carcinomas (66, 67, 72, 73) implying that the multiple gene copies have downstream effects on *EGFR* mRNA and protein. Our results are concordant with these findings at both the mRNA and protein level for tumors informative with all methods, although the amplified group was too small to yield statistically significant results. High *EGFR* copy numbers have been related to poor disease free survival (66), which is in contrast to our data that show better DFS and OS for patients with higher *EGFR* and CEN7 copies, and with *EGFR* gene amplification. Of note, the cutoff produced by ROC analysis for 3-year DFS in our cases coincided with the

median value obtained for *EGFR* and CEN7 copies in the first place, arguing against biased analysis of these parameters. Higher *EGFR* and CEN7 copies may reflect genomic instability in the tumor, *e.g.*, as has previously been described for CEN17 copies (74) while genomic instability may sensitize tumors to cytotoxic agents (75). Whether our finding on CEN7 and *EGFR* copies as a favorable prognosticator upon conventional adjuvant cytotoxic chemotherapy is TNBC specific needs further investigation in cohorts involving all breast cancer subtypes. Plausibly, because of the retrospective nature of our study and the small number of patients with *EGFR* amplification, our findings should be regarded as hypothesis generating prompting for validation in larger studies.

Except for studies in Asian women demonstrating classic *EGFR* mutations (66, 76), all other relevant studies reported the absence of activating *EGFR* mutations in TNBC (48, 67, 70, 77-79). In line with the latter, the *EGFR* mutations identified herein were not classical and most probably not activating, especially regarding the truncation at an early phosphorylation site in one case. Further, these occurred at low frequency within the samples, which excluded their orthogonal validation with routine dd-sequencing as applied on FFPE DNA. In fact, since samples were composed out of tumor and non-tumor cells, the observed low frequency mutations may be stromal or lymphocytic infiltrate specific instead of being tumor specific. In support to this view, *EGFR* mutations among others were identified in breast tumor infiltrating leukocytes (80), while non-classical, often intronic *EGFR* mutations have been described at higher rates in the stroma of tumors from BRCA1/2 carriers as compared to sporadic breast cancers (78). Thus, our results do not support a role for *EGFR* mutations in TNBC.

Finally, we identified a patient with glycogen-rich carcinoma positive for all EGFR parameters and for p53 protein expression, who was event free for more than 9 years. Glycogen-rich clear-cell carcinomas of the breast are characterized by >90% cancer cells having abundant clear cytoplasm containing glycogen (81). There is debate regarding this tumor's behavior and it is difficult to define a prognosis as less than 150 cases have been reported. Most authors have found that this tumor has a poor prognosis but there is no direct comparison to IDC-NOS based on stage (81, 82). Nevertheless, the proposed myoepithelial origin of this type of breast carcinoma (83) is in agreement with the herein observed pathologic EGFR status, especially EGFR protein expression, which is a known marker of myoepithelial cells in the breast (84). To our knowledge, the EGFR geno/phenotype is first reported here for glycogen-rich carcinomas of the breast and may be considered when assessing this rare tumor type.

In conclusion, we found that *EGFR* gene amplification and mutations are rare in TNBC; when present, *EGFR* mutations are not clonal, probably not activating, located outside the druggable ATP binding pocket, and overall do not seem associated with known aggravating TNBC features. We also presented data on a favorable prognostic impact conferred by *EGFR*-related chromosomal aberrations and by combined EGFR/p53 IHC phenotypes to patients with operable TNBC treated with conventional cytotoxic adjuvant chemotherapy. These markers should be regarded as surrogates integrating the broad spectrum of genetic/molecular aberrations widely described for TNBC and need further investigation in this context. Patients with *EGFR*-related chromosomal aberrations seem to benefit from the already practiced taxanes-based adjuvant chemotherapy; whether this feature is an intrinsic good prognosticator for TNBC or whether the observed excellent outcome of these patients is linked to the applied chemotherapy, is an interesting clinical question to be prospectively addressed in appropriately designed clinical trials.

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Conflicts of Interest

The Authors declare that they have no conflict of interest.

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