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# EGFR expression and activation are common in HER2 positive and triple-negative breast tumours

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**Summary.** EGFR has been associated with unfavourable prognosis in patients with triple-negative breast carcinomas, although little is known about EGFR activation in these tumours. In a series of breast carcinomas (archived formalin fixed tumours, n=100), we investigated EGFR phosphorylation status at Tyr992 (pEGFR-Y992) and Tyr1068 (pEGFR-Y1068) by immunohistochemistry, along with EGFR protein expression (extracellular domain), gene amplification status (fluorescent in situ hybridization) and conventional clinicopathologic parameters. EGFR protein was present in 21.9%, while phosphorylation at Y1068 and Y992 was observed in 27.8% and 50.5% of tumours, respectively. None of the tumours showed EGFR gene amplification, whereas 21.1% exhibited chromosome 7 polysomy. The above EGFR parameters were usually not simultaneously detected and were not associated with each other. High grade (p=0.003), lymph node positive (p=0.045), estrogen receptor (ER) negative (p<0.001) tumours often expressed EGFR protein. EGFR-Y992 and Y1068 phosphorylation was inversely associated with ER presence (p=0.023 and p=0.029, respectively) but positively with HER2 expression status (p<0.001 and p=0.002, respectively). The global positivity for any EGFR parameter did not significantly differ between triple-negative and HER2 positive tumours. In conclusion, EGFR phosphorylation is commonly encountered in breast carcinomas, although unrelated to EGFR protein presence and gene amplification. EGFR may appear activated even in cases where the extracellular domain of this protein is not observed with immunohistochemistry. These findings may be useful for further studies aiming at the assessment of EGFR parameters on this type of material.

**Key words:** EGFR, Phosphorylation, Immunohistochemistry, FISH, Breast carcinoma

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## Introduction

Epidermal growth factor receptor (EGFR), a 170kD transmembranous protein, is a member of the HER (ERBB) receptor family, which also includes HER2, HER3, HER4, and belongs to the receptor tyrosine kinase (RTK) superfamily. These growth factor receptors are essential for normal cell function but they are also implicated in human oncogenesis (Gullick and Srinivassan, 1998; Hackel et al., 1999). HER receptors are aberrantly activated by several mechanisms, including excess ligand expression, gene amplification and protein overexpression, activating mutations and failure of inactivation mechanisms (Hackel et al., 1999; Prenzel et al., 2001). Activation of HER receptors by ligand binding results in the formation of homo- or heterodimers with other HER or related RTKs, which are then phosphorylated and activate substrates involved in intracellular signal transduction (McCune and Earp, 1989), thus stimulating proliferation of various cell types (Rajkumar and Guillick, 1994; Wells, 1999).

Six autophosphorylation sites have been identified at the tyrosine kinase domain of EGFR; out of these, three are major (Y1068, Y1148, Y1173) (Downward et al., 1984; Abe et al., 2006) and three minor (Y992, Y1045, Y1086) (Margolis et al., 1989; Walton et al., 1990; Levkowitz et al., 1999). Y992 is critical for substrate phosphorylation and internalization in the context of a truncated receptor only, while it may serve as a regulatory site when the major sites are absent (Sorkin et al., 1992). EGFR phosphorylation activates intracellular

pathways that induce proliferation and angiogenesis while inhibiting apoptosis (Chan et al., 1999; Rampaul et al., 2005). Still, little is known about the incidence of EGFR phosphorylation and its putative implications in breast cancer.

EGFR is important for normal breast development and for the maintenance of adult breast tissue, where it is expressed in myoepithelial and ductal epithelial cells. The involvement of EGFR in oncogenesis was initially evidenced in studies demonstrating that cells overexpressing EGFR become transformed when they are grown in the continuous presence of EGF (Velu et al., 1987). EGFR expression has been associated with increased tumour proliferating activity, angiogenesis and metastatic potential (Pavelic et al., 1993). However, although EGFR overexpression was originally suggested to be involved in later stages of breast carcinogenesis, during the metastatic process (Lichtner et al., 1993; Biscardi et al., 2000), more recent data show that EGFR expression in fact declines with tumour progression in comparison to normal breast tissue (Choong et al., 2007).

EGFR immunoreactivity has been correlated with unfavourable prognosis in patients with triple-negative invasive ductal carcinomas (Rakha et al., 2007; Viale et al., 2009). This subtype of breast cancer is immunohistochemically defined by a lack of estrogen receptor (ER), progesterone receptor (PR) and HER2 expression (Bauer et al., 2007; Rakha et al., 2007), accounts for about 10–17% of all breast carcinomas (Bauer et al., 2007; Dent et al., 2007; Rakha, et al., 2007) and has a higher incidence in younger women (<50 years) (Bauer et al., 2007; Dent et al., 2007; Tan et al., 2008).

Because EGFR expression is a common feature of various malignancies, it has long been investigated as a possible pharmaceutical target for inhibiting cancer growth. Two major therapeutic approaches have been developed for this purpose and are already in clinical use: blocking EGFR activation by targeting its extracellular domain with monoclonal antibodies, such as cetuximab and panitumumab, and inhibiting tyrosine phosphorylation at the intracellular kinase domain of EGFR with small molecule inhibitors, such as gefitinib and erlotinib (Humblet, 2004; Ciardiello and Tortora, 2008). Whether EGFR targeting will be of benefit for breast cancer patients, at least for those with specific carcinoma subtypes, is still under investigation. In any case, because in order to target EGFR we first need to understand whether this molecule is functional in the tumour itself, it appeared to us worth investigating EGFR gene status, as well as protein expression and phosphorylation in breast carcinomas in comparison to classical clinicopathologic parameters.

## Materials and methods

Previously diagnosed invasive breast carcinomas from 100 female patients were included in this study. Patient age ranged from 30 to 87 years. Histological tumour type, grade, stage and conventional marker data are summarized in Table 1.

### *Tissue microarray (TMA) construction*

Hematoxylin and eosin stained sections from each tumour were examined and the areas of invasive breast carcinoma were marked (T.K.). Seven cores (0.6mm diameter) from different areas in the same breast carcinoma, along with 3 cores from matched non-cancerous breast tissue were used from each case for TMA construction (T.K.). Low density TMAs (14-16 cases per array) were constructed with a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). Cores from normal skin and colon adenocarcinoma were also included as controls and for TMA section orientation.

### *Immunohistochemistry (IHC)*

IHC for estrogen receptor-alpha (ER, clone 6F11 [Novocastra, Newcastle upon Tyne,UK]), progesteron receptor (PR, clone 16 [Novocastra, Newcastle upon Tyne,UK]) and for HER2 intracellular domain (HercepTest [DAKO, Glostrup, DK]) was performed on 2micron TMA sections in an automated system according to standard immunohistochemical procedure protocols. Immunodetection of EGFR extracellular domain (EGFR Pharm DX [DAKO]), was performed manually, according to the manufacturer's instructions. The antibodies used and their specifications were: anti-

**Table 1.** Frequencies and percentage of tumour clinicopathologic parameters.

| | | cases | % |
|---|---|---|---|
| Tumor type | Ductal NOS | 78 | 78 |
| | Lobular | 9 | 9 |
| | Mixed | 2 | 2 |
| | Medullary | 3 | 3 |
| | Mucinous | 6 | 6 |
| | Comedo | 1 | 1 |
| | Anaplastic | 1 | 1 |
| Grade | 1 | 4 | 4.2 |
| | 2 | 38 | 40 |
| | 3 | 53 | 55.8 |
| Size | 1.1-2 cm | 28 | 34.2 |
| | >2 cm | 54 | 65.8 |
| LN status | 0 | 15 | 31.3 |
| | 1 | 10 | 20.8 |
| | >1 | 23 | 47.9 |
| ER | Neg | 33 | 34.4 |
| | Low | 16 | 16.7 |
| | Medium | 23 | 23.9 |
| | High | 24 | 25 |
| HER2 | 0 | 51 | 51.5 |
| | 1+ | 26 | 26.3 |
| | 2+ | 9 | 9.1 |
| | 3+ | 13 | 13.1 |

posphoEGFR TYR992 (pEGFR-Y992), polyclonal, diluted 1:150 and anti-phosphoEGFR TYR1068 (pEGFR-Y1068), clone 1H12, diluted 1:50, both from Cell Signaling Technology, Beverly, MA. For all the phosphorylation-specific antibodies, epitope unmasking procedure was accomplished with the DAKO Target Retrieval Solution at pH 6.1-6.2. All experimental antibodies were tested and standardized on excess paraffin sections from breast and colon adenocarcinoma and matched normal tissues. Immunohistochemistry was repeated at least twice on TMA sections.

### *IHC evaluation*

ER / PR status was evaluated by using the common diagnostic scoring system (negative, low, medium, high staining and percentage of positive cells [HistoScore]). HER-2 results were interpreted using the HercepTest (0, 1+, 2+, 3+) (Table 1).

A case was considered as positive for EGFR PharmDX, pEGFR-Y992 and pEGFR-Y1068 when more than 10% of tumour cells expressed these markers. The staining pattern was characterized as membranous (partial or complete staining of any intensity), cytoplasmic or membranous/cytoplasmic (V.K., T.K.). pEGFR-Y1068 exhibited aggregated cytoplasmic staining in dispersed cells within the same tumour, in accordance with receptor internalization upon phosphorylation at this site (Wiley, 2003).

### *Fluorescence in situ hybridization (FISH)*

The commercially available probe set LSI® EGFR/CEP 7 Dual Color Probe (Vysis, U.S.A.) was used for the assessment of EGFR gene copy number. FISH signals in at least 100 non overlapping nuclei were simultaneously assessed by two observers (T.K. and I.K. / G.K.). Based on this 100-nuclei cut-off we finally succeeded in obtaining results in 57 cases. Images were captured with a computer-controlled digital camera and processed with the FISH Imager software. FISH patterns were considered normal when the ratio of EGFR vs chromosome 7 (centromere) signals in each case was between 0.9-1.2. EGFR gene amplification was considered when the above ratio was >2.0. In cases with ≥3 centromere signals per nucleus in >20% of assessable nuclei trisomy or polysomy of chromosome 7 was considered.

### *Statistical analysis*

Associations between immunohistochemical and FISH results and clinicopathological parameters were assessed with the Mann Whitney test. A P-value <0.05 was considered significant.

## Results

Sixty nine out of 89 (77.53%) breast carcinomas

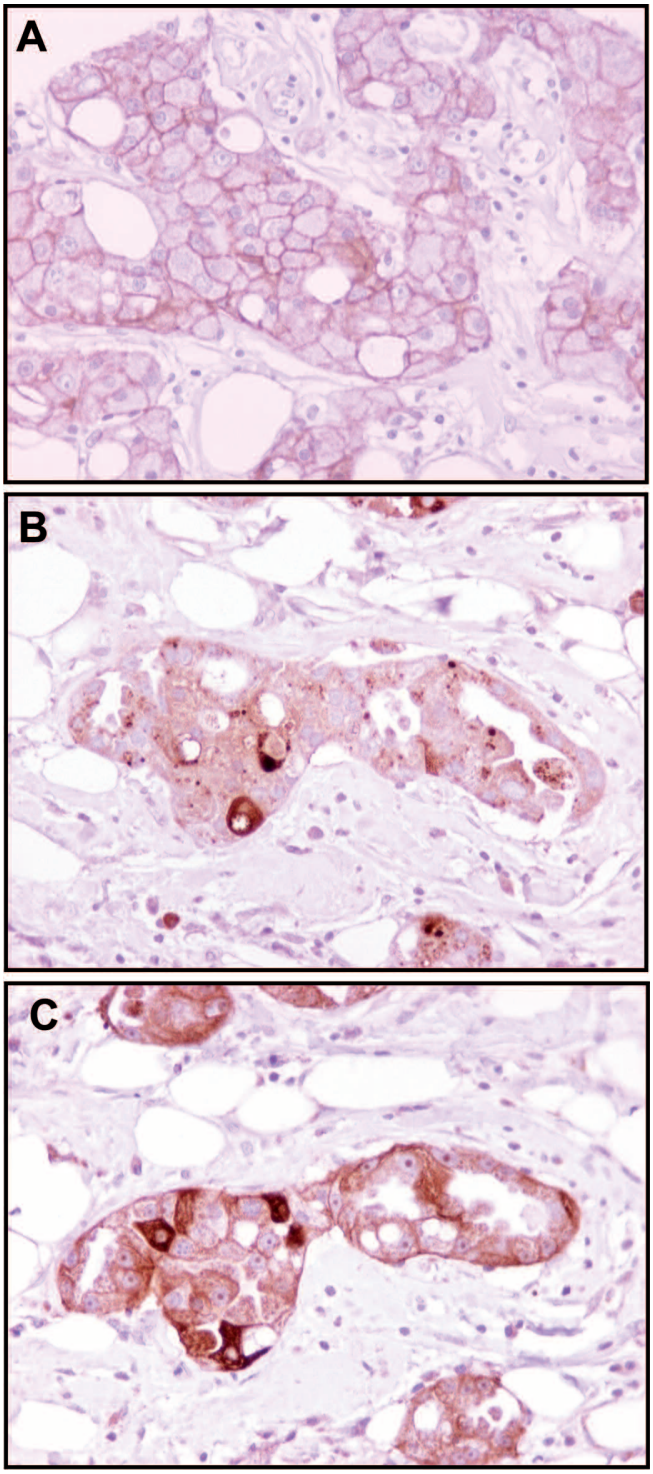


**Fig. 1.** Coexpression of EGFR and phosphoEGFR in the same breast carcinoma. Positivity for the presence of EGFR protein in **A**, phosphoEGFR at Tyr1068 in **B** and phosphoEGFR at Tyr992 in **C**. The staining pattern is clearly membranous for EGFR protein, membranous and cytoplasmic for EGFR-Tyr992 and exclusively cytoplasmic for EGFR-Tyr1068. x 400

were positive for hormone receptors (any positive HistoScore for ER and PR), while 44/91 (48.35%) showed some degree of HER2 expression. The number of cases differs per marker due to missing cores on the TMA sections.

The majority of the tumours were negative for EGFR protein (78.1%) and pEGFR-Y1068 (72.2%); by contrast, 50.5% were positive pEGFR-Y992. EGFR protein positivity was mainly membranous, in contrast to pEGFRY-1068 which was characterized mainly by cytoplasmic staining, while pEGFR-Y992 was detected in the membrane and cytoplasm in 28.9% and 21.6% of the cases, respectively (Fig. 1). Two thirds of pEGFR-Y1068 positive tumours were also positive for pEGFR-À992.

Eleven tumours in our series were triple negative (ER-, PR-, HER2-); five of them showed immunopositivity for EGFR protein. The EGFR/phosphoEGFR immunoprofile of these tumours is presented in Table 2. Interestingly, 9 out of 11 of these triple negative tumours exhibited immunopositivity for at least one EGFR

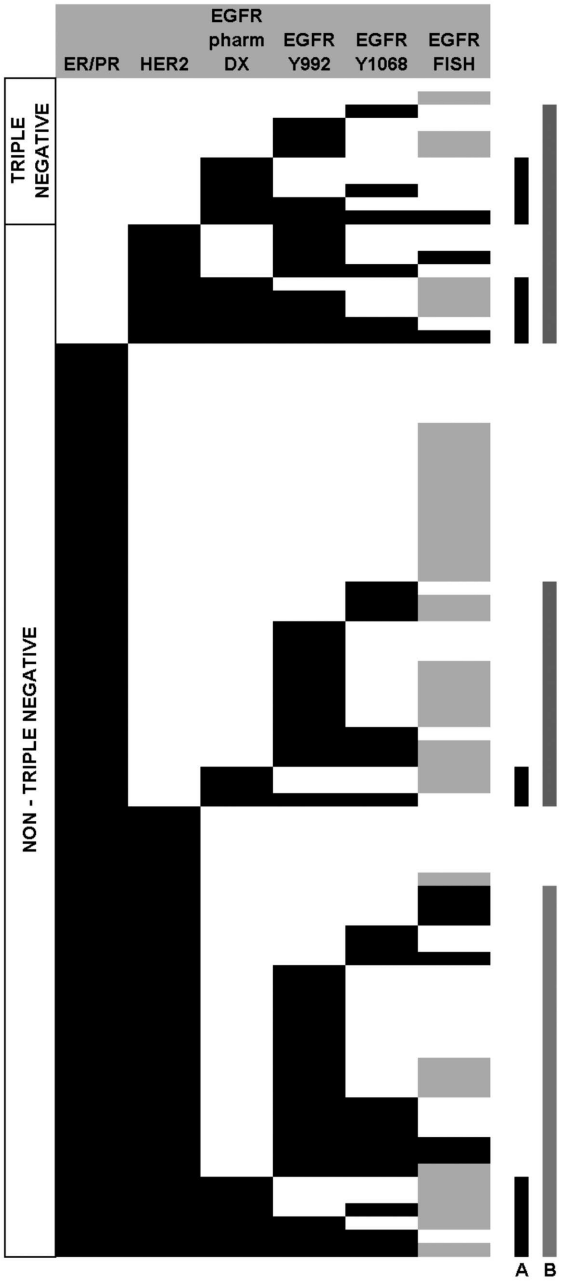


**Fig. 2.** EGFR gene status and protein expression/activation in breast carcinomas. Although EGFR protein is detected in a limited number of breast carcinomas (A bars), EGFR parameters denoting activation of the receptor or corresponding genomic alterations are observed in the majority of these tumours (B bars). Similar EGFR parameter profiles are observed in tumours characterized as triple negative but also in those expressing HER2 in the absence of hormone receptors. black = positive; white = negative, grey (FISH) = no data. A bars = EGFR protein positive tumours; B bars = tumours positive for any EGFR related parameter.

**Table 2.** EGFR/phosphoEGFR immunoprofile in the subset of triple-negative tumours. With the exception of two cases, positivity for at least one of the markers was observed.

| Case | EGFRpharmDX | pEGFRY992 | pEGFRY1068 | FISH result |
|---|---|---|---|---|
| 1 | neg | neg | neg | neg |
| 2 | neg | neg | neg | NA |
| 3 | neg | neg | pos | neg |
| 4 | neg | pos | neg | neg |
| 5 | neg | pos | neg | NA |
| 6 | neg | pos | neg | NA |
| 7 | pos | neg | neg | neg |
| 8 | pos | neg | neg | neg |
| 9 | pos | neg | pos | neg |
| 10 | pos | pos | neg | neg |
| 11 | pos | pos | pos | polysomy |

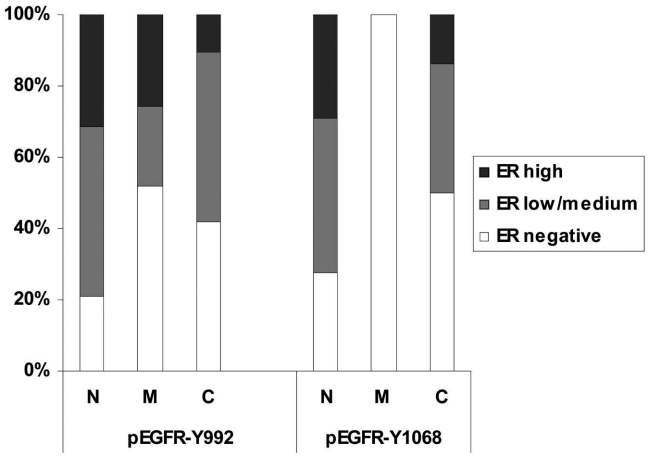


**Fig. 3.** An inverse correlation between ER expression and EGFR activation is observed. N = negative; M = membranous; C = cytoplasmic.

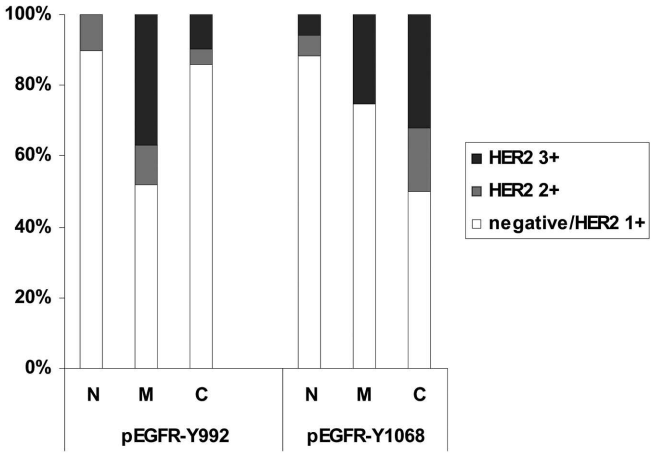


**Fig. 4.** HER2 expression was mainly observed in membranous pEGFR-Y992 and cytoplasmic pEGFR-Y1068 positive cases. N = negative; M = membranous; C = cytoplasmic.

related parameter, while the only tumour with polysomy also showed strong membranous EGFR protein localization and activation. However, we observed one HER-2 1+ positive tumour with EGFR polysomy and strong EGFR, Y992 and Y1068 immunoreactivity as well. Overall, there was no statistical difference concerning EGFR related parameters between triple-negative and HER2 positive tumours. Thirty seven out of 43 (86%) HER2 positive tumours with variable ER positivity, but only half (17/35 [48.6%]) of hormone receptor positive/HER2 negative tumours, appeared positive for at least one EGFR related parameter (Fig. 2), a difference that was found to be statistically significant (p=0.0005).

There was no correlation between EGFR staining and EGFR phosphorylation (Fig. 2). Seven cases only were positive for all three markers. There was an adverse correlation between ER and HER2 (p=0.0027, Mann

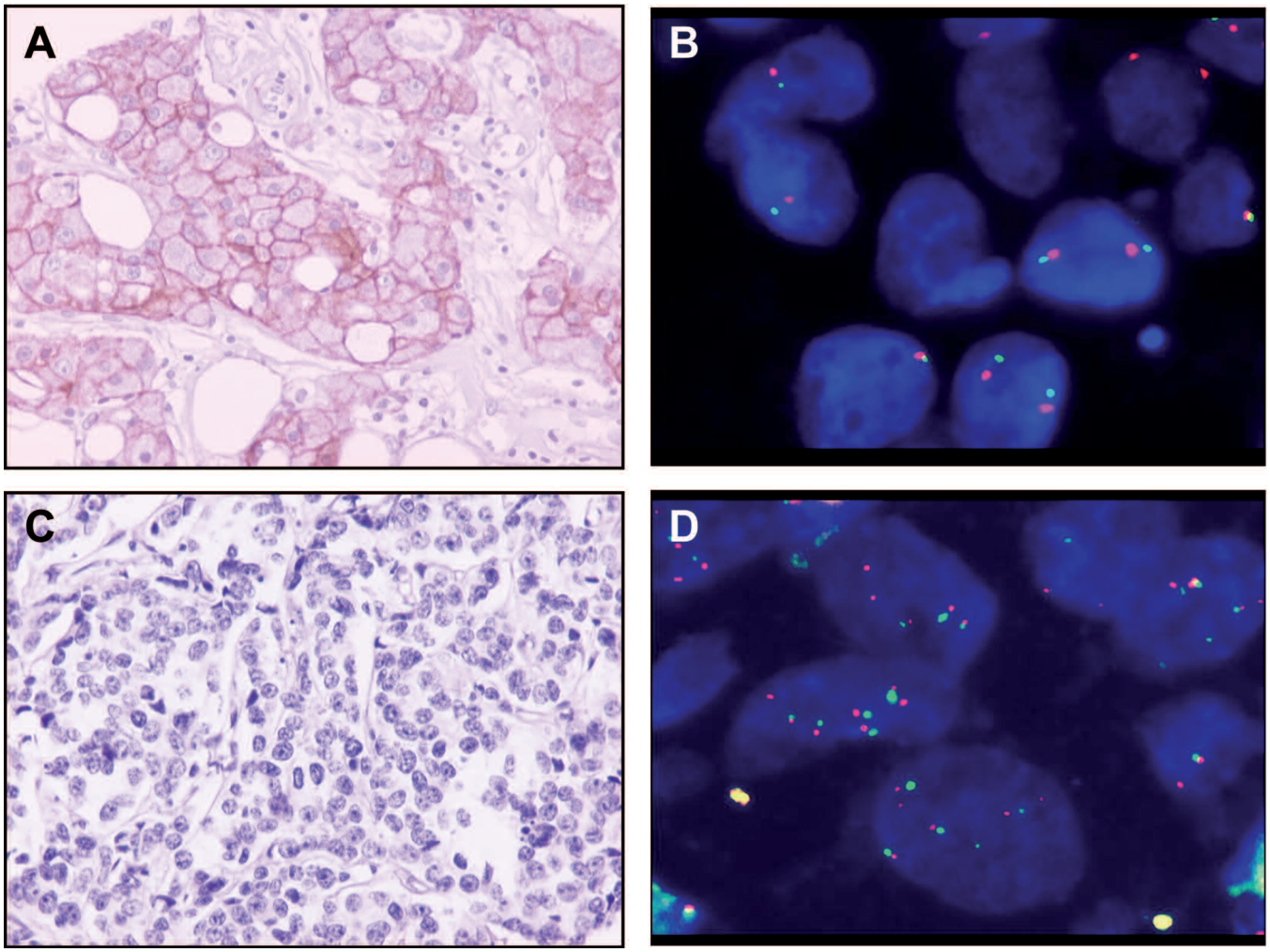


**Fig. 5.** EGFR gene status does not correspond to EGFR protein overexpression. Some cases with normal FISH pattern exhibited strong membranous immunopositivity for EGFR protein **(A, B)**, whereas others with polysomy 7 were negative for EGFRpharmDX marker **(C, D)**. x 400

Whitney), EGFR ($p<0.001$, Mann Whitney), pEGFR-Y1068 ($p=0.029$, Mann Whitney) and pEGFR-Y992 expression ($p=0.022$, Mann Whitney) (Fig. 3).

Most ER negative tumours expressed HER2 and EGFR. In the majority of HER2 3+ tumours (83.3%) a membranous positivity for pEGFR-Y992 was revealed (Fig. 4) ($p<0.001$, Mann Whitney). Cytoplasmic pEGFR-Y1068 positivity was observed mostly in HER2 2+ and 3+ tumours ($p=0.04$ and $<0.001$ respectively, Mann Whitney) (Fig. 4). A correlation between EGFR and histologic grade was observed ($p=0.003$, Mann Whitney), since most EGFR positive tumours (88.9%) were evaluated as grade III. Tumours with metastatic lymph nodes exhibited cytoplasmic positivity for pEGFR ($p=0.034$ for pEGFR-Y992 and $p=0.016$ for pEGFR-Y1068, Mann Whitney).

None of the 57 tumours with informative FISH results showed EGFR gene amplification. Forty five of these (78.9%) were normal and 12 (21.1%) exhibited polysomy. There was no correlation between EGFR gene status and EGFR protein expression in IHC (Fig. 5), including the phosphorylated forms. Three tumours that expressed phosphorylated EGFR only, but not the extracellular domain of this receptor, exhibited EGFR gene polysomy.

## Discussion

Although targeted therapy against EGFR has been developed and used in some types of cancer, such as lung and colorectal, conflicting data have been reported regarding the value of EGFR protein assessment by immuhistochemistry as a predictive marker of response to anti-EGFR drugs (Goldstein and Armin, 2001; Tsao et al., 2005; Dziadziuszko et al., 2007; Theodoropoulos et al., 2009). Controversial data about the prognostic value of EGFR have been reported in breast carcinomas, since some authors have observed a significant association of EGFR expression with poor prognosis, increased risk of recurrence (Sainsbury et al., 1987; Nicholson et al., 1989; Fox et al., 1994; Tsutsui et al., 2002; Tan et al., 2004; Dihge et al., 2008; Magkou et al., 2008) and poor response to hormonal therapy (Nicholson et al., 1994; Arpino et al., 2004), whereas others have not found any such correlation (Ferrero et al., 2001; Rampaul et al., 2004; Gori et al., 2009). In triple-negative breast cancer, EGFR protein is usually expressed in tumours with BRCA1 mutations (Lakhani et al., 2005; Turner and Reis-Fihlo, 2006), which are also classified as basal-like carcinomas because their phenotypic characteristics resemble those of the basal cells in the normal ductal epithelium. EGFR is assessed as part of an immunohistochemical panel for the identification of basal-like breast cancers, where it is present in 60-70% of the cases (Turner and Reis-Fihlo, 2006; Yehiely et al., 2006). In our study, almost half of the triple-negative tumours expressed EGFR protein. Although EGFR expression remains of questionable prognostic value in non-triple negative tumours, it has been associated with worse prognosis in triple negative carcinomas (Viale et al., 2009). Hence, it may be worthy assessing EGFR protein expression at least in this subset of breast tumours.

Controversial data on the incidence of EGFR positive breast carcinomas have been reported (Suo et al., 2002; van Poznak et al., 2002; Tsutsui et al., 2003; Witton et al., 2003). This might be due to the different methods, antibodies and cutoff levels that have been used (Corzo et al., 2005; Rampaul et al., 2005). In this study, we found that almost 20% of breast tumours exhibited membranous EGFR positivity. This rate is in accordance with some previous reports and especially with the findings of Abd El-Rehim et al., who evaluated EGFR expression in more than 1500 breast carcinomas (Abd El-Rehim et al., 2004).

An inverse association between ER and EGFR has been well established in various studies (Fox et al., 1994; Nicholson et al., 1994; Ioachim et al., 1996; Tsutsui et al., 2002; Witton et al., 2003; Nieto et al., 2007). In the same line, both EGFR phosphorylated forms were inversely associated with ER status in the present study. Although no significant association between EGFR and HER2 expression was observed, a subset (26.3%) of the tumours co-expressed EGFR and HER2 (HER2 2+ or 3+), which may indicate EGFR/HER2 heterodimerization. Furthermore, most of the HER-2 3+ cases showed membranous positivity for pEGFR-Y992 and cytoplasmic positivity for pEGFR-Y1068, again suggesting EGFR activation through EGFR/HER2 heterodimers. It is known that HER2 overexpression can potentiate EGFR signalling, resulting in EGFR-mediated transformation and tumour progression (Gross et al., 2004). Additionally, it has been noted that patients with tumours co-expressing these receptors have a worse outcome (Suo et al., 2002; DiGiovanna et al., 2005; Nieto et al., 2007).

Currently, investigations on EGFR overexpression are mostly carried out by using antibodies detecting the extracellular domain of the receptor. However, EGFR activation corresponds to (auto)phosphorylation at the tyrosine kinase domain that is located near the intracellular carboxyl terminus of the receptor. The discrepancy between EGFR protein (extracellular domain) and phosphorylation findings may be described in the following patterns: Pattern 1: EGFR protein positive – phosphorylation negative: EGFR protein does not necessarily reflect the status of receptor activation; phosphorylated sites other than Y992 and Y1068 may be activated in the tumour. Pattern 2: EGFR protein negative – phosphorylation positive: extracellular domain epitope detected by the antibody used for IHC may not be present (protein may be truncated) or its conformation may be altered due to mutations or polymorphisms. It should be noticed, however, that the above patterns might simply reflect the impact of formalin fixation on the preservation of conformational epitopes. The latter include, for example, the epitope in the extracellular domain (S2) targeted by the DAKO

EGFR antibody that was used in this study and practically all phosphorylated epitopes targeted by the corresponding antibodies, since phosphorylated amino acids and proteins are characterized by 3-dimensional conformational changes of the corresponding epitopes in vivo. Conformational epitopes are in most cases formalin-sensitive and maybe unpredictably altered during fixation. Hence, the inability to detect the presence or activation of EGFR protein is strongly dependent on the (in)efficiency of the antibodies to bind to their target epitopes in fixed tissues.

Considering that future therapeutic agents for breast cancer may target the three major phosphorylation sites (Y1068, Y1148 and Y1173) (Abe et al., 2006, 2008), it will be interesting to investigate if there is any association between the presence of activation in these sites with clinical outcome. To our knowledge, there have been no prior reports of the activation status of EGFR in breast cancer, concerning the two phosphorylation sites studied herein. Reports on different phosphorylation sites are controversial. In particular, Magkou et al. considered the simultaneous expression of both EGFR and pEGFR-Y1173 as an independent prognostic indicator for overall survival (Magkou et al., 2008), while Nieto et al. found no prognostic effect of pEGFR-Y1086 (Nieto et al., 2007).

In our study, EGFR gene amplification was not found, although 21.1% of the cases presented with chromosome 7 polysomy. However, there was no correlation between chromosome 7 polysomy and immunohistochemical EGFR/pEGFR protein expression. Polysomy of chromosome 7 has been reported in breast carcinomas but its role in the biology of this disease remains unknown (Unemura et al., 2005). EGFR amplification in breast carcinoma has been reported as absent (Corzo et al., 2005; Unemura et al., 2005) or ranging between <0.5 to 6% (Al Kuraya et al., 2004; Kersting et al., 2004; Bhargava et al., 2005; Laakso et al., 2006). EGFR gene amplification was demonstrated in approximately one-third of metaplastic breast carcinomas (Reis-Filho et al., 2005), while up to 5-10% of invasive ductal carcinomas with a basal-like phenotype may harbour EGFR gene amplification (Lambros et al., 2007). In the present study, EGFR polysomy was not associated with the histological / biological subtype of breast carcinomas, although this may be due to the small number of triple-negative tumours examined. Nevertheless, the question still remains whether these patients would benefit from EGFR inhibitors.

In conclusion, EGFR and phosphoEGFR are usually not simultaneously detected and do not provide any information on chromosome 7 status in breast carcinomas. If examined globally, positivity for any EGFR related parameter is a common feature in breast carcinomas without any significant difference between triple negative and HER2 positive tumours, although it seems related to HER2 expression. These findings may be worth considering in studies investigating the clinical value of EGFR in specific subsets of breast carcinomas.

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