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Triple negative breast carcinoma *EGFR* amplification is not associated with *EGFR*, *Kras* or *ALK* mutations

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Background: The amplification of epidermal growth factor receptor (*EGFR*) in triple negative breast carcinomas (TNBC) suggests its potential therapeutic application, as for *HER-2*, using standardised methods of measurement. In this regard, we aimed to compare several methods for evaluating *EGFR* amplification along with potential mutations for suitability in clinical practice.

Methods: Tissue sections of 138 TNBCs were used (1) to compare *EGFR* amplification and expression by silver *in situ* hybridisation (SISH) to qPCR and immunohistochemistry (IHC) and (2) to search for *EGFR* mutations, along with *Kras*, *PI3K*, *Braf* and *HER-2* mutations and echinoderm microtubule associated protein like 4-anaplastic lymphoma kinase (*EML4-ALK*) translocation.

Results: (1) Amplification of *EGFR* was observed in well-characterised TNBCs (up to 92%); (2) qPCR correlated with SISH with 94% specificity and 75.6% sensitivity; (3) IHC correlated with SISH with 97% sensitivity and 78% specificity; (4) no *EGFR*, *Kras* mutations or *EML4-ALK* translocations were found, but *PI3K* and *Braf* mutations were observed in 26% of cases; and (5) small, acentric circular extrachromosomal DNA similar to 'double minutes' in glioblastomas was observed in 18% of SISH sections.

Conclusions: SISH and IHC are methods that are suitable in clinical practice to screen for *EGFR* amplification and overexpression, which are frequently observed in TNBC. Patients with TNBC are potential candidates for *EGFR*-targeted therapy combined with *PI3K* and *Braf* inhibitors.

Triple negative breast carcinomas (TNBC), defined as tumours lacking oestrogen and progesterone receptors and *HER-2* gene amplification, account for 10–20% of all breast carcinomas in Asian and Western populations (Thike *et al*, 2010; De Ruijter *et al*, 2011). TNBC are usually of high histoprognostic grade and are associated with distinctive, metastatic patterns, particularly with high rates of brain metastases, shorter time-to-recurrence and earlier mortality (Thike *et al*, 2010). TNBC also commonly express basal markers such as cytokeratins 5/6 and epidermal growth factor receptor (*EGFR*) (Nielsen *et al*, 2004; Charpin *et al*, 2009a;

El Guerrab *et al*, 2011), and are therefore generally accepted as clinical surrogates for basal-like breast cancers (BLBC), although not all basal-like breast carcinomas are triple negative (Corkery *et al*, 2009). The TNBC phenotype is also acknowledged as an aggressive breast carcinoma molecular subtype with an early age of cancer onset and a high probability of metastases at presentation. Sustained, complete remission in TNBC is rare and additional treatments directed at appropriate molecular targets, such as poly (ADP-ribose) polymerase inhibitors (Rios and Puhalla, 2011), are urgently needed.

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Likewise, patients with *EGFR*-amplified and *EGFR*-over-expressing TNBC (30–52%) and BLBC (60%) (Reis-Filho, Tutt, 2008) should benefit from *EGFR*-targeted therapy, as well as from anti-HER-2 therapies. Ongoing clinical trials are exploring the roles of monoclonal antibodies against *EGFR*, such as cetuximab in TNBC. However, the use of anti-*EGFR* drugs in TNBC management is still controversial and only a few preliminary studies have been reported to date. The failure of *EGFR*-targeted therapy in TNBC trials is likely due to an insufficient knowledge of the various types of molecular dysfunction as a result of abnormal *EGFR* mediation and from inadequate selection of patients for this specific treatment (O'Shaughnessy *et al*, 2011; Carey *et al*, 2012). Particularly, because of discrepant data in literature reports, as for HER-2 targeted treatments, reliable and standardised methods of the measurement of *EGFR* amplification and overexpression that are suitable for the current clinical and pathological practices are also required to properly identify those patients with TNBC, *EGFR* amplification and *EGFR* overexpression (Nakajima *et al*, 2012). Although a recent study (Nakajima *et al*, 2012) has documented comparative methods (chromogen ISH and immunohistochemistry (IHC) procedures) for *EGFR* evaluation within TNBC, no extensive study that has focused on this particular point has been reported for TNBC.

In these regards, we (i) first correlated different procedures, including IHC, silver *in situ*-hybridisation (SISH), and qPCR, to evaluate *EGFR* overexpression and *EGFR* amplification in TNBC (as extensively documented for *HER-2* in the literature), and (ii) searched for *EGFR* mutations in TNBC, which are well acknowledged in non-small cell lung carcinoma (NSCLC) (Lynch *et al*, 2004; Paez *et al*, 2004). In TNBC, *EGFR* mutations rarely occur (Bhargava *et al*, 2005; Reis-Filho *et al*, 2006; Carey *et al*, 2010; Jacot *et al*, 2011) and often account for silent mutations (Generali *et al*, 2007). However, this point is controversial in TNBC. Most studies have shown that no *EGFR*-activating mutations occur in TNBC (Nakajima *et al*, 2012), but one series of 653 Asian patients with TNBC was recently reported to have *EGFR* mutations in 11.4% of cases (70 out of 653) that were independent of *EGFR* expression (Teng *et al*, 2011). This particular point is crucial for a patient's response to anti-*EGFR* therapies. Our study was also designed to evaluate *EGFR* mutations in TNBC of non-Asian patients.

In TNBC, such as in NSCLC and colorectal carcinomas (Siena *et al*, 2009), *EGFR* deregulation and mutations of downstream pathways, in particular *Kras*, *Braf*, *PI3K*, *PTEN*, or *ALK*, have been recently reported (Martin *et al*, 2012) and might be responsible for the impaired efficacy of *EGFR*-targeted drugs. Therefore, we studied mutations in the signalling pathways downstream of *EGFR*, particularly of *PI3K*, *Kras* and *Braf*, in parallel to mutations involved in response to a specific therapy, such as the *ALK* translocation that is currently observed in NSCLC but is exceptionally found in breast carcinomas (BC) (Lin *et al*, 2009).

MATERIALS AND METHODS

Materials. Our series included 161 cases that were distributed into two different tumour sets. The first set ($n = 47$) consisted of current, large sections. The second set ($n = 114$) consisted of tissue microarray (TMA) sections from 915 consecutive, ductal breast carcinomas that were distributed individually as cores (four cores per case) measuring 0.6 mm in diameter.

This study was designed according to the current recommendations of the French National Committee of Cancer INCa (Institut National du Cancer) and the 15189 ISO European recommendations for legal archival procedures in the Pathology Departments and Certification. According to the French law, approval from an

ethical committee was not required for our study. IHC and *in situ* hybridisation tissue arrays were performed from archived, paraffin-embedded and formalin-fixed tissue samples that remained in blocks after current diagnosis in pathology labs. Informed consents from patients who were included in the study for IHC, *in situ* hybridisation, and analysis of gene alterations were systematically obtained before surgical intervention and tissue sampling.

Tumour sample formalin fixation was controlled (24 h for biopsies and lumpectomy less than 3 cm; and 48 h for larger surgical specimens at room temperature) along with paraffin embedding with a constant control, melted-paraffin temperature (60 °C). Sections were obtained using automated devices calibrated to obtain four micron-thick tissue sections 24 h before immunodetection processing.

Current large sections. Large current sections were also evaluated because they included more tissue that was suitable for PCR and mutation, which was in contrast to the small TMA cores of 0.6 mm in diameter. The tumours that were selected ($n = 47$) for the study included the following: (i) TNBC ($n = 24$ out of 47) without any estrogen receptors (ER)- or progesterone receptors (PR)-positive immune expression and without *HER-2* amplification, which was evidenced by a negative SISH test, or (ii) 'TNBC-like' ($n = 23$ out of 47), which had similar unfavourable histoprosthetic criteria and were *HER-2*-negative (SISH test) but expressed low ER levels (<20%) in positive cells and negative PR levels ($n = 23$ out of 47). This 'TNBC-like' group was included in the study to show that depending on the cut-off of the percentage of ER-positive cells (0 to 20%), the rate of *EGFR* amplification in TNBC could considerably vary, as shown by the variability in the literature data. The tumour pTN stages were pT1 and pT2 (mean size 18 mm), and node-positive carcinomas accounted for 38% at the time of diagnosis ($n = 14$ out of 38).

TMA sections. TMA sections were constructed as previously described (Charpin *et al*, 2009a, b) and included 915 breast carcinomas of any type that were collected from 2000 to 2005. The samples included 114 TNBC that were completely ER- or PR-negative and lacked *HER-2* amplification (SISH test). Tumours were not otherwise specified ductal carcinomas.

Tumours measuring from 7 mm to 53 mm (pT1: $n = 398$; pT2: $n = 488$; pT3: $n = 29$) and exhibiting the usual distribution of Ellis and Elston grade (grade 1: 21%; grade 2: 61%; grade 3: 17%) were included, and 667 were node negative.

Methods

IHC. Automated procedures were assessed (Charpin *et al*, 2009a, b) on a Ventana Benchmark XT device, using E-view detection Ventana kits for DAB staining and the following specific monoclonal mouse or rabbit antibodies: monoclonal anti-savage *EGFR* clone 3C6 (Ventana Roche, Meylan Grenoble, France); clone B6 for detection of the E746- A750 exon 19 deletion of the *EGFR* gene (Cell Signaling Technology, St. Quentin, France); clone 43B2 for L858R, for the detection of mutated *EGFR* exon 21 (Cell Signaling Technology); anti-*ALK* clone 5A4 (Abcam, Paris, France) for the detection of the fusion transcript echinoderm microtubule associated protein like 4-anaplastic lymphoma kinase (EML4-*ALK*); clone SP1 anti-ER (Ventana Roche); clone 1E2 anti PR (Ventana Roche); and clone 4B5 anti-*HER-2* (Ventana Roche). The slides were counterstained with hematoxylin and bluing reagent.

The positive controls for mutated or amplified non-*EGFR* consisted of paraffin sections of breast and qPCR-amplified colonic carcinomas, whereas the controls for mutated *EGFR* and translocated *ALK* consisted of paraffin sections of mutated *EGFR*

(exon 19 deletion and exon 21 mutation) or FISH *EML4-ALK*-translocated lung carcinomas.

In situ hybridisation (ISH). The ISH procedures included silver ISH (SISH Ventana Roche, INFORM) for the detection of the *EGFR* gene and chromosome seven amplification and fluorescent ISH for *ALK* translocation (DAKO Histology FISH accessory kit, ABBOTT DNA ALK probe, Trappes, France). The positive controls for savage *EGFR* consisted of the *EGFR*-SISH xenograft control slides that were provided with the Ventana Roche kits. Normal labelling of stromal cells with two *EGFR* silver dots in normal cells also served as a positive control for tissue quality control for *EGFR* and chromosome seven. In some cases, a polysomia was identified that contained more than 2N chromosomes in diploid, normal cells and when more than two spots were observed for chromosome seven within tumour cells. The mean number of *EGFR* spots, which reflected the number of *EGFR* copies was evaluated in 100 tumour cells (HPF x 60 Zeiss Axiophot). Amplified tumours were those with a ratio of the mean number of *EGFR* copies or silver dots versus the mean number of chromosome 7 ≥ 2 .

In addition, a qualitative pattern of silver spot distribution within *EGFR*-amplified tumour cells was recorded as follows: (i) dense clusters of silver dots within the nucleus, (ii) loose nuclear distribution of variable amounts of distinct, silver, small or tiny dots and (iii) a straight or curved linear arrangement of silver spots within the cytoplasm but close to the nuclear membrane that produced the 'double minute' pattern that was observed in glioblastomas (Vogt *et al*, 2004; Gibaud *et al*, 2010).

Analysis of gene alterations

DNA extraction: All analyses were performed on formalin-fixed, paraffin-embedded blocks. The first stage consisted in dewaxing by Microclearing (DiaPath, Martingo, Italy) followed by slow-tissue rehydration in successive ethanol soakings from 100 to 70%. The tumour area was then scraped with a lancet blade and collected in tubes containing 40 μ l of a mixture of Pronase (Roche, Mannheim, Germany), Tris Plug, SDS and CaCl_2 . The tubes were then incubated with endo and exopeptidases overnight at 55 °C. DNA was purified by automated extraction on EVO75 (Tecan, Mannedorf, Switzerland) according to the M&N protocol NR (NucleoSpin 96 Blood; NucleoSpin 8 Viruses Binding Strips). Elution was carried out in bovine serum albumin at 500 μ g ml⁻¹, and the DNA was stored at -20 °C.

High resolution melting analysis and DNA sequencing: Our laboratory routinely uses qPCR-HRM as a pre-screening tool, which prevents useless sequencing of wild-type DNA. The choice of primers, concentrations, temperature and time of reaction were adapted to each set according to MIQE recommendations (Bustin *et al*, 2009). The negative predictive value of qPCR-HRM was validated on 300 samples for each set of primers/exon. The analytical chain was accredited according to the Iso-Norm 15 189 (Guzel and Guner, 2009; Yanikkaya-Demirel, 2009), the sample and mix (HRM Master Mix, Roche) distribution were automated and performed in 96-well plates (Evo75, Tecan), and reactions were performed in duplicate. Mutated reference samples (cell lines), wild-type DNA (placenta), dewaxing, extraction, and PCR negative controls were included with the samples to be analysed.

The PCR was then carried out on a LightCycler 480 (Roche) and the samples dissociation profiles were compared with those of reference samples. The samples were then classified as 'wild type' or 'not wild type' using the gene-scanning software (Roche). The exponential phase of qPCR-HRM allowed for the quantification of the initial DNA and detection of the presence of PCR inhibitors. Only the samples defined as 'not wild type' by qPCR-HRM were

sequenced by the Sanger method (Sanger *et al*, 1977) (MixBigDye 5X, Roche) after DNA purification (ExoSap-IT) and automated on an Evo75, (Tecan). The sequences were analysed using a 3500 or 3130 Dx Genetic Analyser (Applied Biosystems, St. Aubin, France). The search for mutations in exon 9 of the gene *PI3KCA* was performed after removal of a pseudogene that was localised on chromosome 22 and was similar to exons/introns 9–13.

Quantitative qPCR: qPCR was developed following the MIQE recommendations for RT-PCR dosage (Bustin *et al*, 2009). The copy number was normalised to a gene located in the same chromosome, beta-actin (*ACTB*, 7p22.1), and a gene located on another chromosome (glyceraldehyde-3-phosphate dehydrogenase or *GAPDH*, 12p13.31). This strategy has been used in previous studies (Lamy *et al*, 2006; Lamy *et al*, 2011) and allowed us to distinguish real changes in the copy number variation from aneusomy. The qPCR reaction conditions were adapted to each primer-probe set. Each well contained 18 μ l of mix prepared with the Mix 2X LightCycler 480 Honest Master, 10 μ M forward and reverse primers, 2.5 μ M fluorescent probe and sterile water. Two microliters of sample DNA were added to each well in triplicate.

The PCR plate also contained a genomic DNA standard scale (Roche) containing 12 000, 1200, 120 and 12 gene copies. The standard scale allowed for the conversion of the results obtained in Cq (quantitative cycle) into amplicon copy numbers for each gene by integrating the efficiency of the reaction to each reaction. One DNA that was known to have an amplified *EGFR* gene was used as a positive control (*EGFR/ACTB* = 12.67 \pm 2.31) and human placental DNA was used as a normal control (*EGFR/ACTB* = 0.55 \pm 0.06). The theoretical decision threshold of gene amplification for a sample was *EGFR*/beta-actin ($n = 2$). Below this threshold, the sample was considered 'wild type,' and above this threshold, the sample was considered 'amplified.'

Statistical analysis. The associations between qualitative variables was assessed using a χ^2 -test (or a Fisher exact test when appropriate). A two-sided value of $P < 0.05$ was considered statistically significant.

RESULTS

EGFR overexpression and amplification in TMA (cores of TNBC)

IHC. By TMA, 159 out of 915 sporadic breast carcinomas were *EGFR*-positive (17%) and included a majority of TNBC ($n = 97$) and non-TNBC ($n = 62$). With regards to TNBC ($n = 114$ out of 915), which is strictly defined by a lack of HER-2 amplification (ISH or qPCR) and completely negative ER and PR immunoperoxidation, 97 out of 114 were anti-*EGFR*-positive (85%) (Figure 1). In contrast, *EGFR*-positive, non-TNBC ($n = 62$ out of 801; 6.4%) accounted for 19 TNBC-like (19 out of 170; 11%) and 45 'not otherwise specified,' high-grade ductal carcinomas (45 out of 277; 13%).

In situ hybridisation. Among the 114 TNBC of the 159 cases, 92% (105 out of 114) were positive for *EGFR*-SISH, which was similar to the ratio (87%) that was observed in large sections (Table 1).

***EGFR* gene amplification/qPCR.** The evaluation of *EGFR* gene amplification could not be assessed by TMA for the cores of the 114 TNBC that were included in our 915 series of sporadic breast carcinomas. The small sizes of the tumour cores measuring 0.6 mm in diameter (and 4 μ m-thick sections) were not appropriate for qPCR.

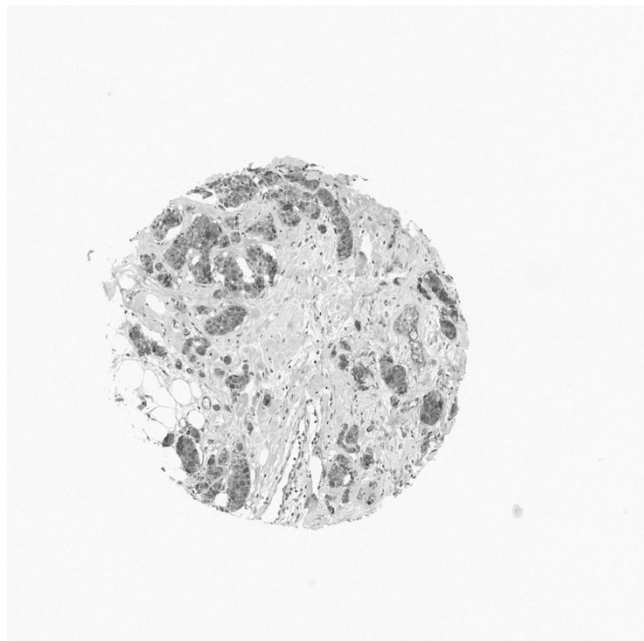


Figure 1. Positive anti-EGFR immunohistochemical reaction in TMA spot of ductal triple negative breast carcinoma.

Correlation between SISH/qPCR/IHC in large sections. In current clinical practice, diagnostic ISH is a cost-effective procedure that is presently recommended for *HER-2* amplification evaluation and requires a small amount of formalin paraffin-embedded tissue that is appropriate to the expertise of the pathologist. We therefore correlated the SISH to qPCR and IHC data.

qPCR versus SISH: The levels of amplification in large sections varied from 2 to 17 copies. In two tumours, the low density of the tumour cells was not sufficient for appropriate measurements. Amplified tumours accounted for 29 out of 45 (64%), and the mean amplification level was 5.6 (0th: 2.0; 25th: 3.1; 50th: 4.7; 75th: 6.2; and 100th: 17). Ninety-one per cent (21 out of 23) TNBC had an increased number of copies of the *EGFR* gene, which was significantly ($P < 0.010$) more than 29% that was observed for TNBC-like (8 out of 22) (Table 1).

Amplification through SISH was observed in 29 out of 47 (62%) tumours, whereas 18 out of 47 were negative. TNBC (21 out of 24 = 87%) were significantly more SISH-positive than TNBC-like tumours (8 out of 23 = 29%) ($P < 0.0005$) (Table 1). A significant correlation was observed between the SISH and qPCR data ($P < 0.0001$). Among the 29 out of 45 EGFR-SISH-positive cases, 23 were also qPCR amplified and six were non-amplified. Among the 17 out of 45 EGFR-SISH-negative, 16 were also qPCR non-amplified, resulting in 75.6% sensitivity and 94% specificity for qPCR when compared with ISH. All qPCR-positive samples were IHC- and SISH-positive.

In large sections, SISH was positive in nine cases that had strong reactions of black dot clustering within the cell nucleus (Figure 2), and in 13 cases several dots were loosely distributed throughout the nucleus (Figures 3 and 4). In 4 out of 24 (18%) of the cases, black dots were distributed as short lines or curves in the cell cytoplasm near the nuclear membrane (Figure 5), which produced a 'double minute' pattern, as described in brain cancer gliomas (Vogt *et al*, 2004; Gibaud *et al*, 2010). The DNA molecules (DMs) were intrachromosomal.

IHC versus SISH: EGFR immunopositivity in the entire series of TNBC and TNBC-like large-tissue sections was observed in 67%

Table 1. EGFR amplification evaluated by SISH, IHC and QPCR

Tumour type	No. of cases	P-value
Large sections:		
TNBC	24	
TNBC-like	23	
Total	47	
TMA: TNBC	114	
IHC		
Large sections:		
TNBC	23 (96%)	<0.0001
TNBC-like	9 (39%)	
Total	32 (67%)	
TMA: TNBC	97 (85%)	
SISH		
Large sections:		
TNBC	21 (87%)	<0.0005
TNBC-like	8 (33%)	
Total	29 (62%)	
TMA: TNBC	105 (92%)	
QPCR		
Large sections:		
TNBC	21 (91%)	<0.0103
TNBC-like	8 (29%)	
Total	29 (64%)	
TMA: TNBC	NT	
Minute		
Large sections:		
TNBC	4 (18%)	
TNBC-like	0	
Total	4 (18%)	
TMA: TNBC	16 (14%)	

Abbreviations: IHC = immunohistochemistry; SISH = silver *in situ* hybridisation; TMA = tissue microarray; TNBC = triple negative breast carcinomas. All data are presented as n (%).

(32 out of 47) of the tumours that were evaluated, and the samples had a significantly ($P < 0.0001$) greater (23 out of 24) EGFR-positive subset in TNBC when compared with TNBC-like that accounted for 9 out of 23 tumours (Table 1). Positive staining (Figure 6) was distributed throughout the cell cytoplasm with enhancements in the cell membrane, but were not found within the nuclei.

Among the 29 out of 47 large-sections, EGFR-SISH-positive cases, 28 were also IHC-positive and one was IHC-negative. Furthermore, among the 18 out of 47 negative cases, 14 were also IHC-negative, which resulted in 78% specificity and 97% sensitivity of the IHC compared with SISH. In addition, among the 114 TNBC, 85% (97 out of 114) were EGFR-IHC-positive, and 92% (105 out of 114) were EGFR-SISH-positive by TMA. High correlations between both tests were observed, with 6 out of 114 being IHC-negative and SISH-positive, and 7 out of 114 being IHC-positive and SISH-negative.

Mutations in large sections

EGFR mutations. None of the 47 large tumour sections in the series exhibited an *EGFR* mutation within exons 18–21. Likewise, the IHC tests for the detection of exon 19 deletions and exon 21 missense mutations were negative in all the 47 cases. In addition, no positive IHC reaction using antibodies for the detection of exon

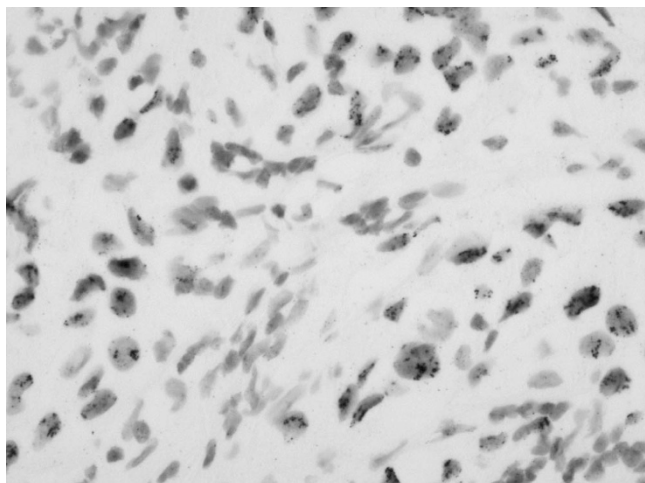


Figure 2. Positive *in situ* hybridisation with black dot clustering within the cell nucleus.

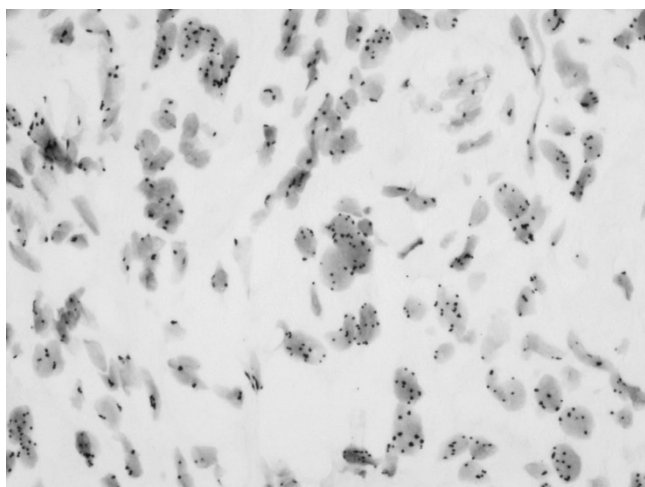


Figure 3. Positive *in situ* hybridisation appearing as separated tiny dots distributed in the nucleus of many cells.

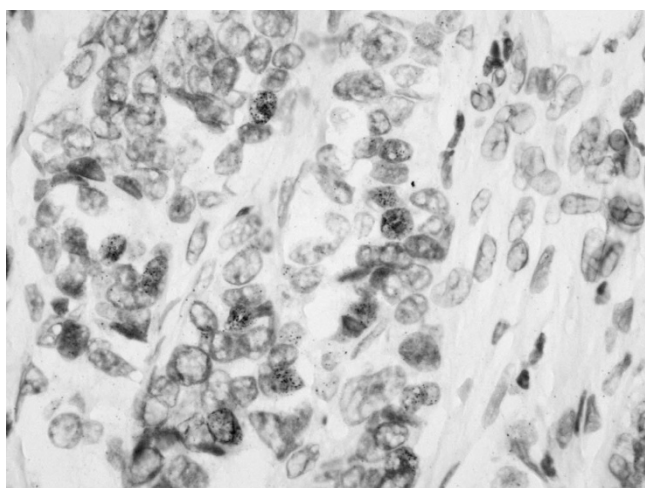


Figure 4. Positive *in situ* hybridisation within some cells in a triple negative breast carcinoma contrasting with the clustering distribution observed in Figure 2.

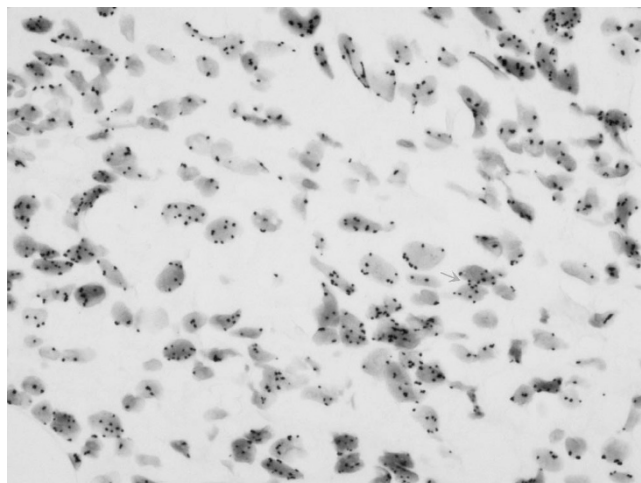


Figure 5. *In situ* hybridisation showing the double minutes chromosome (arrow) appearing as arciform pattern of positive dots within the cytoplasm close to nuclear membrane.

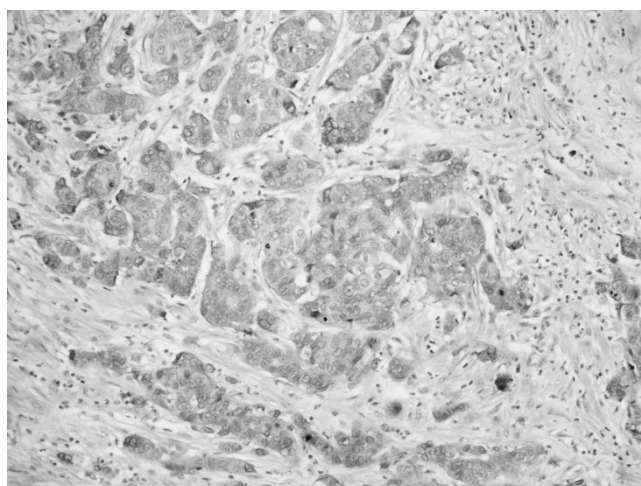


Figure 6. EGFR immunohistochemical positive expression in current large-tissue sections of triple negative breast carcinoma.

19 deletion and exon 21 mutation was observed in the 915 tumours that were included in the TMAs.

EGFR downstream pathway mutations. Among the 34 patients who were tested for mutations in the *EGFR* downstream pathway, 44% (15 out of 34) were mutated and no *Kras* mutation was observed. However, *PI3K* mutations were observed in 35% (12 out of 34) of the tumours and an equivalent proportion of mutants were found in exons 9 and 20 regardless of the gene (6 out of 6). *Braf* mutations were observed in 13.8% (4 out of 29) of cases and *HER-2* mutations were detected in 5.9% cases (2 out of 34). One patient had concomitant mutations in *BRAF* and *PI3K*, and another one had mutations for *BRAF*, *PI3K* and *HER-2*. No significant differences were observed for gene or mutation type between TNBC-like or TNBC (Figure 7).

ALK translocation. None of the 47 TNBC large sections or any of the 114 TNBC by TMA were ALK-positive using the IHC anti-translocated ALK, which was in contrast to the positive control, NSCLC.

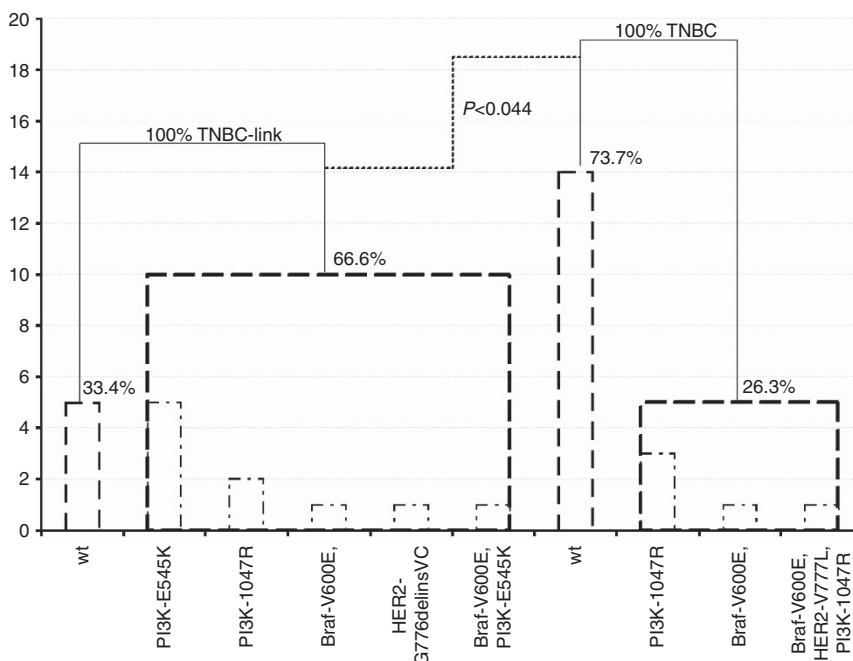


Figure 7. *PI3K* mutations were observed in 35.3% (12/34) tumours. Overall exons 9 and 20, 10 *PI3K* mutations are exclusive of all other mutations (5/exon 9 and 5/exon 20), seven are found in TNBC-like with E545K mutation. H1047R mutations are distributed between both the tumour profiles. *Braf* mutations were observed in 13.8% (4/29) cases, two are exclusive of all other mutations and are distributed between both tumour profiles. *HER-2* mutations were detected in 5.9% cases (2/34), one is exclusive of all other mutations and is found in TNBC-like. One classified TNBC-like patient has concomitant mutations for *Braf* and *PI3K* and another one for *Braf*, *PI3K* and *HER-2*. In total 66.6% (10/15) of TNBC-like are significantly ($P < 0.044$) mutated compared with 26.3% (5/19) of the TNBC. No *EGFR* and *Kras* mutation was detected. wt: Wild type; Association between qualitative variables was assessed with Fisher's exact test.

DISCUSSION

TNBC accounts for 10–20% of all BCs (Thike *et al*, 2010; De Ruijter *et al*, 2011; Teng *et al*, 2011). Accordingly, in our TMA breast cancer set that was tested for *EGFR*-IHC overexpression and *EGFR*-SISH amplification, TNBC accounted for 114 cases (12.5%). Among these 114 TNBC, 105 out of 114 (92%) were found to be *EGFR*-amplified by SISH and 21 out of 24 (87%) of the large sections were found to be amplified. Our results significantly differed from the literature data in which *EGFR* amplification was found in 30–52% (Nielsen *et al*, 2004; Corkery *et al*, 2009; Gumuskaya *et al*, 2010; Lamy *et al*, 2011) of TNBC and in 56–84% of basal-cell breast carcinomas (Da Silva *et al*, 2007).

The discrepancies likely result from variations in the criteria that were used for defining TNBC. In most studies, a different cut-off for ER receptor positivity is used. However, in our study, we used strict diagnosis criteria to define TNBC, and only TN tumours that were completely negative for ER and PR (i.e., with no positive cells) were considered TNBC and were included. Furthermore, the fact that only 29% ($n = 8$ out of 23) of the TN-like tumours in our large sections set were *EGFR*-amplified (versus 92% of properly selected TNBC with 0% of ER-positive cells), does support variations in *EGFR* amplification rates according to the cut-off of ER positivity. Finally, as recommended in the guidelines for ISH that were used for interpreting SISH or CISH for *HER-2*-negative tumours, only tumours with a ratio between the number of spots (copies) and the number of chromosome seven of less than two, were considered as non-amplified, discarding possible false-positive cases due to polysomia in tumours that were only evaluated by IHC (Gumuskaya *et al*, 2010).

Using such strict criteria, we obtained an excellent correlation between the positive IHC staining and SISH in TMA and large sections. As expected, this result suggests that, as was currently

performed for *HER-2*, both methods could be combined for *EGFR* status evaluation in clinical practice. Likewise, the sensitivity and cost efficiency of IHC could serve as the initial tumour screening, and the specificity of SISH could serve to exactly evaluate the degree of amplification and discard polysomia.

Interestingly, we also obtained good correlation in large sections between SISH and qPCR that definitively validated our results and the high frequency of *EGFR* amplification and overexpression in TNBC. The results also indicated that ISH could be considered a cost-effective procedure for *EGFR* amplification evaluation, as was recently assessed for *HER-2*. Bhargava *et al*, reported similar data in a series of 175 TNBC (Bhargava *et al*, 2005). As already acknowledged, discrepant data for *HER-2* ISH, which was reported in the literature concerning rates of *EGFR*-amplified carcinomas was likely a result of the lack of standardisation and variations of ISH methods, such as the use of various kits, procedures and cut-offs (Reis-Filho *et al*, 2006, Ryden *et al*, 2010). However, our results deserve more studies involving additional series.

In clinical practice, ISH, as used for *HER-2* status evaluation, is endowed with significant advantages over qPCR because it can be more rapidly assessed within diagnostic time concomitantly to hormone receptor evaluation in consecutive sections of the tissue; thus, it can be perfectly controlled by the pathologist, who can exclude inappropriate tissue areas such as necrosis, insufficient density of tumour cells and fixation defects. In this regard, it is also noteworthy that all of the tests were performed using paraffin sections of archived blocks that were stored in the libraries of pathology laboratories at room temperature after diagnosis, and are much easier to control and handle than frozen samples.

The clonal and mutational spectrum of primary TNBC has recently been reported (Shah *et al*, 2012). In our study on TNBC, we did not observe, as others have (Lynch *et al*, 2004, Bhargava *et al*, 2005), the *EGFR* mutations in NSCLC in large sections or in TMA using anti-43B2 or anti-6B6 monoclonal antibodies.

However, a recent report (Teng *et al*, 2011) has shown that 11.4% (70 out of 653) of exon 19 deletion or missense exon 21 mutations affected Asian populations more than Caucasian populations in a series of 653 TNBC (Jacot *et al*, 2011; Teng *et al*, 2011). Most literature reports, such as ours, suggest that Caucasian patients with TNBC who lack *EGFR* mutations but have an *EGFR* amplification accompanied by *EGFR* overexpression, could potentially benefit from anti-*EGFR* treatment (such as cetuximab), as reported in some preliminary clinical trials (O'Shaughnessy *et al*, 2011; Carey *et al*, 2012). Nevertheless, further studies are required, particularly those including an accurate selection of patients with true TNBC and *EGFR* amplification that properly predict the potential response of this disease to *EGFR*-targeted therapy.

Anti-*EGFR* therapy of patients with *EGFR*-amplified glioblastomas has been reported to not significantly improve patient survival (Vogt *et al*, 2004; Gibaud *et al*, 2010). In addition, *EGFR*-amplified glioblastomas, in contrast to TNBC, do not often concomitantly overexpress the *EGFR* protein, which could explain the lack of an anti-*EGFR* response when using antibody therapies. Furthermore, those drugs fail to reach their target in the brain (Vogt *et al*, 2010).

Mutations in downstream *EGFR* signalling pathways have been reported to be responsible for the resistance of TNBC to *EGFR* therapies (Martin *et al*, 2012). We show that 44% of TNBC were mutated for *PI3K*, *Braf*, or *Her-2*. *PI3K* mutations have been reported in 25% of breast carcinomas (Martin *et al*, 2012), and we found that 35% of *PI3K* mutations could also explain the failure of patient response to *EGFR*-targeted therapy. The effects of *EGFR* activation of the *MAP kinase* and *PI3Kinase* (AKT-mTOR) signalling pathways can be reduced by inhibitors of these molecules, particularly rapamycin, which is an inhibitor of mTOR. Several ongoing trials are investigating patient's responses to mTOR inhibitors (CCI-779, RAD001, AP 235732) (Macaskill *et al*, 2011). In addition, promising preclinical studies concerning breast cancer cell lines and animal xenografts have recently been reported (Brachmann *et al*, 2009).

In our study of 4 out of 24 large sections (18%) and 16 out of 114 TMA spots (14%), we observed a particular pattern of *EGFR* copy distribution in SISH, as was previously reported in some human tumours such as gliomas or lung cancer, and the amplified sequences were generally localised to DMs (Vogt *et al*, 2004; Gibaud *et al*, 2010). Up to 50% of glioblastomas exhibit *EGFR* amplification with small, acentric, circular, extrachromosomal DNA molecules, which are referred to as DMs and contain from a few hundred kilobases to megabases, and are autonomously replicating DNA fragments. DMs may confer a proliferative advantage to cells, such as by carrying amplified oncogenes or drug-resistance genes (Mondello *et al*, 2010). This accumulation of extrachromosomal DMs has also been observed in some haematological malignancies (Vogt *et al*, 2004; Gibaud *et al*, 2010). We report for the first time, such DMs of *EGFR*-amplified DNA in breast carcinomas and, more specifically, in TNBC.

The *EML4-ALK* fusion gene has been identified as an oncogene in 11.3% of non-small cell lung cancers (NSCLC) and 24% of colorectal carcinomas. *ALK* translocation has also been described in some breast carcinomas (2.4%) (Lin *et al*, 2009). This suggests that *ALK* kinase inhibitors (such as crizotinib) may represent effective treatments for patients whose tumours contain the *EML4-ALK* fusion. Unfortunately, our results showed no TNBC-positive staining with the recently commercially available anti-*ALK* antibody that was used to screen patients with NLCLC for *ALK* inhibitor therapies. Potential false-negative results due to IHC bias were controlled (1) by a positive immunoreaction of NSCLC tumour samples that were fixed, paraffin-embedded, and immunostained according to the same conditions and procedures as that of our breast cancer samples, and (2) by the correlation of ISH

results with visualisation of *EML4-ALK* translocation through FISH in IHC-positive NSCLC.

In conclusion, our study shows that in TNBC, *EGFR* is frequently amplified without mutation but is overexpressed, suggesting that antibody anti-*EGFR* therapy could be a potential therapy for this aggressive breast cancer subset, provided that the tumours are properly characterised and that clinical trials further prove its benefit for appropriately selected patients. Although preliminary reports of preclinical studies have shown that anti-*EGFR* treatment in amplified tumours does not significantly reduce the progression of breast carcinomas, more extensive studies are required that will accurately evaluate *EGFR* amplification by ISH and *EGFR* overexpression by IHC in TNBC. As shown in our study, ISH is a sensitive and specific procedure for the selection of patients for anti-*EGFR* treatment and could be used as a cost-effective screening method that would be suitable in clinical practice, as it is for *HER-2*. In addition, we showed that *PI3K* and *Braf* mutations might be associated with *EGFR* amplification and overexpression, suggesting that combined therapies (vemurafenib) could significantly reduce tumour progression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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