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Molecular characterization of EGFR and EGFR-downstream pathways in triple negative breast carcinomas with basal like features

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Summary. Aims: Triple negative breast cancer with basal like features (TN-BCBL) do not benefit from hormonal and anti-HER2 therapies. As a considerable fraction of TN-BCBLs shows EGFR deregulation, EGFR-targeted therapies have been proposed as an option. The characterization of EGFR and EGFR-downstream members may therefore provide important predictive information. Methods and results: Based on morphological and immunophenotypic features, we identified 38 TN-BCBLs that were subsequently investigated for alterations in EGFR signaling pathways. EGFR and PTEN protein levels were studied by immunohistochemistry, *EGFR* gene status by FISH, *EGFR, H-Ras, K-Ras, N-Ras, BRAF* and *PIK3CA* gene mutations by direct sequencing. EGFR overexpression and loss of PTEN expression characterized the majority of TN-BCBLs (76% and 74% of patients, respectively). *EGFR* gene copy number gain (FISH+) was identified in 51% of analyzable patients. *PIK3CA* gene mutations were detected in three cases (8%), whereas *EGFR, H-Ras, K-Ras, N-Ras* and *BRAF* genes showed no mutations. Overall, out of 17 patients classified as FISH+, 12 cases (70%) showed a concomitant alteration in PI3K/PTEN pathway. Conclusions: These results provide evidence that the efficacy of anti-EGFR drugs in TN-BCBL patients could be impaired by frequent alterations in the PI3K/PTEN axis, and suggest that TN-BCBLs could benefit from tailored treatments against this axis.

Key words: Triple negative, Breast cancers, Basal like features, EGFR-targeted therapy, PTEN, EGFR, Fluorescent in situ hybridization

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Introduction

Triple negative breast cancer with basal like features (TN-BCBL) is a pathological entity, recently defined through molecular profiling as similar to that of the basal/myoepithelial cells of the breast (Sørlie et al., 2001, 2003) and characterized by aggressive clinical behavior and poor prognosis (Nielsen et al., 2004; Fulford et al., 2006; Kim et al., 2006; Rodríguez-Pinilla et al., 2006; Cheang et al., 2008; Rakha et al., 2008). Besides their molecular signature, TN-BCBLs can be fairly characterized by several morphological and immunohistochemical features. In particular, at the histological level, TN-BCBLs show a pushing border of invasion, central geographic necrosis, a prominent stromal lymphocytic response, high tumor grade, marked cellular pleomorphism, high nuclear-cytoplasmic ratio, high mitotic index, frequent apoptotic cells, and scant stromal content (Fulford et al., 2006; Cheang et al., 2008). At the protein level, TN-BCBLs strongly express high molecular weight cytokeratins (e.g. CK5/6, CK14 and CK17), P-cadherin, S-100 protein, p63, c-kit, and vimentin. Most importantly, TN-BCBLs are characterized by the absence of ER, PR expression and HER2 overexpression (Rakha and Reis-Filho, 2009) rendering hormonal therapy and targeted therapy with an anti-HER2 monoclonal antibody ineffective.

As a considerable fraction of TN-BCBLs shows EGFR deregulation, both at the protein (47-57%) and gene levels (Nielsen et al., 2004; Rakha et al., 2006; Reis-Filho et al., 2006; Pintens et al., 2009), the administration of EGFR-targeted therapies has been proposed for patients affected by TN-BCBL, similarly to those affected by advanced non-small-cell lung cancer (NSCLC) or by metastatic colorectal cancer (CRC), where these drugs have efficiently entered into clinical

practice (Campos, 2008; Ciardiello and Tortora, 2008). Due to recent investigations in advanced NSCLC and CRC showing that the deregulation of EGFR and of its downstream pathways may impair the efficacy of EGFR-targeted drugs (Siena et al., 2009), the evaluation of this particular and so far poorly characterized genetic profile is warranted before drug administration in TN-BCBL patients as well. In this study we therefore performed an integrative analysis of EGFR and of EGFR-downstream pathways members (*H-Ras, K-Ras, N-Ras, BRAF, PIK3CA* and PTEN) in a cohort of TN-BCBL patients.

Materials and methods

Patients

The studies were approved by our Institutional Ethical Committee and included 38 consecutive patients with TN-BCBL, and formalin-fixed paraffin-embedded (FFPE) archival material available for immunohistochemical, cytogenetic and molecular analyses. All patients were women who underwent surgery for breast cancer in Ticino, Switzerland, between 2003 and 2007, without preoperative treatment. Informed consent was obtained. Age at diagnosis ranged from 31 to 89 years, and 13 patients (34%) were younger than fifty years. The clinical-pathological features are detailed in Table 1.

All samples were selected for the following criteria: a proficient histological profile, absence of ER and PR expression (defined as no expression, 0%), absence of HER2 overexpression as detected by immunohistochemistry (IHC, i.e. weak and incomplete membrane staining in less than 20% of cells, score 0) and absence of *HER2* gene amplification as detected by fluorescent in situ hybridization (FISH, ratio *HER2* gene versus chromosome 17 centromere less than 2) and a CK5/6 expression at least focally (more than 5% of the tumor cells).

Tumor size ranged from 0.7 to 8.5 cm. Tumor stage was classified as T1 in 15 cases (39%), T2 in 21 cases (55%), T3 in 1 case (3%) and T4 in 1 case (3%). The vast majority of cases (n=36, 95%) were grade (G)3 whereas only 2 cases (5%) were G2.

Immunohistochemical analysis

Immunohistochemical analyses were performed on 3 μm thick tissue sections using a Benchmark automatic immunostaining device (Ventana Medical System, Tucson, AZ, USA). According to the manufacturer's instructions, specimens were incubated after heat induced antigen retrieval with the specific antibody. Positive and negative controls were included in each slide run. Staining evaluations were performed by two independent pathologists (SC and LM).

EGFR protein expression was evaluated by the EGFR PharmDX kit (DakoCytomation, Carpinteria, CA, USA). As positive and negative controls, we used those included in the kit (the colon carcinoma HT29 and the breast carcinoma CAMA-1 cell line, respectively). The reaction was classified as score 0, 1+, 2+ or 3+ on the basis of the percentage of positive cells (0%, <5%, 5-50% and ≥50% of cells, respectively), according to the manufacturer's instructions and our previous experience (Frattini et al., 2007).

PTEN protein expression analysis was performed using the anti-PTEN Ab4 (Neomarkers, Fremont, CA, USA) at a 1:200 dilution as previously reported (Frattini et al., 2007; Molinari et al., 2009). Healthy normal tissue was used as the internal positive control, and normal endometrium was used as the external positive control. PTEN staining intensity scores for invasive tumor and not-neoplastic cells were recorded as described in the literature (Saal et al., 2005) and on the basis of our experience (Frattini et al., 2007). PTEN protein expression was mainly detected at the cytoplasmic level, while very few cases also showed nuclear positivity.

Fluorescent in situ hybridization

EGFR gene status evaluation was performed using the dual color FISH assay LSI EGFR/CEP7 (Abbott Molecular, AG, Baar, Switzerland) on 3 μm thick FFPE tissue sections, according to the manufacturer's instructions and our previous work (Frattini et al., 2007).

Evaluation of FISH signals was performed by two independent operators (VM and FB), providing superimposable results in all cases. Patients were classified using descriptive criteria, taking into account the abnormalities revealed and the percentage of cells involved. In particular, patients showing two copies of chromosome 7 in >50% of tumor cells were classified as

Table 1. Clinical-pathological features of TN-BCBLs resected in Ticino (Switzerland) from 2003 and 2007.

Age at diagnosis		
≥50 y	25	65.8%
<50 y	13	34.2%
tot	38	100.0%
Tumor Stage		
T1	15	39.5%
T2	21	55.3%
T3	1	2.6%
T4	1	2.6%
tot	38	100.0%
Tumor Grade		
G2	2	5.3%
G3	36	94.7%
CK 5/6		
negative	0	0.0%
weak	7	18.4%
moderate	17	44.7%
strong	14	36.8%
tot	38	100.0%

G: grade; y: year.

disomic; patients with 3-4 copies or ≥4 copies of chromosome 7 in ≥40% of cells were classified as low polysomic or high polysomic, respectively; patients with a ratio *EGFR* gene/chromosome 7 centromere greater than 2 in ≥10% of cells were classified as *EGFR* amplified.

Using a FISH stratification criteria that was slightly modified from those defined in NSCLC (Varella-Garcia et al., 2009), patients carrying a high polysomic profile or gene amplification were grouped into a FISH positive class (FISH+); conversely patients revealing low polysomy or disomy of chromosome 7 were classified as FISH negative (FISH-) (Varella-Garcia, 2006; Martin et al., 2009).

A minimum of 100 morphology-clear, non-overlapping nuclei from 10 different areas were scored for each patient.

Molecular analysis

Genomic DNA was extracted from a single representative FFPE tissue block (containing ≥70% of neoplastic cells) using the QIAamp Mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Recently published criteria for block and area selection were applied (van Krieken et al., 2008). We searched for *EGFR* (exons 18-21), *K-Ras*, *H-Ras*, *N-Ras* (exons 2 and 3), *BRAF* (exon 15) and for *PIK3CA* (exons 9 and 20) mutations by direct sequencing. (www.sanger.ac.uk/genetics/CGP/cosmic). The vast majority of mutations occur in these regions. PCR conditions were previously reported (Daniotti et al.; 2004; Pao et al., 2005; Frattini et al., 2007; Sartore-Bianchi et al., 2009). The list of primers used for mutational analyses is available in Table 2. All samples were subjected to automated sequencing by ABI PRISM 3130 (Applied Biosystems, Foster City, CA, USA), and the data were analyzed with Sequencing Navigator Software (Applied Biosystems). All mutated cases were confirmed at least twice starting from independent PCR reactions.

Statistical analysis

The two-tailed Fisher's exact test was used to calculate p values for the association between clinical-pathological and molecular data. The level of significance was set at p=0.05.

Results

EGFR and PTEN IHC

Table 3 summarizes the immunohistochemical, genetic and molecular data.

EGFR protein overexpression was detected in 29 patients (76%). Nine patients (24%) were classified as score 1+, 13 patients (34%) as score 2+ and 7 (18%) as score 3+. Nine patients (24%) did not show EGFR expression and were therefore classified as score 0 (See Figure 1).

PTEN protein expression was documented as normal in 10 patients (26%), whereas loss of PTEN was found in 28 patients (74%) (See Figure 2).

No significant correlation between EGFR overexpression and PTEN loss of expression was revealed (p=0.32, Fisher's exact test).

EGFR FISH

FISH analysis was successful in 33 cases, and failed in 5 cases due to lack of material or poor hybridization

Table 2. List of primers used for mutational analyses.

Gene	Exon	Forward primer	Reverse primer
EGFR	18	CAAATGAGCTGGCAGTGCCGTGTC	GAGTTTCCCAAACACTCAGTGAAAC
	18	CAAGTGCCGTGTCCTGGCACCCAAGC	CCAAACACTCAGTGAAACAAAGAG
	19	GCAATATCAGCCTTAGGTGCGGTC	CATAGAAAGTGAACATTTAGGATGTG
	19	CCTTAGGTGCGGCTCCACAGC	CATTTAGGATGTGGAGATGAGC
	20	CCATGAGTACGTATTTTGAAACTC	CATATCCCCATGGCAAACTCTTGC
	20	GAAACTCAAGATCGCATTCATGC	GCAAACTCTTGCTATCCCAGGAG
	21	CTAACGTTCGCCAGCCATAAGTCC	GCTGCGAGCTCACCCAGAATGTCTGG
	21	CAGCCATAAGTCCTCGACGTGG	CATCCTCCCCTGCATGTGTTAAAC
K-Ras	2	TGGTGGAGTATTTGATAGTGTA	CATGAAAATGGTCAGAGAA
	3	GGTGCACTGTAATAATCCAGAC	TGATTTAGTATTATTTATGGC
H-Ras	2	ATGACGGAATATAAGCTGGT	CTCTATAGTTGGGGTCGTATT
	3	GATTCCTACCGGAAGCAGGTG	CTGTACTGGTGGATGTCCTC
N-Ras	2	ATGACTGAGTACAAACTGGT	CTCTATGGTGGGATCATATT
	3	TCTTACAGAAAACAAGTGGT	GTAGAGGTTAATATCCGCAA
BRAF	15	TCATAATGCTTGCTCTGATAGGA	GGCCAAAAATTTAATCAGTGGA
PIK3CA	9	GGGAAAAATATGACAAAGAAAGC	CTGAGATCAGCCAAATTCAGTT
	20	CTCAATGATGCTTGGCTCTG	TGGAATCCAGAGTGAGCTTTC

signals. *EGFR* gene amplification was revealed in 8 patients (24%); high polysomy of chromosome 7 was observed in 9 patients (27%); low polysomy of chromosome 7 was detected in 11 patients (33%); absence of any *EGFR* gene or chromosome 7 alterations was observed in 5 patients (15%). Grouping FISH results according to the stratification criteria, we considered 17 patients (51%) as FISH+ and 16 patients (49%) as FISH-. Representative examples of *EGFR* FISH profiles are shown in Figure 3.

EGFR FISH and IHC and correlation

We observed no significant correlation between

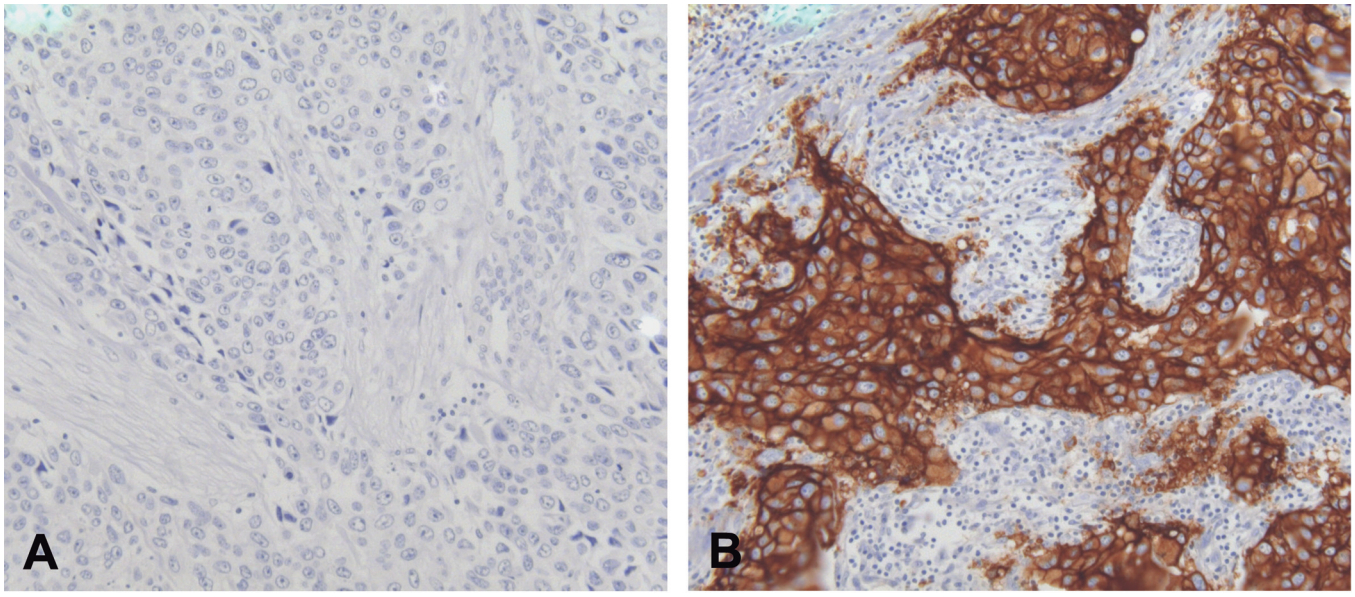

Fig. 1. Representative EGFR IHC staining in TN-BCBLs showing score 0 **(A)**, and score 3+ **(B)**. x 200

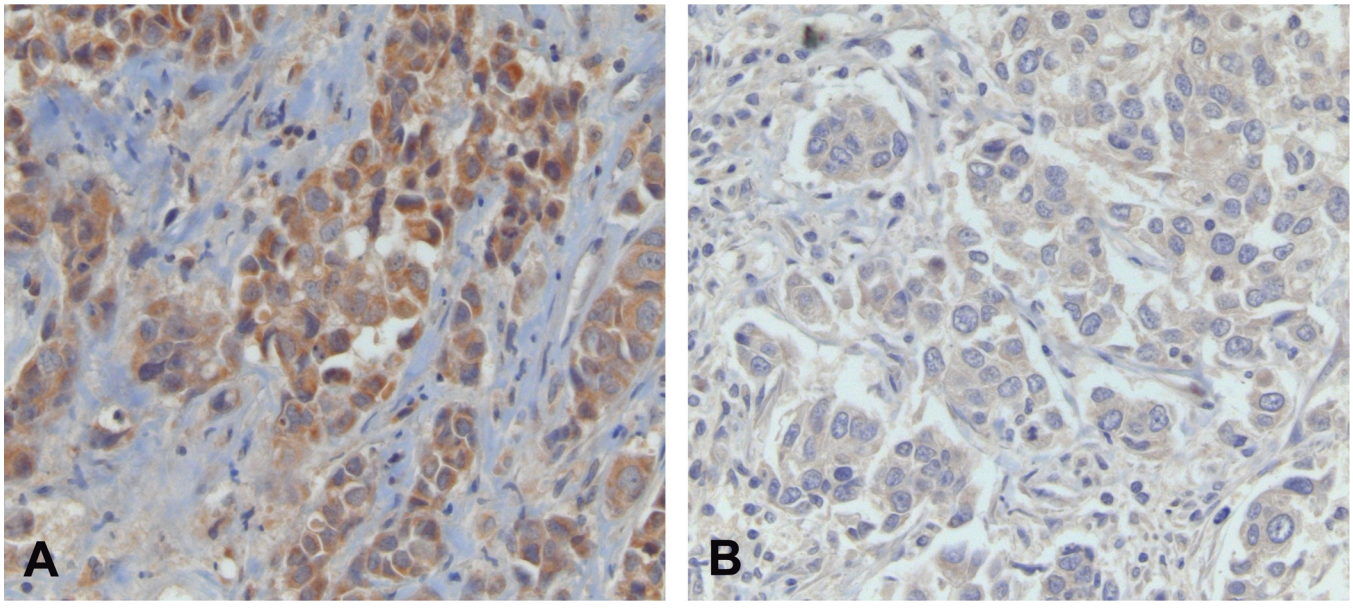

Fig. 2. Representative PTEN IHC staining in TN-BCBLs showing normal expression of PTEN **(A)**, and loss of PTEN expression **(B)**. x 200

EGFR gene status and EGFR protein expression (Table 4). In fact, *EGFR* gene amplification correlated with a score of 0 in 1 out of 8 cases (12%), with a score of 1+ in 3 cases (38%), with a score of 2+ in 1 case (12%), and with a score of 3+ in 3 cases (38%). Conversely, disomy correlated with a score of 2+ in 3 out of 5 cases (60%) and with a score of 0 in 2 cases (40%).

No correlation between *EGFR* gene status and PTEN loss of expression was revealed (p=0.17, Fisher's exact test)

EGFR, K-Ras, N-Ras, H-Ras, BRAF, PIK3CA sequencing

No mutations were found in *EGFR* (0 out of 35 patients, 3 cases were not analyzable), *K-Ras* (0 out of 38 cases), *N-Ras* (0 out of 38 cases), *H-Ras* (0 out of 38 cases) and *BRAF* (0 out of 38 cases) genes. Three patients out of 38 (8%) showed a mutation in exon 20 of *PIK3CA*. In one patient the classical change involving the second base of codon 1047 (CAT→CgT, His→Arg, H1047R) was detected; in one patient the mutation was present in the first base of codon 1049 (GGT→cGT, Gly→Arg, G1049R); in the third patient, the mutation was found in the third base of codon 1040 (ATG→ATa, Met→Ile, M1040I).

Table 3. Immunohistochemical, genetic and molecular features of TN-BCBLs.

	EGFR				
id	IHC	FISH	sequence	PTEN IHC	*PIK3CA* sequence
1	1+	LP	wt	neg	wt
2	3+	LP	wt	neg	wt
3	2+	LP	wt	neg	wt
4	0	LP	wt	neg	wt
5	2+	HP	wt	pos	wt
6	2+	2n	wt	pos	wt
7	2+	2n	wt	neg	wt
8	3+	LP	wt	neg	wt
9	1+	HP	wt	pos	wt
10	3+	HP	wt	neg	wt
11	1+	HP	wt	neg	wt
12	1+	HP	wt	neg	wt
13	1+	HP	wt	neg	wt
14	1+	A	wt	pos	wt
15	1+	A	wt	neg	wt
16	1+	A	wt	pos	wt
17	0	A	wt	neg	ex20
18	3+	A	wt	neg	wt
19	2+	n.e.	wt	neg	wt
20	2+	n.e.	wt	neg	wt
21	2+	LP	wt	neg	wt
22	0	HP	wt	neg	wt
23	0	2n	wt	pos	ex20
24	0	LP	wt	neg	wt
25	0	n.e.	wt	neg	wt
26	2+	LP	wt	neg	wt
27	2+	2n	wt	pos	wt
28	0	2n	wt	neg	wt
29	3+	n.e.	wt	pos	wt
30	3+	A	wt	pos	wt
31	1+	HP	wt	neg	wt
32	2+	LP	wt	neg	wt
33	2+	A	wt	neg	wt
34	2+	n.e.	wt	neg	wt
35	0	LP	wt	neg	wt
36	3+	A	wt	neg	wt
37	2+	LP	wt	neg	wt
38	0	HP	wt	pos	ex20

2n: disomy; A: amplification; ex: exon; HP: high polysomy; LP: low polysomy; n.e.: not evaluable; neg: PTEN loss of expression; pos: PTEN normal expression; wt: wild-type.

Deregulation of EGFR and of its downstream members

Overall, the PI3K/PTEN pathway was altered in 30 patients, with one case showing a concomitant *PIK3CA* mutation (G1049R) and a PTEN loss of expression.

Moreover, by correlating the *EGFR* gene status with alterations in the EGFR downstream pathways, we found that out of 17 patients classified as FISH+, 12 cases (70%) showed a concomitant alteration in PI3K/PTEN axis due to either PTEN loss of expression (10 cases), *PIK3CA* mutation (1 case), or both (1 case); conversely 5 out of 38 patients (13%) displayed *EGFR* copy number gain as the sole abnormality.

Molecular alterations and clinical-pathological features

We did not detect any association between alterations of EGFR and/or of its downstream members and clinical-pathological features, except for *EGFR* gene status and patients' age. In fact, *EGFR* gene amplification has never been detected in patients less than 50 years old (0 out of 10 cases), whereas it has been found in 8 out of 23 cases (34%) of older patients (p=0.03, Fisher's exact test). This correlation remains significant even using the FISH classification criteria for NSCLC (p=0.018, Fisher's exact test).

Discussion

Targeted therapies against EGFR represent new options in the treatment of several neoplastic diseases, such as advanced NSCLC and CRC (Ciardiello and Tortora, 2008). The introduction of these therapies to the management of TN-BCBL patients has been proposed as a consequence of the triple-negative phenotype, and the high frequency of EGFR deregulation, as detected by

Table 4. Correlation between EGFR protein expression (by IHC) and gene status (by FISH).

EGFR IHC	*EGFR* FISH			
	2n	LP	HP	A
0	2	3	2	1
1+	0	1	5	3
2+	3	5	1	1
3+	0	2	1	3

2n: disomy, A: amplification, HP: high polysomy, LP: low polysomy.

immunohistochemistry in these patients (Rakha et al., 2006; Reis-Filho et al., 2006; Campos, 2008; Pintens et al., 2009). We used a combined immunohistochemical, cytogenetic and molecular approach to investigate, in TN-BCBLs, the mechanisms underlying the deregulation of EGFR pathways, which in advanced neoplastic diseases such as NSCLC and CRC are strongly related to therapeutic strategies and responses (Siena et al., 2009). The results of the present study show that, based on immunohistochemical and molecular characteristics, only a small fraction of TN-BCBL patients are likely to benefit from EGFR-targeted drugs. In fact we found that only 13% of cases carry a molecular profile (*EGFR* FISH+, PTEN IHC+, EGFR-downstream members wt) adequate for EGFR-targeted therapies, a percentage in keeping with the percentage of responders in metastatic NSCLC and CRC patients treated with anti-EGFR drugs, and with the little data available on breast cancer patients. To date, in fact, the rare studies investigating the use of anti-EGFR targeted therapies in triple-negative breast cancer showed that only a few patients may benefit from these drugs (Agrawal et al., 2005; Khambata-Ford et al., 2010; www.clinicaltrial.gov).

The absence of point mutations in the *EGFR* gene sequence is indicative of different deregulation mechanisms than those occurring in NSCLC, where tyrosine kinase inhibitors against EGFR are effectively used, thus implying that similar therapies should be avoided for the management of TN-BCBL patients. On the contrary, the presence of an increased copy number of the *EGFR* gene in a considerable fraction of TN-BCBLs, suggests that the administration of monoclonal antibodies, such as cetuximab and panitumumab in these patients may be effective (Campos, 2008; Ciardiello and Tortosa, 2008). We only considered *EGFR* gene status and not EGFR protein expression due to the lack of correlation between these two evaluations (Atkins et al., 2004; Langner et al., 2004; Kersting et al., 2006) and due to the role played by *EGFR* gene status in predicting response to EGFR-targeted therapies in the treatment of colorectal cancer (Chung et al., 2005; Frattini et al., 2007; Martin et al., 2009). In this context, the analysis of EGFR-downstream members did not reveal any mutation in Ras family members (*K-Ras, N-Ras, H-Ras*) and *BRAF* genes, suggesting that MAP-kinase pathway alterations involved in TN-BCBL carcinogenesis presumably occur upstream these genes (Hoadley et al., 2007). Conversely, our results provide evidence that the PI3K/PTEN pathway is strongly involved in the development of TN-BCBL. Previous studies reported that *PIK3CA* mutations and PTEN loss of expression are deregulated with a similar frequency (around 30% each) in a consecutive series of breast cancers (Saal et al., 2005). Here, we show that in TN-BCBL the main factor responsible for PI3K pathway alterations is PTEN loss. In fact, we demonstrated PTEN loss of expression in more than 70% of TN-BCBL patients, similarly to recent reports indicating loss of PTEN expression in 50-82% of basal-like breast cancers (Saal et al., 2008; López-Knowles et al., 2010).

At odds with PTEN, *PIK3CA* mutations were detected in only a small fraction of TN-BCBL cases, confirming a recent study (Gonzalez-Angulo et al., 2009). The high frequency of PTEN loss of expression and the low occurrence of *PIK3CA* mutations in TN-BCBL could support the concept of mutual exclusivity

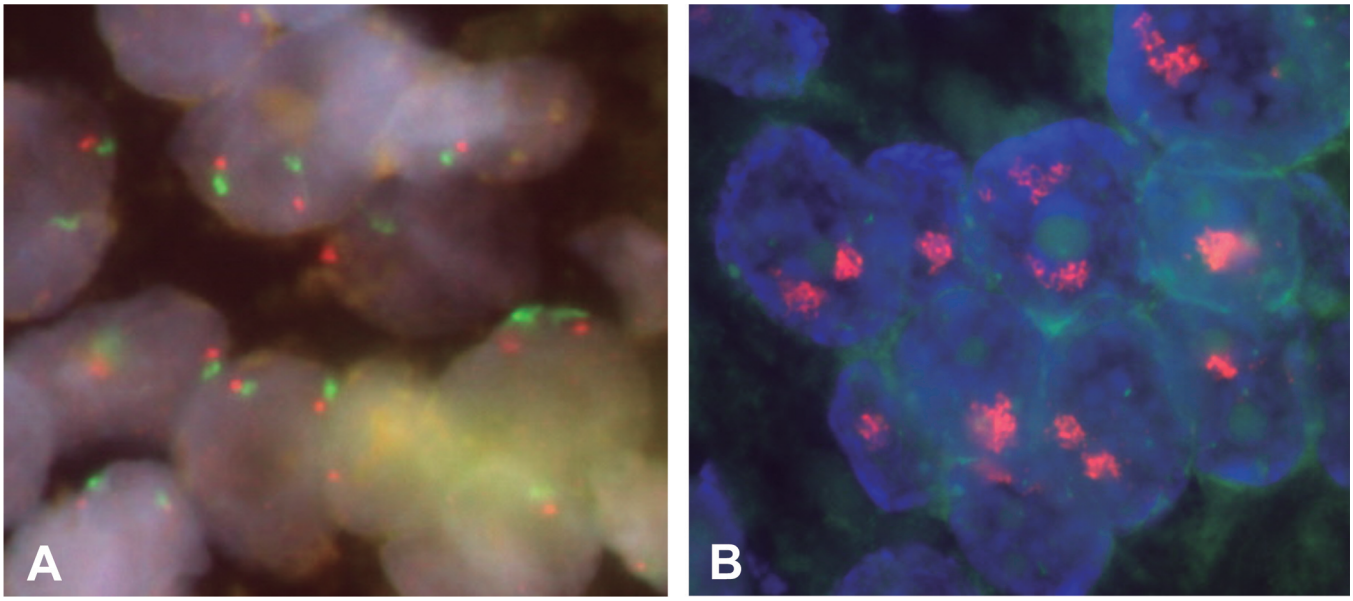

Fig. 3. *EGFR* FISH assay on TN-BCBLs. **A.** FISH negative case showing two copies of the *EGFR* gene (red signals) and disomy of chromosome 7 (green signals). **B.** FISH positive case showing *EGFR* gene amplification in cluster signals. x 1000

between these two alterations, a hypothesis that is still debated in the literature (Saal et al., 2005; López-Knowles et al., 2010). Indeed, in our cohort, out of 31 patients with altered PI3K axis, only one case carried both alterations.

As regards *PIK3CA*, an interesting corollary to our analysis is the presence of point mutations limited to exon 20, which encodes for the catalytic domain of the protein. This evidence seems to be related to the breast cancer histotype as it has been reported that exon 9 mutations in the *PIK3CA* gene mainly occur in lobular invasive carcinomas (Barbareschi et al., 2007). In addition, the *PIK3CA* mutational analysis led us to identify a very rare mutation, the M1040I change, which has never been detected in solid tumors, but has previously been found in a patient affected by a B-cell lymphoma (Abubaker et al., 2007; www.sanger.ac.uk/genetics/CGP/cosmic).

It is important to point out that the results of the present work were obtained in a series of TN-BCBLs defined by morphological features and immunohistochemical studies, as a surrogate of molecular profiling for basal-like breast cancers. It is possible that similar investigations might yield different results according to the design of the studies. Nevertheless, the present results, and an increasing body of evidence in the literature, strongly indicate that PTEN deregulation characterizes the pathogenesis of TN-BCBLs. In this context, our observation that *EGFR* amplification is restricted to patients over 50 years old is also interesting and may indicate that different pathogenetic events are involved in TN-BCBLs.

Overall, our results suggest that, in the future, clinical trials must evaluate the effect of new therapeutic strategies in patient subgroups selected on the basis of molecular profile.

In conclusion, this is the first extensive study simultaneously investigating EGFR and members of EGFR-downstream pathways in the same cohort of TN-BCBLs. Our results indicate that only a small proportion of TN-BCBL patients may benefit from current available EGFR-targeted therapies. Nevertheless, by demonstrating that the PI3K/PTEN pathway is highly deregulated in TN-BCBL, our data suggest that patients affected by this particular breast cancer could benefit from tailored treatments against downstream PIK3CA members, such as mTOR inhibitors, and rapamycin-derivatives (temsirolimus and everolimus), as recently demonstrated in in vivo studies (Liu et al., 2011).

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Competing interest. V.M. and L.M. have participated as invited speakers in Abbott´s Workshops. F.B., E.Z., F.M., S.C. and M.F. have no competing interests.

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