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ORIGINAL ARTICLE

PI3KCA mutations and/or PTEN loss in Her2-positive breast carcinomas treated with trastuzumab are not related to resistance to anti-Her2 therapy

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Abstract The purpose of this study is to evaluate whether activating mutations of the p110α catalytic subunit of class A phosphoinositide 3-kinases (*PI3KCA*) or complete loss of phosphatase and tensin homolog (PTEN) is associated with response to anti-human epidermal growth factor receptor 2 (Her2) treatment in breast cancer (BC). We analysed *PI3KCA* hot-spot mutations and PTEN immunohistochemical expression in 129 Her2-positive infiltrating BC treated with trastuzumab, including 26 cases treated with neoadjuvant therapy, 48 metastatic infiltrating breast cancer (IBC; MBC) and 55 early-stage IBC, with complete clinical information (mean follow-up 37, 66 and 32 months, respectively). *PI3KCA* hot-spot mutations were observed in 25 cases (19 %): 12 (9 %) in exon 9 and 13 (10 %) in exon 20. No correlations were observed between mutations and pathological and biological parameters. In patients treated with neoadjuvant therapy and in MBC, we did not observe any relationship with response to trastuzumab-based therapy. PTEN loss was observed in 24 out of 86 informative cases (28 %), 3 (13 %) of which were also mutated for *PI3KCA*. PI3K pathway activation, defined as *PI3KCA* mutation and/or PTEN loss, was not associated with response to treatment or clinical outcome in MBC. *PI3KCA* mutation and/or PTEN loss should not exclude patients from potentially beneficial anti-Her2 therapy.

Keywords Breast cancer · Her2 · Trastuzumab · *PI3KCA* mutation · PTEN loss

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Introduction

Human epidermal growth factor receptor 2 (Her2) is a tyrosine kinase receptor, which is overexpressed or amplified in 20 to 25 % of infiltrating breast carcinomas (IBC), which may behave more aggressively. Her2 overexpression may lead to increased receptor homo/heterodimerization, which induces phosphorylation of the intracellular domain, leading in turn to activation of many downstream signalling molecules, including class A phosphoinositide 3-kinases (PI3K)/AKT. Activated PI3K catalyzes the phosphorylation of inositol lipids to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn, as a negative feedback mechanism, is dephosphorylated to PIP2 by phosphatase and tensin homolog (PTEN). PIP3 activates the serine/threonine kinase AKT which in turn phosphorylates and regulates the mammalian target of rapamycin (mTOR).

Trastuzumab, a humanized monoclonal antibody targeting the extracellular domain of Her2, has good therapeutic efficacy in patients with Her2-positive IBC. Trastuzumab is effective in neoadjuvant therapy, in cases of metastatic breast cancer (MBC) and in the adjuvant setting in early-stage IBC.

However, not all patients benefit from trastuzumab-based therapy, and a relevant percentage shows an initial resistance to trastuzumab.

The underlying antitumoural mechanisms of trastuzumab are complex and not completely understood [1]. The hypothesized mechanisms include inhibition of Her2 homo/heterodimerization, Her2 endocytosis and degradation or reduced shedding of the extracelluar domain, leading to downregulation of downstream signalling through the PI3K–AKT pathway. An alternative mechanism of action could be related to the fact that trastuzumab, being an intact monoclonal antibody, may recruit Fc-competent immune effector cells and the other components of antibody-dependent cell-mediated cytotoxicity, leading to tumour cell death [2].

Several lines of evidence suggest that Her2-independent activation of the PI3K–AKT pathway may be related to resistance to trastuzumab [3–5]. Aberrations of the components of the PI3K–AKT pathway are frequent in IBC, including activating mutations of the p110α catalytic subunit of PI3K (*PI3KCA*) and PTEN loss, which have been described in 9 to 45 % of cases [6–17] and 4 to 28 % of cases [18–21], respectively. The vast majority of *PIK3CA* mutations, comprising approximately 90 % of cases, are clustered at two hot-spot regions in exon 9 (E542K and E545K) and exon 20 (H1047R and H1047L) encoding the helical and kinase domains, respectively [6–17]. *PI3KCA* mutations are among the most common genetic aberrations in human IBC and have generally been reported as more frequent in node-negative [8, 17], estrogen receptor (ER)-positive and Her2-negative IBC [8–10, 14, 22–24], but data are not consistent. Controversies exist also concerning the prognostic value of *PI3KCA* mutations: most authors report favourable relation with survival [9, 10, 25, 26] but again data are not consistent [14, 27, 28] probably due to the heterogeneity of the investigated series and to possible different pathogenetic significance of mutations occurring at different sites in the molecule [7]. *PI3KCA* mutations have also been indicated as possible markers for poor response to trastuzumab [21, 27–29], but data are still not conclusive [5, 27, 30, 31]. Preclinical studies also suggest that the *PI3KCA* gene could be a target for new drugs [32] suggesting the possibility to combine anti-Her2 therapy with different inhibitors targeting PI3K, TORC1, AKT or EGFR [33, 34].

In the present study, we analyse *PI3KCA* mutations at hot-spots in exon 9 and exon 20 and PTEN expression in a retrospective series of Her2-positive IBC treated with trastuzumab (26 cases treated with neoadjuvant therapy, 48 metastatic IBC and 55 early-stage IBC), investigating their frequency and their relationship with clinical and pathological parameters and response to trastuzumab treatment.

Material and methods

Patients and tumour samples

We retrospectively evaluated 129 Her2-positive IBC which had been treated with trastuzumab at the Santa Chiara Hospital of Trento Italy between 2000 and 2010. The study has been approved by the ethical committee of the Santa Chiara Hospital. The investigated cases include three groups of patients: 26 primary IBC treated with neoadjuvant trastuzumab therapy, 48 advanced MBC and 55 early-stage IBC treated with adjuvant therapy. Complete follow-up information was available for all cases; mean follow-up was 37, 66 and 32 months for the above three groups of patients. Neoadjuvant therapy consisted of doxorubicin and trastuzumab for three courses followed by paclitaxel and trastuzumab for 4 cycles followed by cyclophosphamide, methotrexate, fluorouracil associated with trastuzumab (according to NOAH trial regimen). In MBC patients, the first-line therapy consisted of trastuzumab associated with taxanes (weekly paclitaxel or docetaxel). All early-stage IBC patients received adjuvant trastuzumab for 1 year after surgery. All patients showing positive estrogen receptors also received hormonal therapy with tamoxifen±ovarian ablation if in premenopausal status and with aromatase inhibitors if in postmenopausal status. All cases were reviewed by a pathologist (MB). For each case, one representative tissue block was selected for further analyses. The percentage of tumour cells in each block was visually estimated, and only cases with more that 50 % of tumour cell content were included in the study.

For patients affected by primary IBC treated with neoadjuvant trastuzumab, pathological response at surgery was scored as: (a) *complete*, if no invasive residual disease in breast and axilla was found, (b) *partial*, if the residual tumour burden was inferior to the clinically estimated tumour diameter and (c) *absent*, if the residual tumour burden was identical or higher than the clinically estimated tumour diameter (stable or progressive disease). For MBC, response to therapy was evaluated clinically. The different pathological characteristics of the whole group of cases are shown in Table 1, and the characteristics of the three different series are illustrated in Tables 2, 3 and 4.

Her2 immunoreactivity was evaluated using the HercepTest kit (DakoCytomation, Glostrup, Denmark) according to the manufacturer's FDA-approved procedures, using manual or computer-assisted assessment [35]. All cases with Her2 score 2+ were further investigated with fluorescent in situ hybridization using the *HER2* FISH pharmDx™ kit (DakoCytomation). Tumours with a *HER2*/Cep17 ratio ≥2.2 were considered as amplified.

In all cases, ER, progesterone receptor (PgR) and Ki67 were evaluated using 6 F11 (Leica, Novocastra, Newcastle, UK), Pgr636 (DakoCytomation) and MIB1 (Leica, Novocastra) antibodies, respectively [36]. Ki67 labelling index was

Table 1 Clinical–pathologic characteristics of the whole series of Her2-positive IBC according to mutational status of *PI3KCA*

	Total	mt Ex 9 *PI3KCA*	%	mt Ex 20 *PI3KCA*	%	WT *PI3KCA*	%
Cases	129	12	9.3	13	10	104	81
Histotype							
Ductal	115	11	9.5	11	9.5	93	80.8
Lobular	10	1	10	0		9	90
Other	4	0		2	50	2	50
Tumour size							
T1	42	3	7.1	7	16.7	32	76.2
T2	63	8	12.7	4	6.3	51	81
T3–T4	22	1	4.5	2	9.1	19	83.4
Not known	2	0		0		2	100
Nodal status							
Negative	40	5	12.5	6	15	29	72.5
Positive	88	7	8	7	8	74	84
Not known	1					1	100
Hormone receptor status[a]							
ER positive	73	7	9.6	9	12.4	57	78
ER negative	56	5	8.9	4	7.1	47	84
Not known							
PgR positive	58	4	6.9	6	10.3	48	82.7
PgR negative	71	8	11.3	7	9.9	56	78.8
Proliferative activity[b]							
Ki67 high	119	12	10	13	11	94	79
Ki67 low	7	0		0		7	100
Not known	3	0		0		3	100

[a]Hormone receptor status was defined as positive for ER and PgR if at least 1 % reactive cells were seen

[b]Proliferative activity was scored as high if Ki67-reactive cells were at least 25 %

evaluated manually in the peripheral areas of all tumours. In case of marked heterogeneity, Ki67 labelling was also evaluated using computer-assisted image analysis [37]. Cases with at least 1 % reactive cells were considered positive for ER and PgR. Ki67 labelling was scored as high if at least 25 % reactive cells were counted. A subset of 34 cases out of the series of advanced MBC and 52 out of 55 cases treated with adjuvant therapy were immunostained for PTEN, using the 138G6 antibody (Cell Signaling Technology, Celbio, Italy). PTEN immunohistochemistry was scored as absent (complete PTEN loss) when no immunostaining was observed in spite of positive internal controls, or positive, if any degree of cytoplasmic and nuclear staining was observed. In 14 cases, PTEN could not be evaluated because of lack of available histological samples or inadequate internal control immunostaining.

Mutational analysis

Sample DNA extraction

Five 10-μm sections were cut from each paraffin-embedded tumour block. Slides were deparaffinized twice with 1 mL of xylene, vortexed and centrifuged. The supernatant was removed and then washed with 1 mL absolute ethanol. The samples were resuspended in 180 μl lysis buffer containing 20 μl proteinase K. DNA was isolated with the Qiamp DNA mini-kit (Qiagen, Valencia, CA) and was incubated overnight at 56 °C at 300 rpm.

Real-time polymerase chain reaction amplification

Briefly, DNA fragments were amplified using RT-PCR with primers specific for exon 9 and exon 20 of the *PI3KCA* gene, designed to allow the identification of the following mutations: E542K, E545K and Q546K in exon 9 and M1043I, H1047R, H1047L, G1049S and G1049R in exon 20 (Diatech Pharmacogenetics, Jesi, Italy). Each mix contained 5× buffer Mg^{2+}-free, 50 mM Mg^{2+} solution, 10 mM of each dNTP, 1 μl of specific primers, EvaGreen dye (20× in water), 5 U/μl Hot Star Taq DNA Polymerase and 100 ng genomic DNA in a 50-μl final volume. The mixture was denatured for 3 min at 95 °C and underwent 40 cycles: at 95 °C for 20 s, at 57 °C for 30 s and at 72 °C for 30 s, followed by a final step for 6 min at 60 °C for the acquisition the green signal.

Table 2 Clinical–pathologic characteristics of the series of Her2-positive IBC treated with neoadjuvant trastuzumab therapy according to mutational status of *PI3KCA*

	Total	mt Ex 9 *PI3KCA*	%	mt Ex 20 *PI3KCA*	%	WT *PI3KCA*	%
Cases	26	1	3.8	3	11.5	22	84.6
Mean follow-up	37						
Histotype							
Ductal	26	1	3.8	3	11.5	22	84.6
Tumour size							
T1							
T2	15	1	6.6	1	6.6	13	86.6
T3–T4	11	0		2	18.2	9	81.8
Nodal status							
Negative	7	1	14.3	1	14.3	5	7.1
Positive	19	0		2	10.5	17	89.4
Hormone receptor status							
ER positive	13	0		2	15.4	11	84.6
ER negative	13	1	7.7	1	7.7	11	84.6
Not known							
PgR positive	11	0		1	9.1	9	81.8
PgR negative	15	1	6.6	2	13.3	13	86.6
Proliferative activity							
Ki67 high	23	1	4.3	3	13	19	82.6
Ki67 low	2	0		0		2	100
Not known	1	0		0		1	100

Pyrosequencing

Each sample submitted for pyrosequencing was prepared utilizing 3 μl streptavidin sepharose (GE Healthcare, Buckinghamshire, UK), 37 μl PyroMark binding buffer (Qiagen), 20 μl MB water (Qiagen) and 20 μl biotinylated PCR product. The plate was mixed and incubated for 15 min on a shaking table at 1,400 rpm. In the second step, the binding solutions were released into 40 μl of annealing buffer containing the respective sequencing primer. Primers were annealed to the target after incubation at 80 °C for 2 min and allowed to cool to room temperature prior to pyrosequencing. PyroGold reagents were used for the pyrosequencing reaction, and the signal was analysed using the PyroMark Q96 ID system (Qiagen). The software analyses the bioluminescence produced at the end of the enzymatic reactions, of which the intensity is proportional to the number of incorporated nucleotides. Positive controls were cases with known *PI3KCA* mutational status. The results of the experiments were interpreted according to the cut-offs provided by the manufacturer (Diatech Pharmacogenetics). For exon 9 mutations, the cutoff nucleotide substitution values to define a case as mutated were: E542K A>5 %, E545K A >10 %, Q546K G>10 % and Q546K A>5 %. For exon 20 mutations, the cut-off nucleotide substitution values to define a case as mutated were: M1043I T>10 %, M1043I A>10 %, H1047R C>12 %, H1047L A>10 %, G1049S G>6 % and G1049R T>5 %.

Statistical methods

Statistical analysis was performed using the SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL). In the groups of primary IBC treated with neoadjuvant trastuzumab therapy and MBC groups, we evaluated the possible association of *PI3KCA* mutations/PTEN loss with response to therapy; in the series of MBC, we evaluated also their relation with overall and disease-free survival (DFS). No survival analysis was performed in early IBC because of the short follow-up (all patients are alive and disease free).

Contingency tables were used to analyse the relationships between the categorical variables: absence/presence of mutation/loss of PTEN vs. pathological and biological parameters. Fisher's exact test (2×2 tables) and chi-square test (general contingency tables) were used to test the significance of the association between two classifications. Kaplan–Meier survival analysis with log-rank test was used to determine the difference in DFS and overall survival between groups. All tests were two sided. The statistical significance level was chosen at 5 %. All analyses were performed blinded to the study endpoints.

Table 3 Clinical–pathologic characteristics of the series of Her2-positive MBC treated with trastuzumab therapy according to mutational status of *PI3KCA*

	Total	mt Ex 9 *PI3KCA*	%	mt Ex 20 *PI3KCA*	%	WT *PI3KCA*	%
Cases	48	3	6.25	2	4.1	43	85.6
Mean follow-up	66						
Histotype							
Ductal	43	3	7	2	4.6	38	88.4
Lobular	3	0		0		3	100
Other	2	0		0		2	100
Tumour size							
T1	15	1	6.6	2	13.3	12	80
T2	22	1	4.5	0		21	95.4
T3–T4	10	1	10	0		9	90
Not known	1	0		0		1	100
Nodal status							
Negative	10	1	10	2	20	7	70
Positive	37	2	5.4	0		35	94.6
Not known	1					1	100
Hormone receptor status							
ER positive	23	2	8.7	1	4.3	20	87
ER negative	25	1	4	1	4	23	92
Not known							
PgR positive	16	1	6.6	0		15	193.7
PgR negative	32	1	3.1	2	6.3	29	90.6
Proliferative activity							
Ki67 high	43	3	7	2	4.6	37	86
Ki67 low	3	0		0		3	100
Not known	2					2	100

Results

Clinical outcome of the patients

In the series of patients treated with neoadjuvant trastuzumab therapy, 11 (42 %) showed complete pathological response, 14 (54 %) partial pathological response and 1 (4 %) showed stable disease. At a mean follow-up of 37 months, 3 patients died of disease, 3 were alive with relapsing disease and 20 were alive without evidence of disease.

In the series of patients affected by MBC treated with trastuzumab therapy, 4 (8 %) showed complete clinical response, 18 (38 %) partial clinical response with more than 50 % reduction of the tumour, 14 (29 %) partial clinical response with less than 50 % reduction of the tumour, 8 (17 %) stable disease and 3 (6 %) showed progressive disease. In responsive cases (complete and partial responses), median response duration was 9.5 months with two patients having durable complete response over 3 years of trastuzumab treatment. At a mean follow-up of 66 months, 39 patients died of disease, 7 were alive with disease and 2 were alive without evidence of disease. In the series of 55 early-stage IBC treated with adjuvant therapy at a mean follow-up of 32 months, all patients were alive without evidence of relapse.

Frequency of *PI3KCA* mutations and relationships with pathological and biological characteristics

In the whole series of 129 IBC, *PI3KCA* mutations were observed in 25 cases (19 %): 12 (9 %) in exon 9 (2 Q546K and 10 E545K) and 13 (10 %) in exon 20 (1 H1047L and 12 H1047R; Fig. 1). *PI3KCA* mutations were observed in 15 % of locally advanced IBC treated with neoadjuvant therapy, in 10 % of MBC and in 29 % of early-stage IBC treated with adjuvant trastuzumab therapy. The relationships between *PI3KCA* mutation and pathological and biological characteristics are shown in Table 1 for the whole series and in Tables 2, 3 and 4 for the three different groups of patients. No statistically significant correlations were observed between mutations and pathological and biological parameters. In particular, we did not observe any relationship with histological type, nodal status and hormone receptor status.

Table 4 Clinical–pathologic characteristics of the series of Her2-positive IBC treated with adjuvant trastuzumab therapy according to mutational status of *PI3KCA*

	Total	mt Ex 9 *PI3KCA*	%	mt Ex 20 *PI3KCA*	%	WT *PI3KCA*	%
	55	8	14.6	8	14.6	39	70.9
Mean follow-up (months)	32						
Histotype							
Ductal	46	7	15.2	6	13	33	71.7
Lobular	7	1	14.3	0	0	6	85.7
Other	2			2	100	0	
Tumour size							
T1	27	2	7.4	5	18.5	20	74
T2	26	6	23	3	11.5	17	65.3
T3–T4	1					1	1
Not known	1					1	1
Nodal status							
Negative	23	3	13	3	13	17	74
Positive	32	5	15.6	5	15.6	22	68.8
Not known							
Hormone receptor status							
ER positive	37	5	13.5	6	16.2	26	70.3
ER negative	18	3	16.6	2	11.1	13	72.2
Not known							
PgR positive	31	3	9.7	5	16.1	23	74.2
PgR negative	24	5	20.8	3	12.5	16	66.6
Not known							
Proliferative activity							
Ki67 high	53	8	15.1	8	15.1	47	88.7
Ki67 low	2					2	100

Association of *PI3KCA* mutation with response to therapy and clinical outcome

In the series of patients treated with *neoadjuvant therapy*, *PI3KCA* mutations were observed in 3 out of the 11 cases with complete pathological response (three exon 20 H1047R mutations with 14.1, 33.9 and 71.2 % substitution frequency, respectively) and in 1 out of 14 cases with partial response (one exon 9 Q546K mutation with 19.5 % substitution frequency) (Table 5). These data suggest that *PI3KCA* mutations are not a major determinant of trastuzumab resistance because even cases with very high percentages of the mutated allele showed complete pathological response.

In the series of *metastatic breast carcinomas*, *PI3KCA* mutations were observed in 3 out of the 29 cases with partial clinical response (two exon 9 E545K mutations with substitution frequency of 11.6 and 38.4 % respectively, and one exon 20 H1047R mutation with 28.1 % substitution frequency) and in 1 out of 10 cases with stable/progressive disease (exon 20 H1047R mutation with 21.1 % substitution frequency; Table 6). No gene mutations were observed in the four patients showing complete clinical responses. These data suggest that *PI3KCA* mutations are not a major determinant of trastuzumab resistance in metastatic IBC treated with trastuzumab. Survival analysis in metastatic IBC showed that *PI3KCA* mutations are not related to adverse clinical outcome. However, the survival analysis has limited statistical power due to the limited number of investigated cases.

PTEN expression and PI3K mutation

PTEN immunohistochemical expression was evaluable in 86 cases and ranged from complete loss of reactivity in tumour cells, in spite of positive internal controls, to various degrees of immunoreactivity (Fig. 2). PTEN loss was observed in 24 (28 %) cases, 3 (13 %) of which were also mutated for *PI3KCA*. PTEN immunostaining was observed in 62 (72 %) cases, 17 (27 %) of which showed PI3K mutation. These data show that in the present series of IBC, PTEN loss, although less frequent in cases with concurrent *PI3KCA* mutation (13 versus 27 %), was not mutually exclusive with *PI3KCA* mutation.

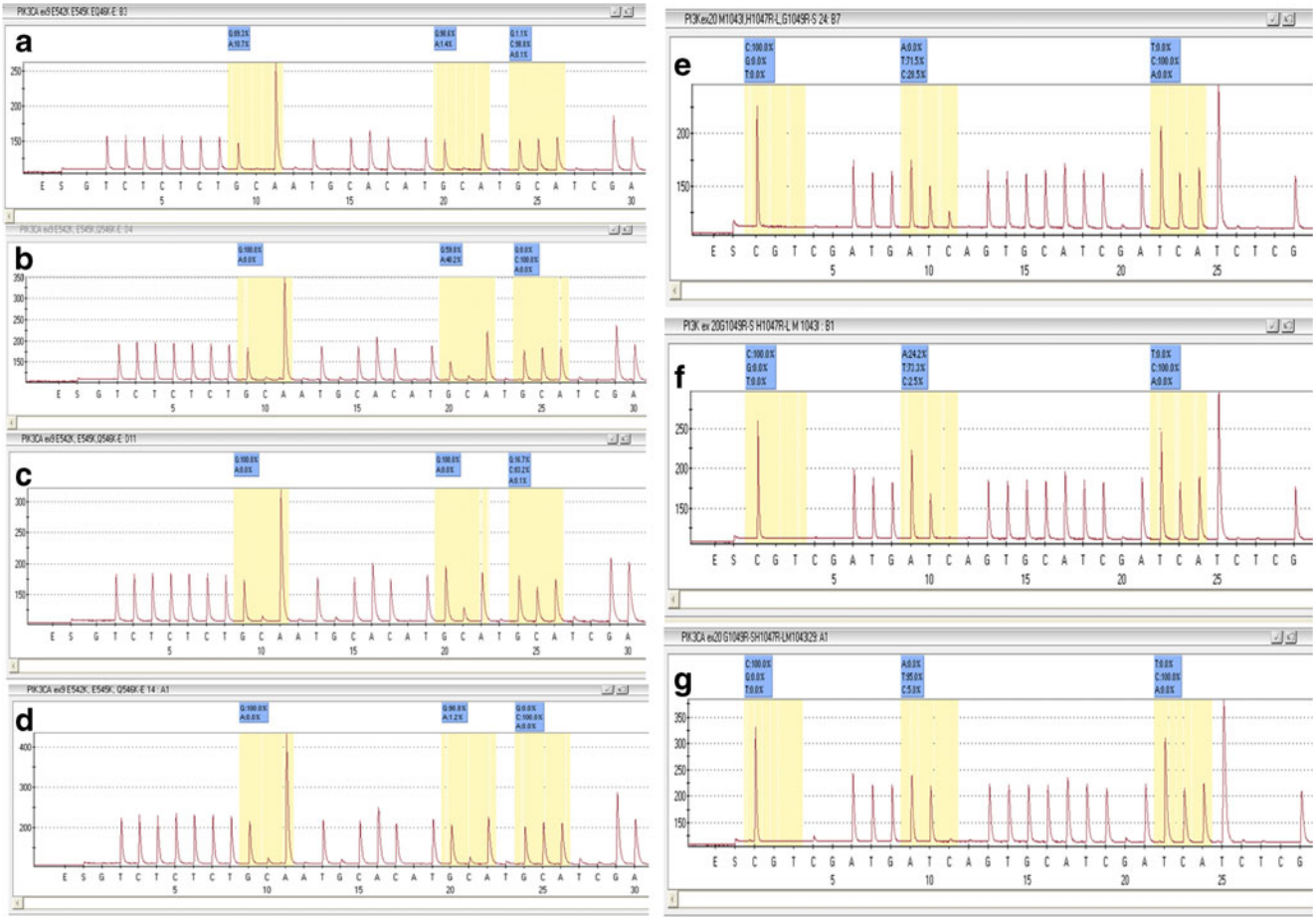

Fig. 1 Pyrosequencing reports of five different cases with **a** exon 9 E542K mutation, **b** exon 9 E545K mutation, **c** exon 9 Q546E mutation, **d** exon 9 wild-type genotype, **e** exon 20 H1047R mutation, **f** exon 20 H1047L mutation and **g** exon 20 wild-type genotype

Combined evaluation of *PI3KCA* mutation and PTEN loss in relation to clinical outcome in metastatic breast carcinomas

PTEN immunostaining could be evaluated in 34 of the 48 MBC, 10 of which showed complete PTEN loss. *PI3KCA* mutations were observed in 1 out of these 10 cases with complete PTEN loss and in 3 of the 24 cases with PTEN expression. PI3K pathway activation, defined as PTEN loss and/or *PI3KCA* mutation, was seen in 13 out of these 34 cases, including 1 case with complete clinical response, 5

Table 5 Relation between *PI3KCA* mutations and therapy responses in the series of patients treated with neoadjuvant trastuzumab

	Total	mt Ex 9 *PI3KCA*	mt Ex 20 *PI3KCA*	wt*PI3KCA*
Pathological response	26			
Stable disease	3 (11.5 %)	0	0	3
Partial responder	12 (46.1 %)	0	1	11
Complete responder	11 (42.3 %)	1	2	8

mt Ex 9 PI3KCA mutation in exon 9 of the *PI3KCA* gene, *mt Ex 20 PI3KCA* mutation in exon 20 of the *PI3KCA* gene, *wtPI3KCA* wild-type *PI3KCA* gene

Table 6 Relation between PI3K mutation and response therapy in the series of 48 patients with MBC treated with trastuzumab

Clinical response	Total	mt Ex 9 *PI3KCA*	mt Ex 20 *PI3KCA*	wt*PI3KCA*
Progressive disease	3 (6.2 %)			3
Stable disease	8 (16.6 %)		1	7
Partial responder >50 %[a]	18 (37.5 %)	1		17
Partial responder <50 %[b]	14 (29.2 %)	1	1	12
Complete responder	4 (8.3 %)			4
NV	1 (2.1 %)	1		0

mt Ex 9 PI3KCA mutation in exon 9 of the *PI3KCA* gene, *mt Ex 20 PI3KCA* mutation in exon 20 of the *PI3KCA* gene, *wtPI3KCA* wild-type *PI3KCA* gene

[a] Partial response with residual tumour burden inferior to 50 % of the clinically estimated initial tumour diameter

[b] Partial response with residual tumour burden greater than 50 % of the clinically estimated initial tumour diameter

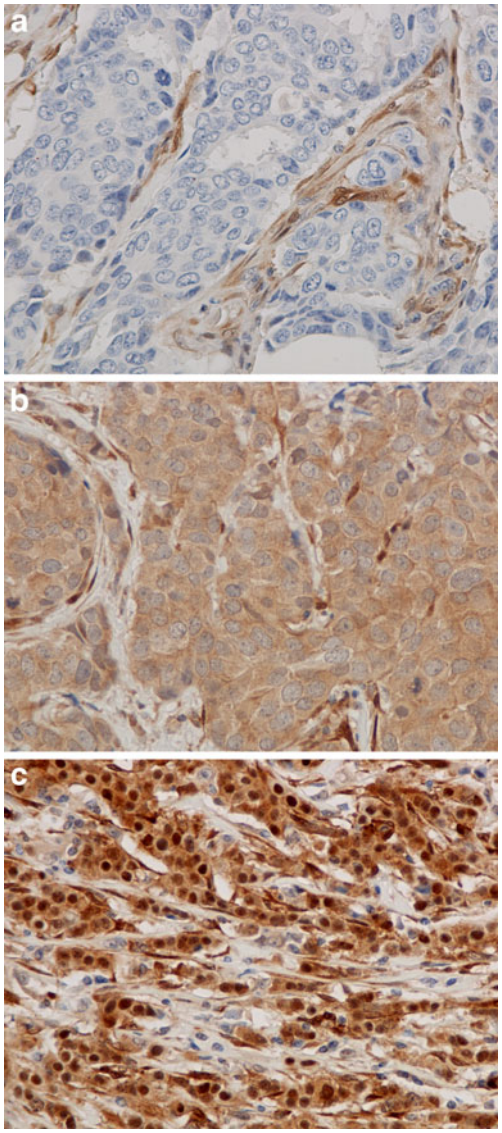

Fig. 2 Different levels of PTEN immunohistochemical expression in breast carcinomas, ranging from complete absence in tumour cells with concurrent positive internal controls (**a**) to mild cytoplasmic positivity (**b**), to strong cytoplasmic and nuclear reactivity (**c**)

cases with prominent clinical response (>50 % reduction in tumour burden) and 5 cases with partial clinical response (<50 % reduction in tumour burden), and in 2 cases with stable disease (Table 7). These data suggest that PI3K pathway activation is not a major determinant of trastuzumab resistance in MBC.

Discussion

Our present study on 129 Her2-positive IBC treated with trastuzumab, analysed with the highly specific and sensitive pyrosequencing technique, shows that *PI3KCA* mutations occur in 19 % of cases and are not related to pathological features of the tumours. In retrospective analyses of small groups of MBC and locally advanced IBC treated with neoadjuvant trastuzumab, we show that *PI3KCA* mutations are not related to response to treatment. In a subset of cases, we also show that *PI3KCA* mutations are not mutually exclusive with PTEN expression loss, and that, in MBC, PI3K pathway activation, defined as *PI3KCA* mutation and/or PTEN loss, is not associated with response to treatment. Survival analysis in the group of MBC shows that neither *PI3KCA* mutations nor PI3K/PTEN pathway activation is related to clinical outcome, but the limited number of investigated cases could have precluded the study to reach statistical significance.

Table 7 PI3K pathway activation, defined as *PI3KCA* mutation and/or PTEN loss in relation with clinical response in the series of MBC (34 informative cases)

	Cases with wt*PI3KCA* and PTEN expression	Activated PI3K pathway (mt*PI3KCA* and/or PTEN loss)
Progressive disease	3	–
Stable disease	6	2
Partial responder >50 %[a]	13	5
Partial responder <50 %[b]	9	5
Complete responder	3	1

wtPI3KCA wild-type *PI3KCA* gene, *mtPI3KCA* mutated *PI3KCA* gene

[a] Partial response with residual tumour burden inferior to 50 % of the clinically estimated initial tumour diameter

[b] Partial response with residual tumour burden greater than 50 % of the clinically estimated initial tumour diameter

The reported frequency of *PI3KCA* mutation in literature is quite variable, ranging from 15 [30] to 29 % [38]. This wide range of frequencies in Her2-positive IBC could in part be related to the limited number of investigated cases in some studies, to the different analytical methods used, or could be related to differences between population groups. The latter is a well-known phenomenon, which has been observed not only between different ethnic groups, such as for example frequency of EGFR mutations lung carcinoma in Western and Japanese populations, but also within the same ethnic group, such as *B-raf* mutations and *PTC/Ret* translocations in thyroid carcinomas [38, 39]. In a previous study of a consecutively collected series of IBC from the same geographical area (Trentino, Northeast Italy), we observed a frequency of *PI3KCA* mutation (26 % mutated cases) which is slightly higher than the one observed in the present study [7]. This difference might be related to the different composition of the two series, as the previous series included mainly Her2-negative ER-positive IBC, while the present one includes only Her2-positive cases,

with a higher percentage of ER-negative cases. Several studies have indeed shown that *PI3KCA* mutation is more frequent in ER-positive and Her2-negative IBC [8–10, 14, 22–24] suggesting that in these cases PI3K/AKT pathway activation occurs through a Her2-independent mechanism. However, discordances exist, and other studies have either not found such associations or even provided opposite results, such as an association between *PI3KCA* mutations and Her2 overexpression [13]. Comparison between the different studies is extremely difficult as the inclusion criteria are different. Moreover, since the pathogenetic role of mutations in different regions of the *PI3KCA* gene could provide different activating effects, with potentially different pathogenetic and clinic relevance [7], further studies, and possibly a meta-analysis of currently available data, should further investigate these relationships.

The most intriguing aspect of the study of *PI3KCA* mutations is their possible role as predictive markers of resistance to anti-Her2 therapy, either using trastuzumab or the reversible dual kinase inhibitor lapatinib, which is active also against EGFR. Preclinical and preliminary data suggest that *PI3KCA* mutations might predict resistance to anti-Her2 therapy [5, 21, 27–29]. The study of Razis et al. [28] is of particular interest as it includes a series of partly Her2-positive and partly Her2-negative metastatic IBC treated with trastuzumab: in Her2-negative cases, *PI3KCA* mutations were not related to time to progression, while in the Her2-positive group, a strong and independent association between *PI3KCA* mutations and worse prognosis was found. However, data are still not conclusive, and some studies suggest that *PI3KCA* mutations are not associated with response or clinical outcome [31, 39] or are of limited predictive value [5, 30]. Our present study shows that *PI3KCA* mutations occur in patients responding to trastuzumab treatment (i.e. four neoadjuvant patients and five MBC patients), further supporting the hypothesis that *PI3KCA* mutations do not exclude the possibility of clinical benefit to trastuzumab therapy.

Previous reports suggested that *PI3KCA* mutation and PTEN loss are mutually exclusive [13]. However, in our study 3 (12.5 %) out of 24 PTEN-negative IBC showed also *PI3KCA* mutation, as also reported by Perez-Tenor et al. [10] and Wang et al. [31]. The reason for this redundancy in the activation of the PI3K pathway is not clear, but could be related to different yet unknown cross-talk mechanisms between different pathways, or to an additive effect of these two activating hits along the same metabolic pathway.

The lack of a clear-cut relationship between *PI3KCA* mutation and therapeutic response to anti-Her2 agents can be interpreted in the light of the complex and poorly understood mechanisms of action of trastuzumab. First, resistance could be related to the fact that the PI3K/AKT signalling pathway is very complex and may be improperly constitutionally activated at several levels with different mechanisms, which, beside *PI3KCA* mutation, potentially include loss of PTEN expression, *PI3KCA* gene amplification, mutations or amplification of *AKT*, deregulation of mTOR activity, altered expression of p70S6K, etc. In particular, PTEN loss has been associated with resistance to anti-Her2 therapy in cell lines and patients [21, 27, 28, 30, 38]. The combined evaluation of alterations of the PI3K/AKT pathway at several levels could provide a rationale for combining anti-Her2 therapy with PI3K/AKT inhibitors [29, 40]. Preliminary data indeed show that concurrent evaluation of *PI3KCA* mutations and PTEN and p70S6K expression may identify patients without PI3K/AKT pathway activation who could benefit more from trastuzumab-based therapy [27, 28, 30, 31]. However, in our small series of MBC, PI3K/PTEN pathway activation was not related to clinical response to trastuzumab, and further studies are needed to clarify this issue. Alternatively, the fact that *PI3KCA* mutated cases may respond to trastuzumab therapy could be interpreted in the light of the possible role of trastuzumab in determining antibody-dependent cell-mediated cytotoxicity. Trastuzumab is an intact monoclonal antibody which, binding to the Her2 molecule, could elicit antibody-dependent cellular cytotoxicity which could lead to tumour cell necrosis and hence clinical/pathological response [41–43].

In conclusion, our study shows that *PI3KCA* mutations are frequent in Her2-positive breast cancer and that there is no significant correlation with response to trastuzumab-based therapy. The study also shows that PI3K pathway activation, defined as PTEN loss and/or *PI3KCA* mutation, is not associated with response to treatment or with clinical outcome. Since most reported series of IBC treated with anti-Her2 treatment are small and the possible variables influencing the results are many, including the confounding effects of the patients' background treatments [5], an effort should be undertaken to pool all data together to perform a meta-analysis and possibly to study PI3K pathway activation mechanisms in a more comprehensive way, including also less frequently studied biomarkers such as *AKT* mutation and phosphorylation status, mTOR and p70S6K expression levels. More importantly, the value of the combined use of trastuzumab with different inhibitors targeting the PI3K pathway at different levels needs to be tested. Finally, our data show that *PI3KCA* mutation and/or PTEN loss are not criteria to exclude patients from potentially beneficial anti-Her2 therapy.

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Conflict of interest We declare that we have no conflict of interest.

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