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Phosphorylated HER-2 tyrosine kinase and *Her-2/neu* gene amplification as predictive factors of response to trastuzumab in patients with HER-2 overexpressing metastatic breast cancer (MBC) ☆

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ABSTRACT

Aim: Trastuzumab (T), a humanised monoclonal antibody against HER-2, is active in HER-2-positive MBC patients. However, nearly 60% of the patients do not benefit from T, stressing the need for additional predictive markers. The following markers could be implicated in response to T: (1) the magnitude of *Her-2* gene amplification; (2) the co-expression of the other HER family receptors, possibly responsible for HER-2 trans-activation; (3) the activated status of HER-2; (4) the activated status of downstream effectors as mitogen-activated protein kinases (MAPKs), p38 and p27.

Methods: Medical files of patients with MBC treated with T either as a single agent or in combination with chemotherapy (CT) were reviewed. HER family members (EGFR, HER-2, HER-3, HER-4), the phosphorylated forms of EGFR (p-EGFR), HER-2 (p-HER-2) and of the downstream effectors were evaluated in the archival tumours. The correlation between clinical outcome and the expression of these markers was investigated.

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Results: (1) Increasing values of *Her-2* amplification were associated with a higher probability of achieving an objective response; (2) no statistical significant correlation between the expression of the HER family receptors was found; (3) p-HER-2 was predictive of response in patients treated with T+CT; (4) a statistically significant correlation between p-ERK 1/2, p-p38 and p-HER-2 emerged, pointing to the activated vertical pathway p-HER-2 → p-MAPKs. *Conclusions:* p-HER-2 and the magnitude of *Her-2* amplification were predictive of response to T and their role deserves to be analysed in larger and more homogenous T-treated populations such as those from large phase III trials.

1. Introduction

The epidermal growth factor receptor family (HER family) belongs to the receptor tyrosine kinases (RTKs) superfamily, which accounts for about 20 subclasses extensively implicated in proliferation, differentiation and survival pathways, both in normal and in cancer cells. The HER family includes four members, the EGFR or ErbB1/HER-1, ErbB2/HER-2, ErbB3/HER-3 and ErbB4/HER-4, which share a high homology in their structure. Deregulation with consequent aberrant function of the four HER receptors may be mainly related to their overexpression due to gene amplification, their transactivation as a consequence of heterodimerisation or to intragenic mutations.

HER-2 overexpression is found in 25–30% of human breast cancers (BC) and is associated with worse outcome in terms of high risk of relapse, disease progression and shorter survival.[1–5]

Monoclonal antibodies directed against the external domain of the HER receptors and small tyrosine kinase inhibitors (TKIs) targeting their cytoplasmic part represent the main attempts at blocking abnormal signals promoted by these receptors. The first anti-HER-2 agent to be approved for use in clinical practice is the humanised monoclonal antibody T (Herceptin®; Genentech, South San Francisco, CA, USA).[6] An important body of data substantiates T efficacy in patients with HER-2 overexpressing MBC, both when the drug is given as a single agent and in combination with CT, with the latter modality improving survival.[7–9] Given alone as first line treatment to MBC patients, T shows an overall response (OR) rate of 38% and a CB of 48%.[8] Therefore, more than half of HER-2 amplified MBC patients exhibit primary resistance to T.

Resistance to HER-2 targeting drugs can be: (1) 'primary' or 'pan-HER-2': resistance to all anti-HER-2 drugs from the start; (2) 'agent-selective': tumours rely on HER-2 signalling but are resistant only to specific therapies, e.g. tumours are sensitive to T but resistant to TKIs or vice versa; (3) 'acquired': resistance occurs after an initial response to T.[10]

To understand the mechanisms of resistance to T, it is important to take into account its mechanisms of action. Although not completely defined they include: (a) down-regulation of the receptor, (b) blockade of the interaction with the other HER, thus avoiding the heterodimerisation responsible for HER-2 trans-activation, (c) antibody-dependent cell-mediated cytotoxicity, (d) reduction of the proteolytic cleavage of the ectodomain, thus preventing the formation of a truncated highly active receptor remnant, (e) the induction of p27 with consequential cellular arrest in G1 phase, (f) anti-angiogenic activity,[11–16] and (g) inhibition of PI3K/Akt and MAPKs pathways.[17–19]

The additive/synergistic effect of T with cytotoxic drugs, such as cisplatin, paclitaxel and anthracyclines, has been reported[20] and there is consistent evidence that HER-2 modifies the sensitivity of BC cells to several CT drugs.[21] For taxanes, preclinical data have shown that by treating the intrinsic taxane-resistant $p185^{erbB2}$ overexpressing BC cell lines with T, the sensitivity to these drugs could be restored.[22] HER-2 overexpression inhibits $p34^{Cdc2}$, a critical kinase in paclitaxel-induced apoptosis. T-induced downregulation of HER-2 causes upregulation of $p21^{Cip1}$, with consequent $p34^{Cdc2}$ activation and paclitaxel-induced apoptosis.[23,24] These data were confirmed in clinical studies with better RR, time to progression (TTP) and overall survival (OS) with the combination of T with taxanes.

In the clinical setting, no other biomarkers besides HER-2-positivity have been found to be predictive of response to T. However, since only about 40% of HER-2 overexpressing/amplified tumours respond to this agent, additional markers are urgently needed particularly in view of its foreseen wide use in the adjuvant setting.

The aim of this retrospective analysis was to identify these potential predictive factors of response to T. Our hypothesis was that the co-expression of other HER receptors, the phosphorylation of HER-2 tyrosine kinase and the activation of downstream effectors could provide a phenotype of resistance to T. Particularly, higher activity of the T was expected when intracellular pathways that it can block were activated. Moreover, based on data suggesting that in HER-2 transfected BC cells the magnitude of overexpression/amplification could play a role in responsiveness to T,[25] we explored whether a threshold value of *Her-2* amplification was required for T efficacy; the possible correlation between levels of *Her-2* gene amplification and response was also analysed.

2. Patients and methods

2.1. Study design

In Belgium, until May 2002, T (Herceptin®) was provided by F. Hoffman-La Roche Ltd., Basel, Switzerland, through two national compassionate use programmes, namely the Expanded Access Programme (EAP) and the Identified Patient Programme (IPP). Patients with MBC pretreated with at least

two CT lines were allowed to receive T (EAP open until September 2000) as a single agent or in combination with CT, mainly paclitaxel. T as first line therapy was allowed only if given with paclitaxel within the IPP, while the same indications as per EAP were followed in case of administration of T as a single agent.

On site monitoring visits were performed to collect data from original source data documents: medical files, Case Report Forms (only for EAP patients), imaging material (CT scan, US, X-rays, bone scan, PET-scan).

All the responses were re-evaluated by this study coordinator. Additionally, the T alone population was used to validate the criteria defined for response assessment. An independent response review (IRR) was therefore performed before the final analysis (correlation of the clinical outcome with the expression of the tested markers) by a staff of medical oncologists and the response to treatment was re-assessed on the basis of the collected material, independently from the original assessment of the investigators. Medical oncologists reviewing the cases were blinded to laboratory results and the pathologist and co-workers to the clinical data. Since no major discrepancy was seen, IRR was not required for the T+CT population.

All the study procedures were approved by the Ethics Committee of the Jules Bordet Institute.

2.2. Patients' selection criteria

Criteria to select the study population were strictly defined before starting of the study.

Patients registered in the programmes, but never treated, as well as patients who had one to five weekly administrations of T were not included in the analysis, unless the discontinuation of treatment was unequivocally ascribed to early progressive disease. Patients with previous or concomitant advanced cancer other than BC were excluded. Patients with synchronous or metachronous bilateral BC were included only if a sample of a metastasis was available for analysis. Patients without archival samples of invasive BC were excluded (e.g. material containing exclusively carcinoma in situ, deteriorated archival samples).

The central confirmation of HER-2 overexpression by FISH (fluorescence in situ hybridisation) or IHC (immunohistochemistry; only for tumour blocks whose fixation method did not allow FISH) was mandatory.

2.3. Clinical outcome and distribution of the markers

To explore whether the expression of the selected markers could have a role in predicting response or resistance to T, two binary outcomes were considered: OR and CB rates. CB was defined as the rate of complete responses (CR) plus partial responses (PR) plus stable disease (SD) lasting not less than 6 months.

All the markers were dichotomised in two categories of negative and positive values and every value different from zero was considered positive. Since all patients were HER-2 positive, the dichotomisation of *Her-2* gene amplification was done according to the median value.

For p-p38 score any value >5 was considered positive.

2.4. Evaluation of the selected markers

Pathologic confirmation of invasive BC was performed by a hematoxylin–eosin staining.

2.4.1. Her-2 evaluation by FISH

FISH was performed using the FDA-approved dual colour probe *HER-2/neu* Spectrum orange-CEP17 Spectrum green from Vysis (Pathvision kit, Vysis, Downers Grove, CA, USA), as previously described.[26] Signals from at least 60 non-overlapping nuclei from the invasive tumour were counted. Amplification was defined as >2 ratio between the number of *Her-2* signals and CEP17. The positive and negative controls were SKBR3 and MCF-7 cell lines, respectively.

2.4.2. Growth factors receptors evaluation by IHC

Paraffin-embedded blocks routinely fixed in neutral buffered formalin were cut on poly-L-lysine-coated slides and stained with antibodies to total *Her-2* clone CB-11 (dilution 1/40 for 30 min at 37 °C; Novocastra, Newcastle, UK) and p-Her-2 clone PN2A (5 μg/ml for 3 h at 25 °C; Neomarker, Fremont, CA), to total EGFR using the DAKO EGFR pharmDx kit (DAKO, Carpinteria, CA) and p-EGFR (Tyr 1068; dilution 1/50 overnight at 4 °C; Cell Signalling Technology, MA), to Her-3 clone RTJ1 (1/20 for 1.5 h at 25 °C as previously described;[27] Novocastra) and to Her-4 clone HFR1 (dilution 1/40 for 30 min at 37 °C; Neomarker).

Antigen retrieval was performed in 10 mmol/L ethylene diamine tetraacetic acid buffer (EDTA), pH 8, for antibody to p-EGFR (15 min at sub-boiling temperature) and in citrate buffer, pH 6, for HER-2 (40 min in a water bath at 94 °C) and p-Her-2 (as previously described by Thor et al.[28]). Phospho-HER-2 Receptor (PN2A) is highly specific for activated tyrosine-phosphorylated (p-Tyr 1248) form of HER-2 receptor and does not cross-react with closely related receptors such as EGFR, HER-3 and HER-4. The specificity of clone PN2A for p-HER-2 has already been reported by Thor et al.[28] Phospho-EGF Receptor (Tyr1068) (1H12) mouse mAb detects the levels of EGF receptor only when phosphorylated at Tyr 1068. This antibody does not recognise EGF receptor phosphorylated at other sites, but may cross-react with other activated ErbB family members.

HER-4 immunostaining was performed using Ventana automated system with the highly sensitive Nexes reagents (Enhanced Nexes reagent, Ventana).

Membranous staining was required for positivity for HER-2, p-HER-2, p-EGFR and HER-3. For HER-2, the Herceptest scoring was used (DAKO, Carpinteria, CA); positive and negative controls were SKBR3 and MCF-7 cell lines, respectively. For HER-3 and p-HER-2, positive controls were a kidney biopsy and a breast specimen provided by the firm Neomarker, respectively. For EGFR and HER-4, positivity was defined as membranous and cytoplasmic staining. For EGFR >10% of cells with membranous staining was considered positive. Any percentage of cells with clearly discernible membranous staining different from 0 was considered as positive, for both p-HER-2 and pEGFR.

The positive and negative controls for EGFR were those provided with the DAKO kit [EGFR negative control: CAMA-1

cell lines (0); positive control: HT-29 cell lines (2+)]. For HER-4, the positive control was a skin biopsy.

2.4.3. *Effectors of intracellular signalling pathway: evaluation by IHC*

Paraffin-embedded sections were stained with antibodies to phosphorylated p44/42-MAPKs ERK1/2 (Thr202/Tyr 204) (p-MAPK) clone E10 (1/50 overnight at 4 °C), phosphorylated p38 (Thr 180, Tyr 182) (p-p38) clone 28B10 (1/100 overnight at 4 °C) (Cell Signalling Technology, Beverly, MA) and p27 clone 1B4 (dilution 1/50 overnight at 4 °C; Novocastra, Newcastle, UK). Antigen retrieval was performed in 10 mmol/L EDTA, pH 8, for antibody to p-MAPK (as described by Albanell et al.[29]) and in citrate buffer, pH 6, for p-p38 and p27 (as described by the manufacturer). Phospho-44/42 MAP Kinase (Thr202/Tyr204) E10 mAb detects endogenous levels of p44 and p42 MAP kinase (Erk1 and Erk2) dually phosphorylated at threonine 202 and tyrosine 204. This antibody does not cross-react with the corresponding phosphorylated residues of either SAPK/JNK or p38 MAP kinase. Phospho-p38 MAPK (Thr180/Tyr182) (28B10) mAb detects p38 MAP kinase only when activated by dual phosphorylation at threonine 180 and tyrosine 182. This antibody does not significantly cross-react with the corresponding phosphorylated forms of either p44/42 MAPK (Erk1/2) or SAPK/JNK. It does not detect non-phosphorylated p38 MAP kinase. This antibody has been extensively used.[34]

Nuclear staining defined positivity for p-MAPK, p-p38 and p27. For p27, $\geqslant$50% of cells with nuclear staining was considered positive.[30–33] For p-p38, an intensity-adjusted scoring system (combining % and intensity of staining) was used according to Esteva et al.[34] Briefly, this score system was set by multiplying the value corresponding to the percentage of positive cells (1 = less than 10%, 2 = 10–50%, 3 = more than 50%) and the degree of intensity (1 = weak, 2 = moderate, 3 = intense staining). A score >5 was defined as positive. The positive control for p-MAPK was a synovial-sarcoma. For p27, tonsil and MCF-7 cells were used as positive control and MDA-MB-231 cell line as negative control.

2.4.4. *Hormone receptors evaluation by IHC*

ER and PgR immunostainings were performed with mouse monoclonal antibodies clone 6F11 (dilution 1/40) and 1A6 (dilution 1/20), respectively (Novocastra, Newcastle, UK). Antigen retrieval was performed in citrate buffer, pH 6, as previously described.[35] The Ventana Nexes automated immunostainer (Ventana Medical Systems, Tucson, AZ) was used with standard reagents for ER staining and highly sensitive reagent for PgR staining. ER and PgR scores were expressed as percentage of tumour cells with positive staining. The tumours were defined as ER- or PgR-negative if <10% of tumour cells had positive immunostaining. Positive control was MCF-7 cell line.

2.5. *Statistical analysis*

The correlation between the expression of the markers, assessed as continuous variables, was measured through the calculation of non-parametric Spearman's rank correlation coefficients (the assumption of normality of the distributions was not verified for some markers, preventing the use of parametric methods). The statistical significance of these coefficients was assessed by testing their equality to zero. The same method was applied to evaluate the correlation between percentage of stained cells and intensity of staining for every marker.

As all the included patients overexpressed HER-2, the association between *Her-2* gene amplification (FISH values) and clinical outcomes (CB and OR) was estimated considering *Her-2* as a continuous variable by fitting the data with a logistic regression model; odds ratio for an increment of 1 unit in the value of FISH was assessed and its significance tested using a likelihood ratio test. A binary assessment was also made splitting the patients into two groups according to the median FISH value.

For the analysis of the relationship between all the other markers and clinical outcome, the markers were analysed as continuous variables or were dichotomised by considering a case as positive if expression was >0. For p-p38 MAPK an intensity-adjusted scoring system was also taken into account with the marker defined as positive when >5. The influence of the markers on the clinical outcomes was assessed by using Mann–Whitney tests chi square tests (when for ordered variables, both percentage of stained cells and intensity of the staining were taken into account) or Fisher exact tests.

OS and TTP distributions were estimated by the non-parametric Kaplan–Meier method and compared using log-rank tests.

All reported p values are two sided: a p value <5% was considered as significant, a p value between 5% and 10% was considered as highlighting a trend for significance.

3. Results

We reviewed 248 medical files, with 122 patients registered in the EA and 126 in the IP programmes. Two populations were identified: (1) 105 patients that received single agent T and (2) 123 patients that received T+CT.

Among the patients who started the combination T+CT (mainly with paclitaxel), six patients received only one dose of CT (weekly regimens) and single agent T for the rest of their treatment; therefore for response to treatment analysis they were included in T-alone population.

3.1. *I: Study population 1. Patients treated with single agent T*

Among the patients who received single agent T, 46 met the inclusion criteria, with confirmed HER-2 overexpression, either by IHC (3+: seven patients) or FISH (39 patients).

Only three received T as first-line treatment. Median number of previous CT lines was two (range 1–4), and 46% of patients had received hormonal therapy (HT) for MBC.

Time on T treatment was 5.7 months (range 1.1–24.1 months), with a median number of 24.5 administered cycles (range 6–87).

Thirty-three of the collected archival samples corresponded to primary BC (71.7%), three to a local relapse (6.5%) and 10 to a site of MBC (21.7%).

3.1.1. *Response to treatment and survival times*

One CR (2.2%) and nine PR (19.6%) were reported for an ORR of 21.7% (95% CI: 10–34%). In 21 patients (45.7%), the best response to treatment was SD, which lasted not less than 6 months in 11 patients (23.9%) and up to 5 months for the rest of them (10 patients, 21.7%). The CB rate was 45.7% (95% CI: 31–60%).

Median TTP was 6.2 months (95% CI: 4.1–8.2) and median OS was 26.3 months (95% CI: 18.8–33.7).

None of the 12 patients excluded from the analysis due to lack of *Her-2* amplification by FISH or low level of expression by IHC ($\leqslant$2) responded to T.

3.2. *II: Study population 2. Patients treated with T+CT*

This population consisted of 57 evaluable patients, whose archival samples showed HER-2 overexpression by IHC (two patients) or amplification by FISH (55 patients). Median age of the study population was 51 years (29–76), ECOG PS 1 (0–3); 22 patients (40%) received adjuvant HT and 38 (65.5%) adjuvant CT. Median number of metastatic sites was two (range 1–4) and 40 patients (69%) had visceral involvement. Median number of treatment lines for MBC was one for HT and two for CT. Median duration of T+CT treatment was 8.1 months (0.5–35.2 months). Forty-five (79%) of the 57 collected archival samples were from the primary BC, two (3.5%) from local relapses and 10 (17.5%) from metastatic sites.

3.2.1. *Response to treatment and survival times*

Ten patients had a CR (17.5%) and 21 a PR (36.8%), for an ORR of 54.3% (exact 95% CI: 41–54%). SD was documented in 17 patients (29.8%), of at least 6 months in eight patients (14%). CB rate was 68.4%. PD was seen in nine patients (15.8%).

Actuarial median TTP was 6.1 months, actuarial median survival time was not reached, with a survival rate of 75% of patients at 13 months.

3.3. *Markers expression in the entire population of 103 patients*

Of 103 evaluable patients with HER-2 overexpressing BC, 94 cases were centrally confirmed to be FISH+, and nine 3+ by IHC. The median value of FISH was 7.26 (range 2.33–13.6) in population 1 and 6.90 (range 2.2–13.96) in population 2.

The paucity of available tissue limited the assessment of the selected markers in the entire group of 103 patients. Therefore, for each marker the number of positive cases out of the number of samples on which the test was performed is specified.

HER-2 was phosphorylated in 15 of 100 tumour samples (15%), six of 46 samples (13%) in population 1 and nine of 54 cases (16.6%) in population 2 (in this group p-HER-2 was not assessable in 3 samples, due to paucity of cancer tissue).

Nineteen of 87 samples (21.8%) tested for EGFR were positive. In EGFR positive samples, a complete concordance between the membranous and the cytoplasmic staining was observed. EGFR was phosphorylated in 12 of the 19 EGFR positive cases (63.1%). Fifty-nine of 87 samples (67.8%) and 51 of 86 (59.3%) were positive for HER-3 and HER-4, respectively.

Positivity for phospho-ERK 1/2 MAPKs was seen in 47% of cases (43/91), and p27 was positive in 86.5% of the cases (77/89), with a percentage of stained cells $\geqslant$50% in 35/77 cases (45.4%).

Thirty-three of 82 cases (40.3%) showed a staining for p-p38, but when the score system was applied only five samples were considered positive (6%).

ER was positive in 34.1% (29/85) of cases and PgR in 23.5% (21/89).

The correlation coefficients between markers were calculated and the full panel of results is reported in Table 1.

As expected, p-HER-2 correlated positively with p-MAPKs (coefficient: 0.299, $p = 0.004$), and both with p-p38 (coefficient: 0.275, $p = 0.012$) and p-p38 score (coefficient: 0.313, $p = 0.0042$), whereas a negative correlation with PgR emerged (coefficient: -0.216, $p = 0.0042$).

EGFR was positively correlated with its activated form, p-EGFR, and both inversely correlated with hormonal receptors.

HER-3 was co-expressed with p-p38 and p-p38 score. No correlation emerged for HER-4 with any other marker.

As far as MAPKs are concerned, they correlated positively with p27 and p-p38, other than with HER-2. Conversely, MAPKs, p-p38 and p-p38 scores were all inversely correlated with EGFR.

p27 positively correlated with ER, p-p38 and p-p38 scores (inversely with EGFR).

A positive correlation of ER with PgR was also found.

When the expression of the markers was analysed separately in each population, the positive correlations of EGFR with p-EGFR, HER-3 with HER-4 and ER with PgR were found in the single agent T group. The inverse correlations of HER-3 with MAPKs, EGFR with both ER and PgR, and p-EGFR with ER were also observed.

In the T+CT group, a statistically significant ($p < 0.05$) correlation between p-MAPKs ERK1/2 and p-HER-2 was observed (0.318, $p = 0.031$), and p-MAPKs were also correlated to the p-p38 score, whereas no correlation appeared between p-HER-2 and p-p38.

3.4. *Association of phosphorylated HER-2 with the other markers*

The distribution of the markers was analysed according to the phosphorylated status of HER-2. The expression rate of each marker in the group with phosphorylated HER-2 (hereafter referred as positive p-HER-2) was compared with the same value in the group with unphosphorylated HER-2 (negative p-HER-2). MAPKs were significantly more expressed in patients with positive p-HER-2 than those with negative p-HER-2 (79% versus 42%, $p = 0.02$), as well as p-p38 (75% versus 34%, $p = 0.01$). Conversely, PgR was exclusively associated with negative p-HER-2 (27% versus 0%, $p = 0.06$). None of the other markers showed a preferred pattern of expression according to the p-HER-2 status.

3.5. *Clinical outcomes and distribution of the markers*

Both in the single agent T and in the T+CT populations, no difference was observed in CB rates according to the expression of the markers, when every variable was

Table 1 – Correlations coefficients in 103 patients

		p-HER-2	EGFR	p-EGFR	HER-3	HER-4	p-MAPK	p27	p-p38	p-p38 score	ER	PgR
Spearman's rho												
p-HER-2	Correlation coefficient	1.000	.009	.050	.108	.060	**.299** (**)	.090	**.275** (*)	**.313** (**)	−.091	**−.216** (*)
	Sig. (2-tailed)		.937	.649	.318	.586	**.004**	.403	**.012**	**.004**	.406	**.042**
	N	100	87	85	87	86	91	89	82	82	85	89
EGFR	Correlation coefficient	.009	1.000	**.787** (**)	.007	.023	**−.265** (*)	**−.258** (*)	**−.264** (*)	**−.261** (*)	**−.339** (**)	**−.292** (**)
	Sig. (2-tailed)	.937		**.000**	.951	.833	**.014**	**.018**	**.019**	**.020**	**.002**	**.006**
	N	87	88	86	87	86	85	84	79	79	85	87
p-EGFR	Correlation coefficient	.050	**.787** (**)	1.000	−.124	.036	−.113	**−.231** (*)	**−.267** (*)	**−.268** (*)	**−.267** (*)	**−.228** (*)
	Sig. (2-tailed)	.649	**.000**		.259	.744	.307	**.036**	**.019**	**.019**	**.014**	**.035**
	N	85	86	86	85	84	84	83	77	77	84	85
HER-3	Correlation coefficient	.108	.007	−.124	1.000	−.014	−.083	.185	**.306** (**)	**.263** (*)	−.007	−.013
	Sig. (2-tailed)	.318	.951	.259		.899	.452	.092	**.006**	**.020**	.950	.908
	N	87	87	85	88	87	85	84	78	78	85	87
HER-4	Correlation coefficient	.060	.023	.036	−.014	1.000	−.095	.121	.048	.061	−.008	−.007
	Sig. (2-tailed)	.586	.833	.744	.899		.392	.274	.681	.597	.939	.946
	N	86	86	84	87	87	84	83	77	77	85	87
p-MAPK	Correlation coefficient	**.299** (**)	**−.265** (*)	−.113	−.083	−.095	1.000	.109	**.344** (**)	**.344** (**)	.033	.090
	Sig. (2-tailed)	**.004**	**.014**	.307	.452	.392		.316	**.002**	**.002**	.766	.408
	N	91	85	84	85	84	92	87	80	80	84	86
p27	Correlation coefficient	.090	**−.258** (*)	**−.231** (*)	.185	.121	.109	1.000	**.244** (*)	**.244** (*)	**.270** (*)	.094
	Sig. (2-tailed)	.403	**.018**	**.036**	.092	.274	.316		**.029**	**.029**	**.013**	.392
	N	89	84	83	84	83	87	90	80	80	83	85
p-p38	Correlation coefficient	**.275** (*)	**−.264** (*)	**−.267** (*)	**.306** (**)	.048	**.344** (**)	**.244** (*)	1.000	.973 (**)	.192	.044
	Sig. (2-tailed)	**.012**	**.019**	**.019**	**.006**	.681	**.002**	**.029**		.000	.094	.701
	N	82	79	77	78	77	80	80	83	83	77	79

p-p38 score	Correlation coefficient	**.313 (**)**	**−.261 (*)**	**−.268 (*)**	**.263 (*)**	.061	**.344 (**)**	**.244 (*)**	**.973 (**)**	1.000	.201	.016
	Sig. (2-tailed)	**.004**	**.020**	**.019**	**.020**	.597	**.002**	**.029**	**.000**		.079	.887
	N	82	79	77	78	77	80	80	83	83	77	79
ER	Correlation coefficient	−.091	**−.339 (**)**	**−.267 (*)**	−.007	−.008	.033	**.270 (*)**	.192	.201	1.000	**.361 (**)**
	Sig. (2-tailed)	.406	**.002**	**.014**	.950	.939	.766	**.013**	.094	.079		**.001**
	N	85	85	84	85	85	84	83	77	77	86	86
PgR	Correlation coefficient	**−.216 (*)**	**−.292 (**)**	**−.228 (*)**	−.013	−.007	.090	.094	.044	.016	**.361 (**)**	1.000
	Sig. (2-tailed)	**.042**	**.006**	**.035**	.908	.946	.408	.392	.701	.887	**.001**	.
	N	89	87	85	87	87	86	85	79	79	86	90

Evidence of a trend or a significant correlation between the expression of different markers is marked in bold.
* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

examined independently from the others. There was therefore no indication to perform a recursive analysis to define possible clusters of markers predictive of response to treatment.

All the markers have also been studied as continuous variables, and again, no difference emerged when the CB group was compared to its counterpart.

Similar results were found in the single agent T group when overall RR was examined (Fig. 1).

Conversely, in T+CT group, p-HER-2 showed a statistically significant impact on response (next paragraph and Fig. 2).

3.6. Clinical outcomes and p-HER-2

In the T+CT population p-HER-2 was associated with a higher RR. Particularly, eight patients out of nine (89%) with activated p-HER-2 experienced a response to treatment (six PR and two CR), versus 22 out of 45 patients with negative p-HER-2 (49%, $p = 0.03$). These findings were not observed when CB rate was considered.

It was noted that it was not necessary to have p-HER-2 in order to find activated MAPKs (ERK 1/2), but seven of the eight responders with p-HER-2 also had activated MAPKs, although this association did not reach statistical significance ($p = 0.11$).

In the single agent T population p-HER-2 was not predictive of response. Particularly, one patient out of six (17%) with p-HER-2 derived a benefit from treatment (SD for 7 months), versus 20 out of 40 patients with negative p-HER-2 (50%, $p = 0.20$). The same data emerged when only OR to T was analysed.

In the T+CT subgroup, actuarial median TTP in patients with p-HER-2 positive was 6.7 months versus 6.1 months for p-HER-2 negative. Taking into account the small sample size, no formal comparison was performed. In the T alone subgroup, actuarial median TTP was 3.1 months in the p-HER-2 positive group compared to 6.7 months in the non-phosphorylated group. Although these data seemed to be consistent with higher OR rate observed in patients with non-phosphorylated HER-2, a formal comparison was not carried out because of the small sample size. OS data were not mature at the time of the analysis.

3.7. Clinical outcomes and FISH amplification

Her-2 amplification was regarded as a continuous variable and its possible impact on responsiveness to T was explored in the entire group of 103 patients, and separately in each population. Among the 103 patients, the estimated odds ratio for progressive increases of 1 unit of FISH values was 1.23 (95% CI: 1.05–1.43, $p = 0.009$) for OR and 1.13 (95% CI: 0.98–1.31, $p = 0.09$) for CB. When adjusted for treatment, T+CT versus single agent T, the odds ratios were 4.72 (95% CI: 1.80–12.34, $p = 0.002$) for OR and 2.4 (95% CI: 1.01–5.70, $p = 0.09$) for CB.

The same analysis was also run in each population separately. In the single agent T group a statistical trend to significance emerged, with an incremental odds ratio of 1.31 (95% CI: 0.96–1.78, $p = 0.09$, Fig. 3a). In the T+CT group the association of increasing FISH values with response was statistically

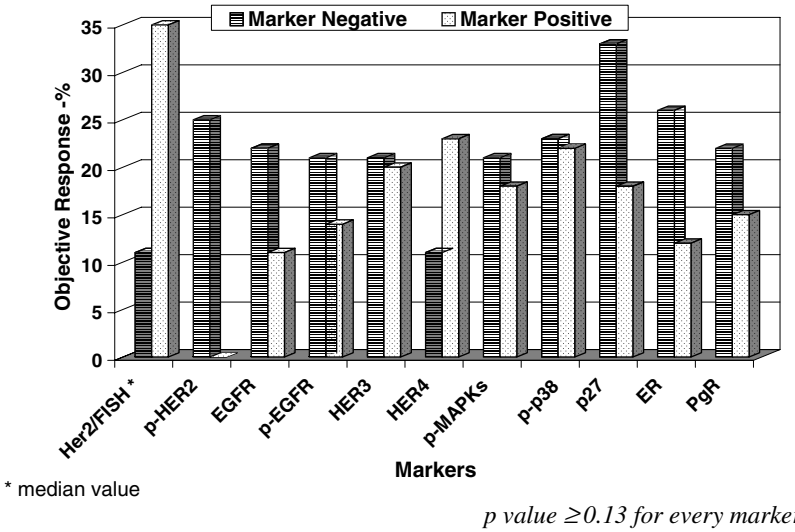

Fig. 1 – Association between markers and objective response in the study population 1.

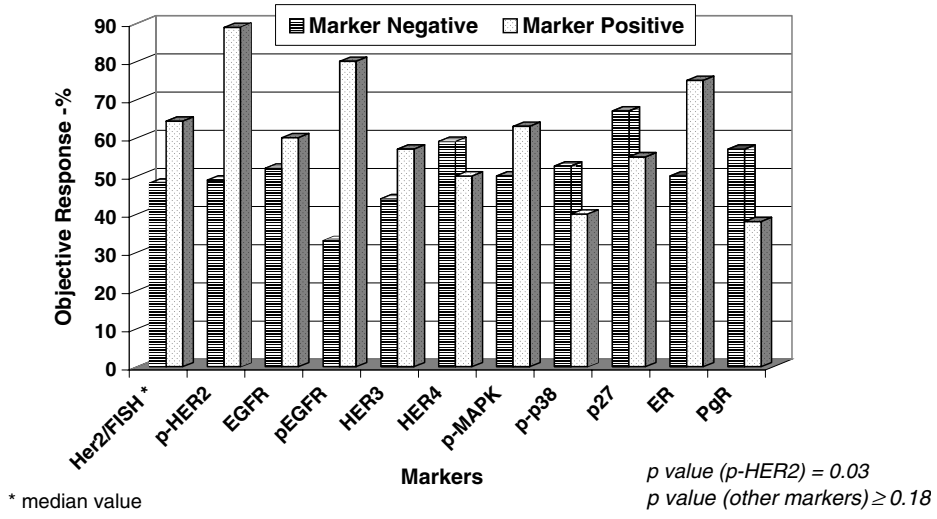

Fig. 2 – Association between markers and objective response in the study population 2.

significant ($p = 0.05$, Mann–Whitney test), with an incremental odds ratio of 1.20 (95% CI: 1.01–1.43, $p = 0.04$, Fig. 3b).

Unlike the findings in the entire group of 103 patients, no statistical significant association of FISH values was observed in both populations with respect to CB rate.

It should also noted that (1) the combination of the two variables, progressive FISH values and positive p-HER-2, did not yield a better response prediction; (2) no significant predictive cut-off value of FISH was identified.

4. Discussion

We report the results of a retrospective study aiming at finding better predictive factors of response/resistance to T, in 103 HER-2-overexpressing MBC patients treated with single agent T (46 patients) or with T+CT (57 patients).

More than half of the initially identified population was not eligible mainly due to technical problems related to the

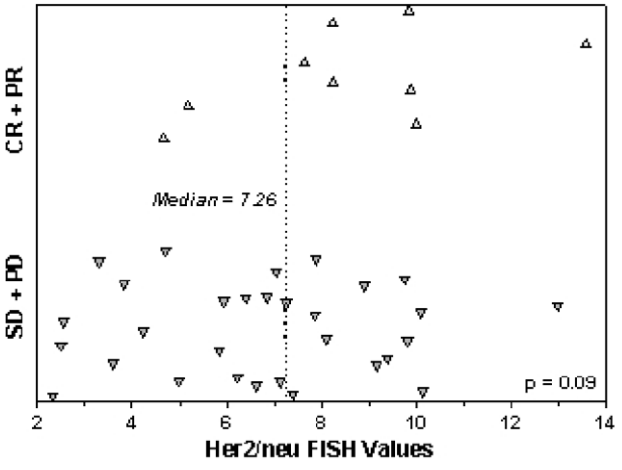

Fig. 3a – Association between Her2/neu gene amplification and objective response (CR + PR) in population 1.

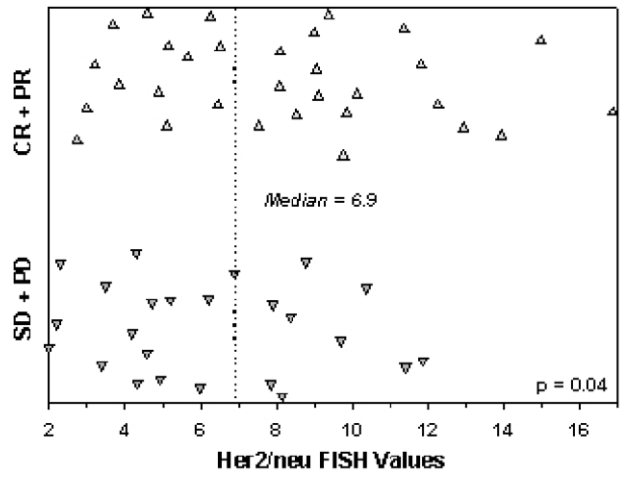

Fig. 3b – Association between Her2/neu gene amplification and objective response (CR + PR) in population 2.

archival samples (e.g.: HER-2 overexpression not confirmed centrally, no remaining invasive cancer) or to the lack of the objective evaluation of the clinical outcome (patients treated within compassionate programmes). However, no statistical significant difference was found between the TTP and OS of the eligible patients and those of the 29 excluded patients ($p = 0.41$) (data not shown). Additionally, the OR and the p-HER-2 positive rates indicate that no major selection bias occurred. Indeed, (1) the rates of OR and CB in the eligible patients are comparable to those of previously published prospective trials;[7,8,36] (2) p-HER-2 was present in 15% of the global population (103 patients), in 13% of single agent T patients, and 16.6% of T+CT patients. These rates agree with those reported in a previous study of 816 primary breast cancers where p-HER-2 positivity was seen in 12% of cases.[28]

The main goal of our study was to evaluate the role of p-HER-2 as a predictive marker of response to T. We hypothesised that T effect would be higher in tumours dependent on the HER-2 pathway. Therefore, p-HER-2 was expected to be higher expressed in responsive patients.

The predictive value of p-HER-2 in BC was previously reported in only one retrospective study including 69 cases, 54 of which were evaluated only by IHC as HER-2 3+. The majority of patients had been treated with T in combination with different CT, whereas only six received T as a single agent. The authors reported a trend for statistical significance when OR and CB rates were correlated to p-HER-2.[38]

In our study, the role of p-HER-2 was evaluated separately in two populations, T alone and T+CT, presuming that the interaction between T and CT could favour one mechanism of action of T over the others. In our single-agent population, no statistically significant differences were found in terms of OR and CB rates between p-HER-2 positive and p-HER-2 negative patients, but the finding that five out of six p-HER-2 positive patients did not benefit from T was quite surprising and against our initial hypothesis. On the contrary, in the T+CT population the activated axis p-HER-2 and p-MAPKs was essential for T efficacy, with 89% of p-HER-2 positive patients obtaining a response versus 49% of p-HER-2 negative ($p = 0.03$). Although the characteristics of the two populations could partly account for the contrasting results, other reasons can be speculated, such as a different mechanism of action of T when given as a single agent or in combination with CT.

It is known that HER-2 proteolytic cleavage results in the formation of two constitutively activated (i.e. phosphorylated) fragments: the intracellular and the aminoterminal 95kDA. Moreover, several studies have proposed the shed HER-2 ectodomain (ECD) as a poor prognosis marker. T efficacy may depend also on the ability to prevent the shedding of HER-2 ECD, with increasing data supporting the predictive role of HER-2 ECD.[39–43] Unfortunately, our study did not allow the testing of the abovementioned hypothesis or other possible mechanisms of action of T, such as its antibody dependent cellular cytotoxicity.

In the 46 patients receiving single agent T, the analysis of the expression of the markers showed a high incidence of HER-3 (56.8%). This result is not unexpected, since it is known that HER-3 is highly co-expressed in HER-2+ tumours. In addition, heterodimers HER-2-HER-3 are preferentially formed and represent the most powerful dimers in terms of capability to initiate an intracellular cascade.[29,37] It has been postulated that the HER-3 heterodimers interact preferentially with PI3K/Akt, because of the presence in the intracellular domain of HER-3 of six docking sites for the p85 adaptor subunit of PI3K. Interestingly, a strong inverse correlation between HER-3 and MAPK (-0.444, $p = 0.003$) was found in our study, which may be in favour of a preferential coupling of HER-3 with PI3K/Akt.

The second key-finding of our study concerns the role of quantitative *Her-2* gene amplification as predictive marker of OR both in single-agent T and in T+CT populations. Data from *in vitro* models of transfected breast and ovarian cells led to speculate that HER-2 overexpression should reach a threshold value in order to induce resistance to taxanes[44] and implied that the magnitude of *Her-2* amplification could impact differently on the efficacy of T alone or T+CT. Interestingly, while in the single agent population only a trend to significance emerged, in the T+CT group the association between *Her-2* and increasing probability of OR was statistically significant.

Disappointingly, the combined use of the variables associated with a better clinical outcome, namely *Her-2* amplification and p-HER-2, did not result in a more powerful prediction of response. This study was designed to analyse a clinical short-term endpoint, i.e. ORR. Data on TTP and OS were collected, but the length of the follow-up and the sample size of each population did not allow us to perform meaningful analysis on TTP and OS according to p-HER-2 status.

Some recently published papers[45–47] highlighted the potential predictive role of HER receptors mutations. It was also shown that loss of the tumour suppressor gene *PTEN* confers resistance to T in HER-2+ BC.[48] These markers could not be evaluated in our study.

New technologies allowing for the simultaneous measurement of key actors of these pathways, such as dimers identification[49] and gene expression analysis, are in development and may prove superior to current assays for predicting response to anti-HER therapies.

Finally, it could be argued that more than two-thirds of the centralised archival samples in both populations (71.7% and

79%, respectively) correspond to primary and not MBC; although there is evidence of very high concordance in HER-2 status between primary and metastatic sites,[26,50] it is not known whether this is also applicable to p-HER-2.

In conclusion, the role of p-HER-2 and quantitative *Her*-2 gene amplification in predicting response to T could be of interest, especially when T is given with CT. The number of patients, their characteristics (e.g. slightly more pre-treated patients in the T alone than in the T+CT group) and the retrospective design of the study could partly account for the divergent findings concerning the role of p-HER-2 in the two populations. The confirmation of these interesting data in larger and more homogenous T-treated populations such as those from large phase III trials is warranted.

Conflict of interest statement

None declared.

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