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From Cell Biology to Tissue Engineering

Novel biomarkers in primary breast core biopsies to predict poor response to neoadjuvant chemotherapy and appearance of metastases

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Summary. Drug resistance has been one of the major obstacles limiting the success of cancer chemotherapy. In two thirds of breast cancer patients, large (>1cm) residual tumors are present after neoadjuvant chemotherapy (NCT). The residual tumor and involved nodes have been indicators of relapse and survival very important in breast cancer.

The goal of this preliminary study was to assess the predictive significance of a panel of molecular biomarkers, related with the response to treatment or drug resistance to NCT, as determined on the diagnostic tumor. The expression of 22 proteins was examined using immunohistochemistry in tissue microarrays (TMA) from 115 patients of stage II-III breast cancer, treated with NCT. Among studied proteins, there are some that are anti-apoptotic, pro-proliferative, cancer stem cell markers and the Vitamin D Receptor. Other proteins are involved in the identification of molecular subtype, cell cycle regulation or DNA repair. Next, a predictive signature of poor response was generated from independent markers of predictive value. Tumors that expressed four or five conditions (biomarkers of chemoresistance with a determinated cutoff) were associated with a 9-fold increase in the chances of these patients of having a poor response to NCT. Additionally, we also found a worse prognostic signature, generated from independent markers of prognostic value. Tumors which expressed two or three conditions of worst prognostic, were associated with a 6-fold reduction in Distant Disease Free Survival. In conclusion, finding biomarkers of chemoresitance (ypTNM II-III) and metastases can become a stepping stone for future studies that will need to be assessed in a bigger scale.

Key words: Biomarkers, Chemoresistance, Metastases, Immunoexpression, Core biopsy

Introduction

Currently, there has been an important reduction of breast cancer (BC) mortality and recurrences due to modern neoadjuvant chemotherapy (NTC) and investigating tumor characteristics (age, nodal status, tumor differentiation, estrogen receptor [ER] status, use of tamoxifen) (Peto et al., 2012). Despite improved treatment, drug resistance has been one of the major

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Abbreviations. ALDH-1, Aldehyde deshidrogenase-1; BrCa, Breast Cancer; Bcl-2, B-cell lymphoma -2; CTSL, Cathepsin L; DDFS, Distant Disease Free Survival; ERα, Estrogen Receptor α; FGFR-2, Fibroblast growth factor receptor-2; Hsc, Histo-score; HR, Hazard Ratio; HER-2, human epidermal growth factor receptor-2; HER-4, human epidermal growth factor receptor -4; IHC, immunohistochemistry; OR, Odds-ratio; p-Akt, phospho serine/threonine-specific protein Kinase; pHER-3, phosphorylated human epidermal growth factor receptor-3; PTEN, Phosphatase and tensin homolog; PR, progesterone receptor; NCT, neoadjuvant chemotherapy; TMA, tissue microarrays; VDR, vitamin D receptor; ypTNM, pathological TNM after neoadjuvant treatment

obstacles limiting the success of cancer chemotherapy (Jiang et al, 2012). The residual tumor and involved nodes have been indicators of relapse and survival very important in breast cancer (Corben et al., 2013). In fact, it was well known that about 35% of patients with a macroscopic residual tumor (>1cm) relapsed with 3 years, and less than 30% of patients achieved an optimal or complete response to NTC (Buzdar, 2007).

One-third of patients did not benefit from chemotherapy (estrogen-receptor-positive disease) and were only treated with endocrine therapy, which presented 10-year survival without chemotherapy (Peto et al., 2012), as in the case of luminal A tumors.

In recent years, it has been reported that steroid hormones and their receptors might decrease the therapeutic efficacy of antineoplastic drugs. Approximately, around 65% of breast tumors expressed estrogen receptor alpha (ERα). Binding of estrogen to its receptor induced tumor growth and could also inhibit apoptosis by upregulating Bcl-2 expression. Thus, it is necessary to have information about tumor gene expression markers or quantitative immunohistochemistry that might help to predict tumor with low absolute risk-benefit to NCT (Peto et al., 2012).

Definitely, it has long been known that breast tumors expressing ERα protein (ERα+) behave in a fundamentally different way to ERα-negative (ERα-) tumors (Jiang et al., 2012). For this reason, we also decided to find chemoresistance specific markers for ERα+ tumors.

The pathological measurement of residual disease after NTC is an important prognostic parameter that can influence outcomes in patients with stage II and III disease (Cockburn et al., 2014). We discarded the Miller and Payne classification because it ignores tumor size and nodal status altogether, and estimates only the decrease in cancer cellularity after treatment (Ogston et al., 2003). Hence, we considered using the AJCC classification, based on ypTNM system (which analyses treatment results undergoing surgery following NCT), which is useful for predicting distant relapse and survival, and which assesses residual tumor in the breast and axillary surgical specimens after NCT. Consequently, patients with residual tumors with the highest pathological stages were associated to lower Distant Disease Free Survival (DDFS) rates (Carey et al., 2005; Charpin et al., 2012). On the other hand, patients with pathological complete response (ypTNM0) were associated to long-term outcome. Overall, this association was stronger in patients with aggressive breast cancer subtypes, such as hormone-receptor-negative tumors (Cortazar et al., 2014).

In this study, we evaluated 21 proteins selected because of the reported evidence of their relevance in breast cancer in addition to markers of major signaling pathways. These proteins are involved in: the identification of molecular subtype (Ki67, ER, PR, HER-2, CK5/6, EGFR); blockade of apoptotic pathways (Bcl-2 and survivin), activation of proliferative signaling pathways (NF-κβ p65, p-HER-3, HER-4, FGFR2, E-cadherin, GATA-3 pAKT, and PTEN); cell cycle regulation (Cyclin D1 and p27); the cancer stem cell marker aldehyde deshidrogenase -1 (ALDH-1) and DNA repair proteins (53BP1, cathepsin L and Vitamin D Receptor (VDR)). The purpose of this study was to identify biomarkers of chemoresistance (ypTNM II and III) and biomarkers of metastases, both assessed in the core biopsy before starting to receive NTC.

Materials and methods

Patients

The series was composed of 115 breast cancer patients, stages II / III, diagnosed and treated at Hospital Universitari Arnau de Vilanova, in Lleida (HUAV) Spain (1996-2010). An informed consent was obtained from each patient and the study was approved by the local Ethical Committee.

After pathologic diagnosis in a core biopsy, every patient underwent pre-operative administration of anthracycline and/or taxane based regimen (4 to 8 cycles). More specifically, most patients (85%) were treated with 6 or 8 (tandem) cycles of anthracycles and taxanes. Approximately 6 weeks after NTC, standardized surgery (mastectomy or tumorectomy in function to tumoral size) was performed. After surgery, all patients with luminal A/B tumors received the standard treatment of adjuvant hormonotherapy (tamoxifen for 5 years). Additionally, patients, who had been subjected to a conservative surgery were treated with adjuvant radiotherapy (AR) in both breast and tumoral bed. However, patients who had more 3 involved nodes were treated with AR to axilla. On the other hand, 18% of patients were treated with AR in the chest wall and after in the axilla, because their tumors were larger than 5cm and they had been subjected to radical surgery. Finally, none of the patients received adjuvant chemotherapy and trastuzumab. Response grade to NTC was evaluated according to the AJCC classification, based on ypTNM system (assessment of residual tumor in the breast and axillary surgical specimens after NCT). Thirty-nine patients had good response to NTC (ypTNM 0 and I) with 34.2%, while 75 patients had bad response (ypTNM II and III) with 65.8%.

All tumor tissues were subjected to formalin-fixation and paraffin-embedding. The molecular subtype of the tumors was determined by IHC with the 3 biomarkers (Ki67, HER2, ERα and PR) in the core biopsy. Follow-up data, recorded prospectively for all patients, included: age, primary tumor size, residual tumor size, stage, stage of residual tumor based on AJCC classification (ypTNM of residual tumor after NTC).

Follow-up time was calculated from the date of tumor resection up to the date of death, or up to the last visit for surviving patients. The last follow-up recording

was conducted in November 2011. DDFS was defined as the time free of distant recurrences or cancer-related death after primary treatment and surgery, censoring this time for those patients where these events were not observed.

Preparation of Tissue Microarray (TMAs)

TMAs were built using a tissue arrayer device (Beecher Instrument). Representative areas of all samples were marked in the corresponding paraffin blocks of the core biopsies obtained before starting NAC. In each sample, three cylinders were selected from the tumor area per patient (0.6 mm of largest diameter) for immunohistochemical analysis.

Immunohistochemical analysis

TMA blocks were sectioned at a thickness of 3 μm, dried for 1 h at 65°C after being dewaxed in xylene and rehydrated through decreasing concentrations of ethanol, and washed with PBS. Then, all TMA sections were subjected to antigen retrieval for all antibodies though heat treatment at 95°C for 20 min in buffer (DAKO). Before staining, endogenous peroxidases were blocked. Primary antibodies were: Ki67 (ready-to-use; MIB; DAKO); ER (ready-to-use; 1D5; DAKO); PR (ready-to-use; PgR636; DAKO);Her2 (Herceptest kit; Dako); NF-κβ p65 (1:600,c-20; Santa Cruz Biotechnology, Inc); Bcl-2 (ready-to-use; 124 ; DAKO); PTEN (1:50; 6H2.1; DAKO; incubation 40 min at room temperature); 53BP1 (1:2500; NB100-304; Novus Biologicals; inc); ALDH-1 (1:100; 44; BD Transduction Laboratories); Cyclin D1 (ready-to-use; EP12; DAKO); E-cadherin (ready-to-use; NCH-38; DAKO); p-HER3 (1:200; 21D3; Cell Signaling); pAKt (1:50; 736E11; Cell Signaling); VDR (1:2000; ab3508; Abcam); Survivin(1.50; D-8; Santa Cruz Biotecnology); FGFR2 (1:200; ab58201; Abcam); CK5/6 (ready-to-use; D5/16 B4; Dako); EGFR (ready-to-use; EGFR pharmaDx; DAKO); p27 (1:100; SX53G8; DAKO); and HER-4 (1:2;RB-9045; Thermo Scientific). For all of these antibodies, the reaction was visualized with Envision Flex (Dako). Sections were counterstained with hematoxylin. Appropriate positive and negative controls were tested simultaneously.

Pathologic evaluation of response as well as immunohistochemical evaluation was done by two pathologists. Discrepancies were solved by joint examination.

Histo-score (Hsc) provide semiquantitative measurements of protein expression by taking into consideration both the percentage of positive cells and the intensity of their staining. An Hsc ranging from 0 (no immune reaction) to 300 (maximal immunoreactivity) was obtained with the formula Hsc = 1× (% light staining) + 2× (% moderate staining) + 3× (% strong staining).

Her2 staining was evaluated according to a standard protocol (HercepTest; Dako), considering negative Her2 expression for intensity values of 0, 1+, and 2+ when there was no amplification by FISH. Positive Her2 expression for intensity values of 3+ and 2+, when 2+ was amplified by FISH.

EFGR staining was evaluated according to a standard protocol of EGFR pharmaDx; DAKO) and scored as (i.e., negative = 0; weak = 1+; moderate = 2+; and strong = 3+).

The reliability of TMA immunostaining was confirmed by comparing TMA cylinders and the corresponding whole-sections obtained from the paraffin donor blocks from 20 randomly selected cases (Rimm et al., 2001).

ERα, PR, Ki67 and Her2 stainings were evaluated in whole sections from paraffin blocks from all patients

Sample size

From our sample of 115 patients of whom 75 had a poor response to NCT, we detected biomarkers with minimum statistically significant odds-ratio of 2.9, assuming a minimum prevalence of poor responses of 40% in the group of reference that associated to poor response to NCT leads to a statistical power of 80%.

Statistical analysis

In this validation process, the reliability was studied for quantitative variables through Intra-class Correlation Coefficients in the quantitative Hsc, all of which ranged from 0.612 to 0.975. While categorical variables (membrane HER4 and p-HER3) were validated with a kappa Index, which was of 1. The correlation between Hsc of TMA cylinders and those from whole-sections for all tumor markers has been confirmed.

Univariate and multivariate logistic and Cox proportional hazards regression models were used to evaluate the association of the selected biomarkers and protein expression profiles, calculating odd-ratios (OR, for logistic models) and hazard-ratios (HR, for Cox models) to measure their association with the risk of progression and the distant disease-free survival time and computing 95% confidence intervals. The cutoff point for each biomarker was selected from the value that optimized the logistic model to improve the discrimination of chemoresistant tumors and appearance of metastases.

Survival curves of distant disease-free survival (DDFS) were analyzed using Kaplan-Meier and evaluated with log-rank test. Individually significant biomarkers ($p<0.05$) were used to establish a combination of biomarkers with prognostic and predictive value using a stepwise-based selection algorithm. Sensitivity and specificity analyses, with their corresponding 95% confidence intervals, measured the predictive accuracy of each identified molecular signature. All analyses were

performed using R statistical package and threshold for significance was set at 5% (α=0.05).

Results

Clinical and pathological differences between groups of response to NCT (ypTNM 0, I, II and III).

A total of 115 patients with a mean age of 53 (range 17-86) were studied. The median tumor sizes at diagnosis and after treatment were 3.6cm and 1.6 cm, respectively. The median follow-up was 51 months (ranging from 6 to 150 months) and there were 24 metastatic events reported. The clinical characteristics of the patients are summarized in Table 1.

Response to NTC was assessed for ypTNM classification in 115 cases with 1 case missing, due to either lack of slides or incomplete pathological information on the residual tumor. A good response to NTC was observed in 34.2% patients (ypTNM 0, I; N=39) and poor responses in 65.8% (ypTNM II, III; N=75).

Table 1. Clinical and molecular characteristics of 115 patients with breast cancer treated with neoadjuvant chemotherapy.

Characteristics	N (%)
Mean age (range) years	53 (17-86)
Age <50 years	58 (50.4%)
Age ≥50 years	57 (49.6%)
Pathological stage after treatment (ypTNM)	
0 (Good Response to NCT)	21 (18.3%)
I (Good Response to NCT)	18 (15.7%)
II (Poor Response to NCT)	46 (40%)
III (Poor Response to NCT)	29 (25.2%)
Missing	1 (0.9%)
Diagnostic tumor size (cm)	
<2	23 (20.0)
2-5	71 (61.7)
>5	21 (18.3)
Post-treatment tumor size (cm)	
<1	43 (37.4%)
1-3	52 (44.4%)
>3	21 (18.3%)
Involved nodes	
0	43 (37.4)
1-3	51 (44.4)
>3	21 (18.3)
Molecular subtype	
Luminal A	21 (18.3%)
Luminal B	45 (39.2%)
HER2	23 (20 %)
Triple Negative	26 (22.6%)
Missing	1
Nº metastases	24 (19.3%)
ypTNM 0-I	2 (8%)
ypTNM II-II	22 (92%)
Median follow-up (range) months	51 (6-150)

This study compared one, three and five years of distant disease-free survival (DDFS) between group responses to NTC, which was statistically different (log-rank p-value =0.008). The 1 and 3-years DDFS for the ypTNM0-I (good response group) was the same, 96% (91-100%) and 5-years DDFS was maintained to 93% (85-100%). On the contrary, the poor response group (ypTNM II-III) showed a 1-year DDFS of 94% (88-100%), 3-years DDFS of 75% (64-87%). Finally, the survival of this group was reduced a lot. The percentage of 5-years DDFS for poor response was 54% (36-80%). Kaplan-Meier curves of DDFS of both groups are shown in Fig. 1. Finally, poor response group to NCT showed a higher rate of metastases, with 92% of metastases (22/24).

Biomarkers of chemoresistance in poor response group (ypTNM II-III).

The differential expression of 21 selected proteins in the core biopsy was compared between the tumors from poor response to NTC (ypTNM II-III) with those from tumors from good response (ypTNM 0-I).

In univariate analysis, 7 biomarkers with specific cutoffs, namely ALDH-1 (Hsc≥10), cytoplasmic HER4 (Hsc≥110), nuclear Survivin (Hsc≥115), cytoplasmic Survivin (Hsc<70), Bcl-2 (Hsc≥25), membrane p-HER-3 (+≥1) and nuclear cathepsin L (Hsc≥10), were associated with the lack of response to NTC. Odds-ratio and p-value of these biomarkers are shown in Table 2.

From multivariate logistic regression analysis, we generated a predictive signature of poor response

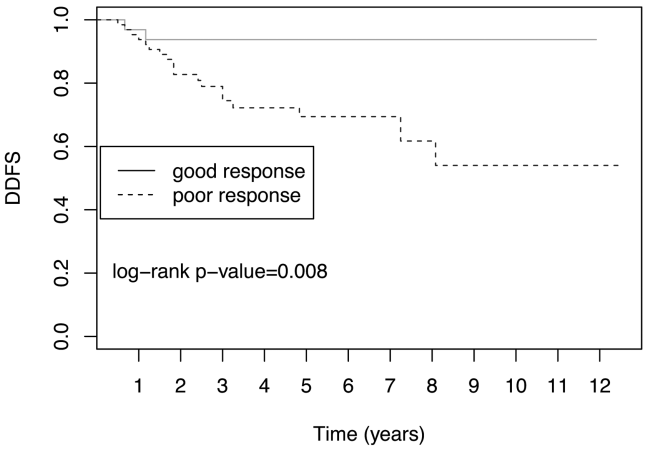

Fig. 1. Comparison of Kaplan-Meier curves of DDFS between good and poor response group to NTC. There were statistically significant differences DDFS between patients of good (ypTNM0-I) and bad (yTNMII-III) response to NCT (log-rank p=0.008). The good response group showed patients with a long survival with a 5-year DDFS of 93% (85-100%). The poor response group had a shorter survival with a 5-year DDFS of 54% (36-80%).

(ypTNM II and III groups), based on independent biomarkers of predictive value, with a determination coefficient of 55.6%. The predictive profile of chemoresistance was composed of five conditions in the primary tumor: nuclear cathepsin L (Hsc<12), cytoplasmic HER4 (Hsc<110), cytoplasmic Survivin (Hsc≥70), ALDH-1 (Hsc=0) and Bcl-2 (Hsc≥25). Only 69 of 115 cases had expression of all 5 biomarkers of profile predicted for chemoresistance. Next, we decided to simplify this profile grouping conditions as is shown in Table 3. Tumors expressing four or five conditions from profile in the core biopsy were associated with 8.6-fold increase in the chances of these patients to have a poor response to NCT (CI95%=1.5-219.1; p=0.01). The sensitivity of this profile was 28% (16-43%) and a specificity of 100% (79-100%). Moreover, multiple studies have shown that the different molecular types respond to NTC differently. Thus, we have done an analysis of the robustness of the signature for different molecular types of breast cancer, even grouping TNBC and HER2 versus luminal cancer, as is shown in Table 4. We cannot assess whether our signature explains better the chemoresistance of a determinated molecular subgroup. This analysis is underpowered to draw conclusions, because of the small sample size of the different molecular subtypes.

Finally, we reanalyzed chemoresistant biomarkers in a subcohort with positive ERα and/or PR tumors (which are the most chemoresitant population). This analysis was with a much smaller sample of only 66 patients of breast cancer, of whom 44 cases had a poor response to NCT (ypTNM II-III).

In univariate analysis, there were 6 biomarkers for positive ERα and/or PR tumors with specific cutoffs, namely ALDH-1 (Hsc≥5) (OR=5.0, 1.1-25.4, p=0.04), nuclear Cyclin D1 (Hsc≥90) (OR=3.9, 1.0-15.8, p=0.05), cytoplasmic HER-4 (Hsc≥110) (OR=16.5, 2.3-344, p=0.02), cytoplasmic p-Akt (Hsc≥110) (OR=7, 1.4-40.9, p=0.02) and membrane p-HER-3 (+≥1) (OR=7, 1.4-42.9, p=0.02).

Biomarkers of metastases in primary breast tumor treated with NTC.

Metastases were detected in 24 out of the 115 patients (19.3%), mostly in the group of poor response to NCT with 22 metastases. Protein expression profiles were compared between metastases-positive and metastases-negative tumors.

In univariate analysis, the presence of metastases was associated with 7 biomarkers in the primary tumor with specific cutoffs: Nuclear Cyclin D1 (Hsc<95), Cytoplasmic PTEN (Hsc <65), Nuclear PTEN (Hsc <65), Cytoplasmic VDR (Hsc≥95), ERα (Hsc<2.5), PR (Hsc<20), Cytoplasmic/Nuclear Survivin Ratio (Hsc≥0.56). Interestingly, Hazard-Ratio (HR) of some novel biomarkers was similar or higher than those of the currently recognized clinical markers of poor prognosis (involved nodes and ypTNM) as shown in Table 5.

Next, we generated a signature of adverse prognostic of 3 or 2 conditions (membrane HER4 (+=0), cytoplasmic VDR (Hsc≥95) and cytoplasmic/nuclear Survivin ratio ≥0.56) with a determination coefficient of 36.4%. This protein profile was obtained from a small sample of 70 patients with a low number of metastases

Table 2. List of chemoresistant biomarkers overexpressed in core biopsy of ypTNM II and III tumors.

N total	Biomarkers of Chemoresistance	Poor Response Propotion (%)	O-R (CI95%)	p-value
100	ALDH (Hsc>0)	11/31 (35.5%)	1	0.002
	ALDH (Hsc=0)	63/69 (91.3%)	5.8 (1.95-18.7)	
79	Cytoplasmic HER4 (Hsc≥110)	22/50 (44%)	1	0.009
	Cytoplasmic HER4 (Hsc<110)	25/29 (86.2%)	4.9 (1.6-18.5)	
89	Cytoplamic Survivin (Hsc<75)	43/59 (72.9%)	1	0.036
	Cytoplamic Survivin (Hsc ≥75)	28/30 (93.3%)	5.2 (1.34-33.3)	
93	Bcl2 (Hsc<25)	13/29 (44.8%)	1	0.006
	Bcl2 (Hsc≥25)	48/64 (75.0%)	3.7 (1.5-9.5)	
89	Nuclear Survivin (Hsc≥113)	31/59 (54.5%)	1	0.014
	Nuclear Survivin (Hsc<113)	24/30 (52.5%)	3.6 (1.3-10.9)	
92	Membrane p-HER3 (+≥1)	23/30 (76.7%)	1	0.05
	Membrane p-HER3 (+=0)	57/62 (91.9%)	3.5 (1.1-5.1)	
111	Nuclear CTSL (Hsc≥12)	20/38 (52.6%)	1	0.01
	Nuclear CTSL (Hsc<12)	53/73 (72.6%)	2.9 (1.3-6.8)	
113	Ki67 (%≥41)	21/39 (53.8%)	1	0.04
	Ki67 (%<41)	49/74 (66.2%)	2.3 (1.0-5.1)	

Table 3. Number and frecuency of response group according to number of expressed conditions in the tumor of core-biopsy.

Nº fulfilled conditions	Poor Response (ypTNM II-III)	Good Response (ypTNM 0-I)
5-4	13 (100%)	0 (0%)
3	23 (82%)	5 (18%)
2	10 (53%)	9 (47%)
0-1	0 (0%)	9 (100%)

Table 4. Parameters of the effectiveness of the signature of chemoresistance for different molecular types of breast cancer, grouping TNBC and HER2 versus luminal cancer.

Molecular Subtypes Breast Cancer	N group	Sensitivity	Specificity
Luminal A group	13	100% (59-100)	0% (0-81)
Luminal B group	32	100% (77-100)	18% (2-52)
HER2 group	12	100% (42-100)	67% (22-96)
TNBC group	12	100% (55-100)	0% (0-81)
HER2 + TNBC	24	80% (52-96)	0% (0-81)
Luminal A + B	45	68% (49-83)	0% (0-41)

(N=12). When tumors expressing 2 or 3 conditions from this profile were associated with 6-fold increase in the chances of these patients to have metastases (CI 95%= 1.9-21.8; p=0.003). This signature of adverse prognosis presented a 66% (34-90%) sensitivity and 87.9% (76.7-95%) specificity.

The 5-year DDFS for patients with primary tumors who presented either 0 or 1 conditions of this profile were 90% (81-100%). However, patients who showed tumors with 2 or 3conditions showed a short survival with a 5-year DDFS of only 56% (34%-85%) (Log-rank. p=0.0006, Fig. 2).

Table 5. List of metastatic biomarkers in tumorcore biopsy: HR and p-value.

N	Biomarkers of metastases	Proportion metastases (%)	H-R (IC95%)	p-value
106	53BP1 (Hsc ≥155)	4/45 (8.9%)	1	0.014
	53BP1 (Hsc <155)	19/61 (31.1%)	3.85 (1.3-11.3)	
80	Cytoplasmic Her4 (Hsc<205)	13/77 (16.9%)	1	0.001
	Cytoplasmic Her4 (Hsc≥205)	2/3 (66.7%)	17.1 (3.0-95.7)	
80	Nuclear Her4 (Hsc <20)	7/57 (12.3%)	1	0.03
	Nuclear Her4 (Hsc ≥20)	8/23 (34.8%)	3.1 (1.1-8.5)	
94	Cytoplasmic PTEN (Hsc≥ 65)	14/83 (16.9%)	1	0.029
	Cytoplasmic PTEN (Hsc <65)	5/11 (45.5%)	3.1 (1.1-8.7)	
102	Nuclear PTEN (Hsc ≥ 65)	14/83 (16.9%)	1	0.014
	Nuclear PTEN (Hsc <65)	8/19 (42.1%)	3.1 (1.25-7.8)	
115	ER (Hsc≥ 2.5)	9/70 (12.9%)	1	0.002
	ER (Hsc<2.5)	16/45 (35.6%)	3.8 (2.7-8.6)	
105	PR (Hsc≥ 20)	5/54 (9.3%)	1	0.003
	PR (Hsc<20)	20/61 (32.8%)	4.4 (1.6-11.7)	
115	Negative Nodes	7/62 (11.3%)	1	0.010
	Involved Nodes	18/53 (34%)	3.2 (1.3-7.7)	
114	ypTNM 0-I	2/39 (5.1%)	1	0.018
	ypTNM II-III	21/75 (28%)	5.7 (1.34-24.2)	

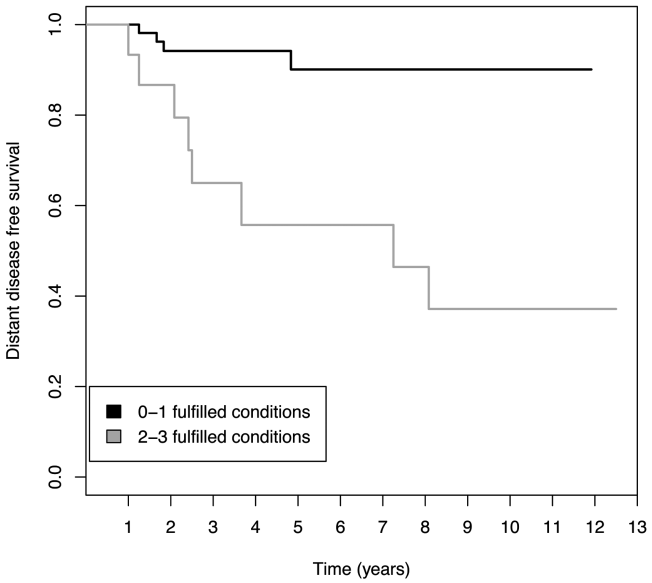

Fig. 2. Comparison of Kaplan-Meier curves of DDFS for the signature for adverse prognosis according to the number of fulfilled conditions. The identified expression profile for adverse prognosis classified patients with longer distant disease-free survival (DDFS), when only one or no conditions were expressed, compared to patients with shorter DDFS, when 2 or 3 conditions were expressed. The differences between Kaplan-Meier curves were statistically significant (Log-rank p=0.0006).

Discussion

This study presents the results of immunohistochemical analyses aiming to identify biomarkers of response to NTC (predictive biomarker) and development of metastases (prognostic biomarker) in breast cancer patients subjected to NTC, which were the two main current clinical challenges for the second most common cancer in women. Biomarkers of the response to treatment might correlate not only with chemosensitivity but also with prognosis, and consequently, they could help predict the effect of a certain treatment, and identify patients at higher-risk, who would benefit little from chemotherapy (Peto et al., 2012).

Our most important contribution was the generation of a predictive signature of chemoresistance based mainly on biomarkers of proliferation and apoptosis. Previous published studies have already reported that chemotherapy worked to kill tumor cells that proliferate faster through apoptosis (Engels et al., 2013; Colleoni et al, 2000). Moreover it has been demonstrated that markers related to cellular proliferation and apoptosis and balance between these two processes in tumor development could be predictive for clinical outcome (Engels et al., 2013). Thus, our predictive signature of chemoresistance related to cellular proliferation (nuclear CTSL, citoplasmic HER-4, ALDH-1) and apoptosis (citoplasmic Survivin and Bcl-2).

Although this signature was obtained from a very small preliminary sample (N=69), it has a high power of discrimination, as shown with its OR of 9. It is necessary to be validated with a larger sample and a higher number of events. On the other hand, it is well known that different molecular types respond to NTC differently. However, our signature does not discriminate for molecular subtype of breast cancer, due to the small sample size of every subgroup.

We focused on analysis of chemoresistant biomarkers in the sub-cohort with ERα and/or PR tumors, which are the most chemoresitant population. Interestingly, their Odds-Ratio (OR) was higher in this sub-study. Our results indicate that these biomarkers of chemoresistance were prevalent in the studied population (patients with positive ERα and/or PR tumors) in comparison with the reference population (patients with negative ERα and/or PR tumors) with a proportion of 75-80% versus 25-20%.

Our second contribution was the identification of

predictive biomarkers of metastases expressed in the diagnostic biopsy of the tumor. Currently, the number of lymph nodes involved and tumor size are the main prognostic factors in the BrCa, which constitutes the basis for the AJCC staging system (pTNM) (Gonzalez-Angulo et al., 2007). Thus, pTNM is the best parameter that correlates with patients' Distant Disease Free Survival (Carey et al., 2005; Charpin et al., 2012; Cortazar et al., 2014). In our results, there were biomarkers with higher or similar HR than pTNM. This finding might be very interesting because they could help the oncologist to make a first classification of the chemoresistant tumors and be likely to develop metastases, before starting treatment.

Finally, we generated a signature to poor prognosis when two or three conditions were fulfilled in diagnostic tumor. In spite of high HR of signature of 6, this needs to be validated with a bigger sample and with a higher number of metastases.

To sum up, this second signature is what allowed the oncologist to obtain a first approximation of survival that the patient might had at the time of diagnosis.

Conclusion

The present study could become a stepping stone for future studies that will need to assess on a larger scale these identified as prognostic factors of pathologic response (or non-response to NTC). The identification of novel biomarkers with the potential to accurately predict responses to treatment and prognosis is highly relevant in clinical practice to act preventively to attenuate both possible resistance to primary therapy and the appearance of metastases. In addition, these biomarkers could help design personalized and more effective strategies to target the main pathways implicated in the adverse clinical outcome.

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