HER2, P53, FAS, FASL, COX2, PGE_2S, EGFR EXPRESSION IN BREAST CANCER AND IN NORMAL PERITUMORAL BREAST TISSUE: POTENTIAL NOVEL RISK BIOMARKERS

Ph.D. Thesis

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Summary

Breast cancer is the most common cancer among women in many parts of the world and early detection is the key to the survival of the patients. There is evidence that changes in HER2 and p53 protein expression might be relevant to breast cancer progression. Furthermore, we have recently reported that malignant breast tumors show an altered expression of Fas and Fas ligand (FasL) compared to normal tissues and these molecular changes are significantly related to patient outcome and COX2 is a relevant new prognostic marker. In this study we hypothesized that these molecular markers might also be useful to evaluate the malignant potential of non-neoplastic breast tissues.
To this end, we analyzed, by using specific antibodies, HER2, p53, Fas and FasL expression in 72 breast carcinomas, the corresponding autologous peritumoral tissues (PTT) sampled at 1, 2 and 3 cm far from the tumor itself and in 44 benign mammary lesions. Ten breast carcinomas and their autologous 1 cm PTT samples were also analyzed by fluorescent in situ hybridization (FISH) to determine if HER2 gene amplification can be demonstrated in the background of cytoplasmic immunohistochemical HER2 staining. Further 186 stage I-II primary BC and 95 autologous metastatic lymphnodes were analyzed immunohistochemically for COX2, hormones receptor, p53, HER2, Fas and Fas ligand (FasL) expression to determine the effect of COX2 expression on the prognosis of breast cancer patients receiving chemotherapy either as a single factor or taken into consideration together with other factors. To understand better the probable role of functional COX2 alteration in developing primary breast cancer and metastases, we also analyzed the expression of PGE_2S in 121 primary breast tumors and of epidermal growth factor receptor (EGFR) in 167 of the cases Because of insufficient quantity of tissue samples no complete analyses for all the 186 cases and their 95 metastases were available.
Results obtained suggest that HER2 gene amplification often underlies even cytoplasmic HER2 staining when analyzed immunohistochemically. Furthermore, breast carcinomas and the closest adjacent uninvolved parenchyma shared an upregulated FasL phenotype which is lost in PTT farther from the tumor. Therefore, among the biological parameters investigated, HER2 and FasL expression seems to represent biomarkers of breast tumorigenesis easily applicable to fine needle aspirates and potentially useful to detect patients at high risk of breast carcinoma. Moreover, in high-risk breast cancer patients the immunohistochemical evaluation of COX2, together with PGE_2S, p53, Ki67, HER2, Fas and FasL, may be of clinical value in distinguishing different responses to adjuvant anthracycline-based chemotherapy.

Introduction

Breast cancer is the most common cancer among women in many parts of the world and early detection is the key to the survival of the patients. There is evidence that changes in HER2 and p53 protein expression might be relevant to breast cancer progression. Furthermore, we have recently reported that malignant breast tumors show an altered expression of Fas and Fas ligand (FasL) compared to normal tissues and these molecular changes are significantly related to patient outcome and COX2 is a relevant new prognostic marker. In this study we have shown that these molecular markers might also be useful to evaluate the malignant potential of non-neoplastic breast tissues.

Epidemiology
According to a worldwide study on cancer mortality, Eastern European men had the second highest rates of cancer (414.2), with extremely high rates in Hungary (566.6) and in the Czech

Republic (480.5). The rates of cancer in Eastern European women were lower than in the other three areas, although as with men, female rates were very high in Hungary (357.2) and in the Czech Republic (333.6). Generally, mortality rates were highest in Eastern European countries, notably in Hungary, reflecting a combination of poorer cancer survival rates and a higher incidence of the more lethal neoplasms.

Aims of the thesis

Since the development and progression of breast cancer, as in other solid tumors, result from the accumulation of genetic alterations, it is likely that some types of benign lesions, precursors for invasive breast cancer and the normal appearing peritumoral tissue, according to the field effect hypothesis, may harbor molecular changes representing signatures of clinical relevance which heralds early stages of cancer development.
The aims of our two studies were

1. To determine immunohistochemically the HER2, p53, Fas, FasL, hormone receptores, COX2, PGE_2S and EGFR expression in breast cancer tissues and in morphologically normal-appearing peritumoral tissue samples taken 1cm, 2 cm and 3 cm far from the tumors.
2. To determine HER2 gene amplification by FISH in HER2 positive 1 cm peritumoral tissue samples to support the field-effect hypothesis.
3. To determine the influence of the investigated markers on the 5-year disease-free survival and overall survival of the breast cancer patients.
4. To determine the combination of markers most useful to determine accurate prognosis.
5. To establish a set of markers for diagnosing high risk of recurrence by determining field-effect in peritumoral tissue samples.

Patients and Methods

Patients
Seventy-two breast cancer specimens, 72 autologous peritumoral tissues sampled at 1 cm, 65 at 2 cm and 43 at 3 cm from the primary breast cancer and 44 mammary benign lesions were prospectively collected for immunohistochemical analysis and fluorescent in situ hybridization (FISH) from patients surgically treated at Regina Elena Cancer Institute.
Further hundred and eighty-six patients receiving anthracycline-based adjuvant therapy were selected for studying the COX2, hormone receptors, HER2, p53, Ki67, Fas, FasL, PGE_2S and EGFR expression in primary breast cancers and in the their autologous lymph node metastases.

Immunohistochemistry
IHC staining was carried out on 5 μm thick sections on silane (APES, Sigma, St. Louis, MO, USA) treated slides for routinely fixed paraffin embedded blocks. The deparaffinized and rehydrated sections were pretreated twice in microwave oven at 750 W for 5 min in citrate buffer pH=6 and incubated for 60 min. at room temperature with primary antibodies. The reaction was visualized using a streptavidin-biotin immunoperoxidase system (LSAB2 kit, DakoCytomaton, Milan, Italy) and 3-amino-9-ethyl-carbazole solution (DakoCytomaton) as chromogenic substrate. Sections were then slightly counterstained with Mayer's haematoxylin and mounted in aqueous mounting medium (Glycergel, Dakocytomaton).
For COX2 immunostaining, antigen was retrieved using microwave (two cycles of 750W, 5 min in citrate buffer) and sections were incubated for 60 min at room temperature with COX2-specific human polyclonal primary antibody. The reaction was visualized using a

streptavidin-biotin immunoperoxidase and chromogenic substrate system (Histostain-Plus, Broad Spectrum (DAB), 85-9643, ZYMED Laboratories Inc., CA, USA). PGE_2S immunohistochemistry (121 specimens) was performed with a PGE_2S-specific rabbit polyclonal primary antibody while EGFR activity (167 specimens) was examined with a commercial mouse primary antibody. In case of EGFR antigen was retrieved using pepsine (Digest-All 3 ready-to-use pepsine solution, 00-3009, ZYMED Laboratories Inc., CA, USA) for 10 min. at 37°C, otherwise the reactions were performed both for PGE_2S and EGFR as described for COX2 immunoreaction.
Antibodies and working solutions used for immunohistochemistry are shown in table 1. Immunostained slides were analyzed and scored independently by 2 investigators.

HER2
Overexpression of HER2 oncogene product was determined using the high affinity monoclonal antibody (mAb) 300G9 mAb CB11 (BioGenex, Menarini, Italy).

Smooth muscle actin
Smooth muscle actin staining was performed with using monoclonal mouse anti-human anti-actin (smooth muscle) antibody 1A4 (DakoCytomation).

p53
p53 protein expression was evaluated using the murine mAb DO7 (DakoCytomation).

Fas
Fas protein was detected by using a commercial mAb (Novocastra).

FasL
FasL expression was evaluated using N-20 mAb (Novocastra).

ER, Pgr
Estrogen (ER) and progesterone (PgR) receptors were assayed with using commercially available antibodies (ER1D5 and 1A6 Immunotech, UCS, Rome, Italy).

Ki67
To assess the proliferative activity of the tumors, immunostaining with monoclonal antibody Mib-1 (DakoCytomation) was performed.

COX2
COX2-specific human polyclonal primary antibody (125 ng/µl, 160107, Cayman Chemical Co., Ann Arbor, MI, USA) was used to determine COX2 expression.

PGE_2S
Immunohistochemistry was performed with a PGE_2S-specific rabbit polyclonal primary antibody (125 ng/µl, 160140, Cayman Chemical Co., Ann Arbor, MI, USA)

EGFR
EGFR activity was examined with a commercial mouse primary antibody (750 ng/µl, 28-0005, ZYMED Laboratories Inc., CA, USA)

Antibody	Working solution	Origin
HER2 300G9	1:200	BioGenex, Menarini, Italy
HER2 CB11	1:200	BioGenex, Menarini, Italy
Actin 1A4	1:300	DakoCytomaton, Milan, Italy
p53 D07	1.300	DakoCytomaton, Milan, Italy
Fas (CD95)	1:50	Novocastra Laboratories Ltd., Milan, Italy
FasL	1:50	Novocastra Laboratories Ltd., Milan, Italy
ER ER1D5	1:300	Immunotech, UCS, Rome, Italy
PgR 1A6	1:300	Immunotech, UCS, Rome, Italy
Mib-1	1:300	DakoCytomaton, Milan, Italy
COX2	1:200	Cayman Chemical Co., Ann Arbor, MI, USA
PGE_2S	1:200	Cayman Chemical Co., Ann Arbor, MI, USA
EGFR	1:200	ZYMED Laboratories Inc., CA, USA

Table 1. Antibodies and working solutions used for immunohistochemistry

FISH

PathVysion HER2 DNA Probe Kit (Abbott Diagnostici, Rome, Italy) was used to determine the HER2 DNA amplification in breast cancer and PTT samples.

The slides were processed with Olympus BX60 fluorescence microscope (Olympus Italia, Segrati, Italy) equipped with a 100-watt mercury lamp. Separate band pass filters were used for the detection of the HER2 probe signals (Spectrum Orange), CEP 17 probe signals (Spectrum Green) and DAPI counter stain. Fluorochrome signals were captured individually and images were generated via computer with Quips Genetic Workstations and Imaging Software (Vysis, Abbott Diagnostici, Rome, Italy). The slides were observed at 1000x magnification.

Statistical analysis

Association between clinical and biopathological variables were evaluated using the chi-square test. All these parameters were treated as dichotomous or categorical variables and described using the Pearson statistics. The disease-free (DFS) and overall survival (OS) curves were estimated by the Kaplan-Meier product-limit method. Log-rank test was used to assess differences between subgroups. Significance was defined at the $p<0.05$ level. The relative risk and the confidence limits were estimated for each variable using the Cox univariate model and adopting the most suitable prognostic category as reference group. A multivariate Cox proportional hazard model was also developed using stepwise regression (backward selection) with predictive variables which were significant in the univariate analyses. Enter limit and remove limit were $p=0.10$ and $p=0.15$ respectively. All analyses were conducted using the BMDP software package (Chicago, IL).

Results

HER2 expression in 72 breast cancer specimens and their autologous peritumoral tissue samples

Nineteen of the 72 breast cancer samples (26.3%) were HER2 positive (2+/3+ score). In 10 PTT independently of the distance from the primary tumor we observed a distinct cell membrane immunostaining prevalently confined to typical ductal hyperplasia, atypical ductal

hyperplasia and florid adenosis. In these PTT we never found a 3+ score immunostaining whereas a weak 1+ score staining was observed in 9 PTT (12.5%) collected at 1 cm, 6 at 2 cm (9.2%) and 5 at 3 cm (11.6%).
The percentage of HER2 overexpression was significantly higher in malignant tumor than in benign lesions ($p=0.007$) and PTT sampled at 1, 2 and 3 cm ($p=0.01$, $p=0.001$ and $p= 0.03$ respectively). No difference in HER2 overexpression was observed among the three PTT analyzed by IHC ($p=0.49$, $p=0.99$ and $p=0.99$ respectively).

p53 expression in 72 breast cancer specimens and their autologous peritumoral tissue samples
Thirty-four BC (47.2%) were p53 positive with variable percentage of nuclear staining ranging from 10% to 90% with a median value of 40%. In contrast we observed p53 nuclear accumulation in only 1 benign lesion. Also in PTT, p53 positivity was seen in a limited number of lesions. 4 cases of TDH and 3 FA displayed p53 nuclear accumulation in a low percentage of epithelial cells ranging from 1% to 10%.
p53 expression was higher in malignant tumors with respect to benign lesions ($p<0.001$) and in malignant tumors versus 1, 2 and 3 cm PTT ($p<0.0001$). No significant difference in p53 positivity was observed among the three PTT ($p=0.21$, $p=0.42$ and $p=0.99$ respectively).

HER2 gene amplification determined by FISH
Five out of the 10 peritumoral tissue samples immunohistochemically HER2 positive demonstrated HER2 gene amplification (ratio >2). In the autologous BC HER2 was amplified in 7 of these 10 cases.

Expression of Fas system molecules in 72 breast cancer specimens and their autologous peritumoral tissue samples
Forty out of 44 benign lesions (90.9%) showed a strong and homogeneous Fas expression prevalently localized on cell membrane whereas only 22% of benign tumors were FasL positive. On the other hand, when the 72 malignant tumors were evaluated, only 41 breast cancer samples (56.9%) showed Fas positivity that was often heterogeneous in intensity and cell distribution whereas FasL was positive in 45.8% of the cases. The rate of cases expressing Fas was significantly lower in breast cancer than in benign lesion ($p<0.0001$) as well as the rate of FasL positive cases was significantly higher in BC than in BL ($p<0.001$). In both cases tumor-infiltrating lymphocytes provided internal controls. In breast cancer, the expression of receptor and ligand antigens appeared to be inversely related ($p<0.0001$) with 37.5% of Fas+/FasL– and 26.4% Fas–/FasL+. Double positive (Fas+/FasL+) and double negative (Fas–/FasL–) phenotypes accounted for 19.4% and 16.7% respectively.
The percentage of Fas expression in normal appearing breast epithelium in the three different samples adjacent to invasive cancer was similar to that observed in benign lesions independently of the distance from the autologous BC (90.9% vs 87.5% at 1 cm, 90.8% at 2 cm, 90.7% at 3 cm). In contrast, FasL was significantly upregulated in peritumoral tissues sampled at 1 cm with respect to benign lesions (22.7% $p=0.05$). Therefore the percentage of FasL positive cases in 1cm peritumoral tissue samples (41.6%) was similar to that found in BC (45,8%) and no statistically significant difference was evidenced between invasive cancer and the closest peritumoral tissue specimens ($p=0.73$). FasL expression in breast specimens collected farther from the autologous breast cancer was similar to that observed in benign

lesions (22.7% vs. 27.7% at 2 cm, 23.3% at 3 cm) and significantly different from BC (p= 0.04 at 2 cm and p=0.02 at 3 cm).

COX2 expression in 186 breast cancer specimens and in their autologous metastatic lymph nodes

Of the 82 COX2 positive primary tumor, 81 (98.8%) had COX2 positive metastatic nodes while in 1 case (1.2%) nodal metastasis was COX2 negative. Among the 13 COX2 negative metastatic primary tumor, 9 (69.2%) had COX2 positive metastatic nodes, while in 4 cases (30.8%) we found no COX2 expression in the metastatic lymph nodes. Altogether, increase in expressing COX2 was found in metastatic lymph nodes regarding primary tumors (93.7% *vs* 86.5%)

PGE_2S expression in 121 breast cancer specimens

Of the 102 COX2 positive samples 94 (92.2%) showed PGE_2S immunoreactivity, 8 cases (7.8%) were PGE_2S negative. Among the 19 cases negative for COX2 expression, 7 cases (36.8%) showed PGE_2S positivity and 12 cases (63.2%) did not express PGE_2S. No metastatic lymph node tissue samples were available for PGE_2S immunohistochemical reaction.

EGFR expression in 167 breast cancer specimens

Sixty-six (46.5%) of the 142 COX2 positive samples expressed EGFR, while 76 samples (53.5%) were EGFR negative. Of the 25 samples showing no COX2 reactivity, 16 (64.0%) were EGFR positive and 9 samples (36.0%) were EGFR negative. No metastatic lymph node samples were available for further analysis.

Predictors of 5-year disease free survival

Tumor size (OR=2.10, CI=1.25-3.51, p=0.05), nodal status (OR=3.76, CI=2.11-6.69, p<0.001), COX2 expression (OR=4.39, CI=1.59-12.11, p=0.004), PGE_2S positivity (OR=3.36 (CI=1.04-10.85, p=0.004), Ki67 expression (OR=1.95, CI=1.18-3.24, p=0.009), Fas positivity (OR=0.18, CI=0.10-0.31) and FasL expression (OR=3.28, CI=1.90-5.66) were found to be significant predictors of DFS in univariate analysis, while a borderline significance was found between DFS and p53 positivity (OR=1.60, CI=0.96-2.65, p=0.069). ER status (OR=1.02, CI=0.62-1.69, p=0.93), PgR expression (OR=1.06, CI=0.64-1.76, p=0.83) and HER2 positivity (OR=1.01, CI=0.59-1.71) had no significant impact on DFS. When considering concomitant expression of different variables, patients with tumors expressing both COX2 and p53 (OR=5.20, CI=1.58-17.13, p=0.007) or COX2 and PGE_2S (OR=7.96, CI=1.09-57.96, p=0.04) were found to have significantly reduced DFS (data not shown).

Tumor size (OR=2.17, CI=1.18-3.98, p=0.013), nodal status (OR=4.61, CI=2.16-3.83, p<0.001), COX2 expression (OR=3.50, CI=1.07-11.46, p=0.039), Ki67 positivity (OR=5.56, CI=1.71-18.09), Fas expression (OR=0.09, CI=0.03-0.29) and FasL expression (OR=1.96, CI=1.19-3.32) were significant predictors of DFS even in multivariate analyses.

Variable	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
Tumor size (< 2 cm vs. > 2 cm)	2.10 (1.25-3.51)	0.005	2.17 (1.18-3.98)	0.013
Nodal status (N_0 *vs.* N_+)	3.76 (2.11-6.69)	<0.001	4.61 (2.16-3.83)	<0.001
Histotype (ductal vs. lobular)	0.89 (0.45-1.75)	0.78	0.89 (0.25-3.19)	0.88
COX2 (positive vs. negative)	4.39 (1.59-12.11)	0.004	3.50 (1.07-11.46)	0.039
PGE_2S expression (positive vs. negative)	3.36 (1.04-10.85)	0.004	2.96 (0.69-12.7)	0.22
EGFR expression (positive vs. negative)	1.44 (0.80-2.59)	0.37	0.84 (0.31-2.32)	0.78
p53 status (positive vs. negative)	1.60 (0.96-2.65)	0.069	2.63 (0.90-7.71)	0.14
Ki67 status (positive vs. negative)	1.95 (1.18-3.24)	0.009	5.56 (1.71-18.09)	0.019
ER status (positive vs. negative)	1.02 (0.62-1.69)	0.93	0.42 (0.09-1.87)	0.34
PgR status (positive vs. negative)	1.06 (0.64-1.76)	0.83	3.35 (0.75-15.08)	0.19
HER2/*neu* (positive vs. negative)	1.01 (0.59-1.71)	0.96	0.61 (0.20-1.93)	0.48
Fas (positive vs. negative	0.18 (0.10-0.31)	<0.001	0.09 (0.03-0.29)	0.001
FasL (positive vs. negative)	3.28 (1.90-5.66)	<0.001	1.96 (1.19-3.32)	0.01

Table 2. Univariate and multivariate analyses of prognostic factors for DFS in 186 breast cancer patients

Predictors of overall survival

In univariate analysis, tumor size (OR=2.38, CI=1.20-4.70, p=0.013), nodal status (OR=3.89. CI=1.77-8.55, p=0.001), p53 positivity (OR=2.17, CI=1.12-4.21, p=0.02), Ki67 positivity (OR=2.43, CI=1.25-4.75, p=0.009), HER2 expression (2.68, CI=1.48-4.86), Fas positivity (OR=0.07, 0.03-0.16) and FasL expression (OR=6.19, CI=3.05-12.53) were significant predictors of OS, while a borderline significance was found between COX2 expression and OS (HR=3.15, CI=0.87-10.27, p=0.05). Histotype (OR=1.65, CI=0.70-3.89), PGE_2S positivity (OR=2.66, CI=0.63-11.29, p=0.19), EGFR expression (OR=1.49, 0.79-2.82), ER status (OR=1.39, CI=0.72-2.69, p=0.33) and PgR status (OR=1.51, CI=0.79-2.91, p=0.22) had no significant impact on OS.

Similarly to DFS, concomitant expression of COX2 and p53 in the primary tumors significantly influenced OS (OR=4.71, CI1.10-20.16, p=0.037), while in contrary to DFS, patients with tumors expressing both COX2 and PGE_2S had not significantly reduced OS (OR=3.80, CI=0.51-28.25, p=0.19) (data not shown).

In multivariate analysis, nodal status (OR=3.66, CI=1.66-8.07, p=0.001), HER2 positivity (OR=4.86, CI=1.12-20.99) and Fas expression (OR=0.03, CI=0.005-0.17) were significant predictors of OS, while a borderline significance was found between OS and tumor size (OR=1.87, CI= 0.93-3.79, p=0.081), p53 positivity (OR=1.89, CI=0.96-3.74, p=0.068), Ki67 positivity (OR=1.83, CI=0.91-3.68, p=0.089) and FasL expression (OR=4.29, CI=0.97-18.88). Histotype (OR=2.37, CI=0.34-16.96), COX2 expression (OR=0.14, CI=0.02-1.26), PGE_2S positivity (OR=1.73, CI=0.18-16.7), EGFR expression (OR=0.88, CI=0.20-3.83), ER status (OR=0.29, CI=0.05-1.70) and PgR status (OR=2.7, CI=0.48-15.4) had no influence on OS.

	Univariate analysis		**Multivariate analysis**	
Variable	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
Tumor size (<2 cm vs. >2 cm)	2.38 (1.20-4.70)	0.013	1.87 (0.93-3.79)	0.081
Nodal status (N_0 vs. N_1)	3.89 (1.77-8.55)	0.001	3.66 (1.66-8.07)	0.001
Histotype (ductal vs. lobular carcinoma)	1.65 (0.70-3.89)	0.34	2.37 (0.34-16.96)	0.47
COX2 expression (positive vs. negative)	3.15 (0.87-10.27)	0.050	0.14 (0.02-1.26)	0.15
PGE_2S expression (positive vs. negative)	2.66 (0.63-11.29)	0.19	1.73 (0.18-16.7)	0.69
EGFR expression (positive vs. negative)	1.49 (0.79-2.82)	0.30	0.88 (0.20-3.83)	0.88
p53 status (positive vs. negative)	2.17 (1.12-4.21)	0.02	1.89 (0.96-3.74)	0.068
Ki67 status (positive vs. negative)	2.43 (1.25-4.75)	0.009	1.83 (0.91-3.68)	0.089
ER status (positive vs. negative)	1.39 (0.72-2.69)	0.33	0.29 (0.05-1.70)	0.25
PgR status (positive vs. negative)	1.51 (0.79-2.91)	0.22	2.7 (0.48-15.4)	0.35
HER2/*neu* (positive vs. negative)	2.68 (1.48-4.86)	0.007	4.86 (1.12-20.99)	0.08
Fas (positive vs. negative	0.07 (0.03-0.16)	<0.001	0.03 (0.005-0.17)	0.002
FasL (positive vs. negative)	6.19 (3.05-12.53)	<0.001	4.29 (0.97-18.88)	0.109

Table 3. Univariate and multivariate analyses of prognostic factors for OS in 186 breast cancer patients

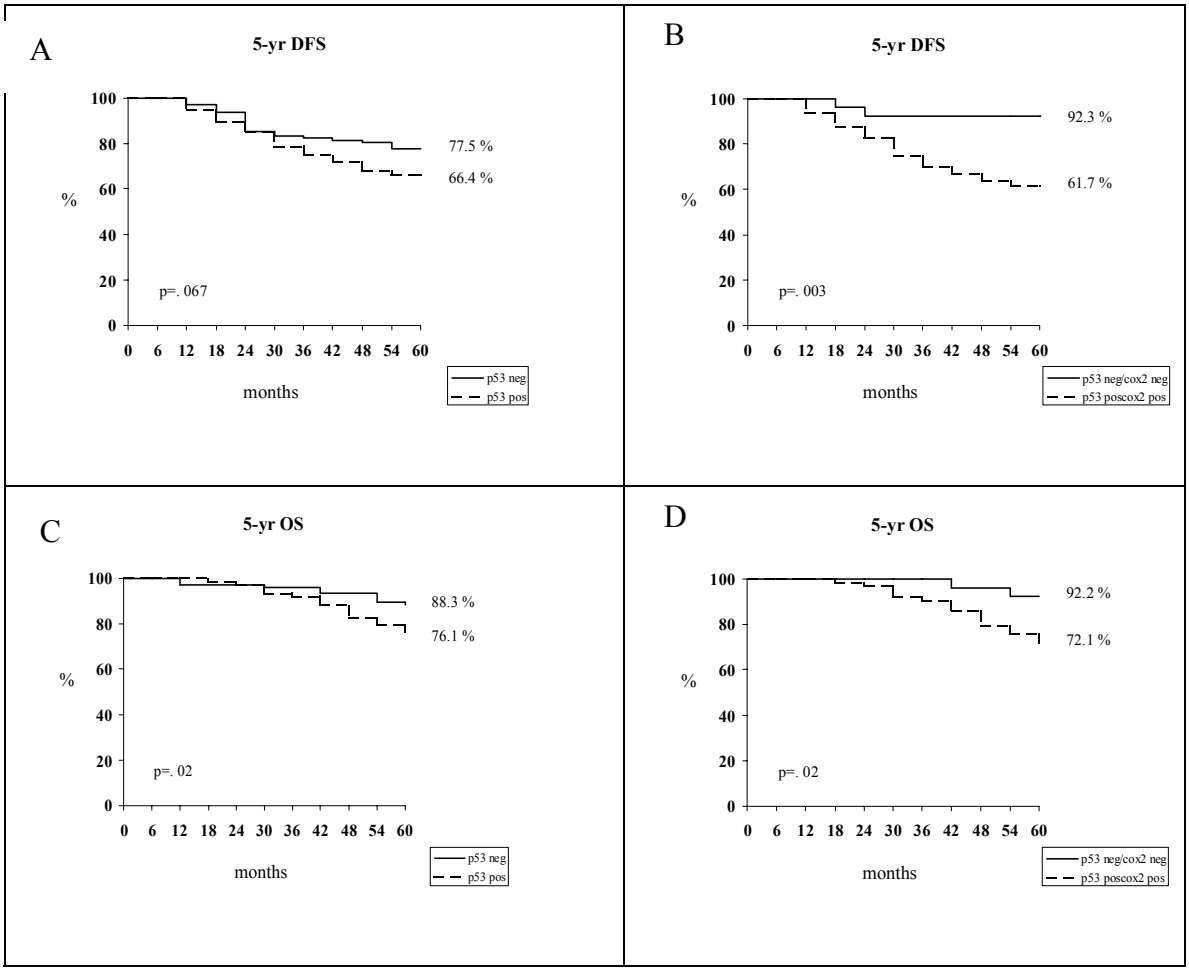

Figure 1. Impact of p53 expression on DFS (A) and OS (C), and concomitant COX2/ p53 expression on DFS (B) and OS (D)

Relationships of different prognostic factors examined in the present study

COX2 expression in primary breast tumors was significantly related to p53 expression (OR=2.917, CI=1.308-6.505) and Ki67 expression (OR=1.993, CI=1.001-3.970). There was a significant relationship between HER2 and FasL (OR=1.687, CI=1.026-2.774), Ki67 (OR=1.765, CI=1.068-2.916) and EGFR (OR=2.314, CI=1.320-4.056) positivity. ER expression was significantly related to Ki67 (OR=0.433, CI=0.264-0.710), p53 (OR=0.427, CI=0.258-0.706), PgR (OR=99.171, CI=40.558-242.489) and EGFR (OR=0.448, CI=0.255-0.786) expression, while PgR expression showed significant relationship to Ki67 (OR=0.360, CI=0.217-0.596), EGFR (OR=0.377, CI=0.214-0.664) and p53 (OR=0.481, CI=0.292-0.792) expression. p53 expression was significantly related to FasL (OR=1.843, CI=1.122-3.028) and to Ki67 (OR=2.540, CI=1.524-4.232) expression, while there was a significant relationship also between Fas and FasL expression (OR=0.245, CI=0.146-0.411).

Variable I	Variable II	OR	5% CI	95% CI
FAS	FASL	0.245	0.146	0.411
Ki67	FASL	0.923	0.569	1.498
EGFR	FASL	0.935	0.575	1.730
PGE2S	FASL	1.306	0.595	2.866
COX2	FASL	1.333	0.675	2.633
p53	FASL	1.843	1.122	3.028
HER2	FASL	1.687	1.026	2.774
PgR	FASL	0.966	0.595	1.570
ER	FASL	1.001	0.618	1.622
Ki67	FAS	1.245	0.763	2.031
EGFR	FAS	1.094	0.628	1.904
PGE2S	FAS	0.831	0.378	1.825
COX2	FAS	1.019	0.515	2.014
p53	FAS	0.781	0.476	1.282
HER2	FAS	1.040	0.632	1.713
PgR	FAS	0.818	0.500	1.338
ER	FAS	0.833	0.511	1.358
EGFR	Ki67	1.226	0.703	2.136
PGE2S	Ki67	0.885	0.407	1.928
COX2	Ki67	1.993	1.001	3.970
p53	Ki67	2.540	1.524	4.232
HER2	Ki67	1.765	1.068	2.916
PgR	Ki67	0.360	0.217	0.596
ER	Ki67	0.433	0.264	0.710
PGE2S	EGFR	0.770	0.304	1.948
COX2	EGFR	1.873	0.845	4.155
p53	EGFR	1.435	0.818	2.515
HER2	EGFR	2.314	1.320	4.056
PgR	EGFR	0.377	0.214	0.664
ER	EGFR	0.448	0.255	0.786
COX2	PGE2S	$>1.7 \times 10^7$	$>1.7 \times 10^7$	$>1.7 \times 10^7$
p53	PGE2S	1.714	0.753	3.905
HER2	PGE2S	0.000	0.000	0.000
PgR	PGE2S	1.275	0.586	2.776
ER	PGE2S	0.000	0.000	0.000
HER2	COX2	1.226	0.608	2.471
PgR	COX2	0.919	0.465	1.816
ER	COX2 TU	0.515	0.256	1.036
p53	COX2 TU	2.917	1.308	6.505
HER2	p53	1.417	0.859	2.336
PgR	p53	0.481	0.292	0.792
ER	p53	0.427	0.258	0.706
PgR	HER2	0.697	0.424	1.145
ER	HER2	0.882	0.539	1.444
ER	PgR	99.171	40.558	242.489

Table 4. Relationship of the investigated biopathological variables detemined by OR

Discussion

HER2 expression and gene amplification

In our series of 72 BC patients HER2 was overexpressed (score 2+/3+) in 26.3% of cases. In addition, we found variable levels of HER2 positivity (score 1+/2+) in a low percentage of benign lesions and in some normal appearing peritumoral tissue samples independently of the distance from the autologous BC.

In order to better understand the biological basis of HER2 immunoreactivity in morphologically uninvolved PTT, we evaluated, by FISH analysis, gene amplification in the 10 PTT sampled at 1, 2 or 3 cm with HER2 immunostaining. Of interest, gene amplification was detected in 5 of these cases, independently from the distance from the autologous BC and in 8 out of 10 corresponding invasive cancer, demonstrating that the IHC HER2 positivity in normal-appearing breast epithelium has often underlying HER2 gene amplification.

p53 expression

We detected p53 nuclear staining in a limited number of benign lesions as well as in multiple PTT. The positivity was not related to the distance from the primary tumor and no statistical significance among specimens collected at 1, 2 and 3 cm ($p=0.21$, $p=0.42$ and $p=0.99$ respectively) was found. These results are of clinical interest since it has been recently reported that p53 immunostaining in benign epithelial cells, even if weak and focal, appears to be associated with a statistically significant increased risk of BC development.

Among the samples of patients receiving anthracycline-based adjuvant chemotherapy, p53 expression alone significantly influenced only OS but not DFS, while when considering concomitant COX2 and p53 expression, this combination had a significant impact both on DFS and OS among our samples. Traditionally, p53 overexpression by immunohistochemistry is thought to represent *TP53* mutation, however, high concordance of increased p53 protein is only seen with missense mutations, which result in protein that is resistant to degradation and has longer half-life than the wild-type (wt) counterpart. Furthermore, our present data imply that breast tumors overexpressing p53 and COX2 have a significantly poorer DFS ($p=0.003$) and OS ($p=0.02$) than those with normal low pattern of p53 expression and no COX2 expression. Because the COX2 gene has been shown to be induced in p53 defective cells and down-regulated by wt p53, there may exist a direct link between a defective p53 pathway and elevated levels of COX2 expression in cancer cells. Though we did not investigate that the expressed p53 protein in our samples were wt or mutant, our results indicate that the p53 protein expressed in the breast tumor samples may be at least partly defected mutant p53 protein thus failing to repress COX2 expression.

Fas system

Fas and FasL interactions, which play an important role in different immune functions, are crucial in the involution of the mammary epithelium preventing cellular accumulation of mutations and neoplastic transformation. It is largely reported that benign and malignant breast lesions are characterized by different expression of Fas and FasL molecules. In agreement with other authors, we demonstrated that altered FasL:Fas ratio in breast carcinomas is related to adverse clinical outcome. Moreover, this is the first report in which Fas and FasL expression was accurately analyzed in malignant breast tumors and autologous normal appearing breast epithelium collected at different distance from invasive cancer. Fas expression was significantly downregulated in our series of primary tumors, but it was homogeneously expressed in PTT independently of the distance from the autologous BC (87.5%, 90.8% and 90.7% respectively). Therefore changes in Fas expression, which in the tumor may be inactivated by different molecular mechanisms such as promoter methylation,

transcriptional repression and histone acetylation, do not seem to be an early event in breast carcinogenesis.
Although we do not know the mechanism/s underlying Fas downregulation in breast cancer, this change may result in resistance to apoptosis i.e. accumulation of genetic changes and decrease of NK cell mediated immunosurveillance. Differently from Fas, FasL was significantly upregulated in malignant tumor samples (45.5%) and peritumoral tissue samples closer (1 cm 41.7%) to the tumor with respect to benign lesion (22.7%) and to peritumoral tissue samples sampled at 2 cm (27.7%) and at 3 cm (23.3%) from the autologous breast cancer. FasL in non-lymphoid tissues is known to be induced by a number of factors among which the response to activated lymphocytes has been extensively documented. Whether this latter mechanism is responsible for FasL upregulation in peritumoral tissues samples nearest to the tumor is unclear at present. One may hypothesize that in some patients FasL expression may be induced by circulating T cells recognizing MHC-peptide complexes on tumor cells. Although we cannot exclude that FasL upregulation in surrounding peritumoral tissue may be induced by a paracrine mechanism, the same immune-mediated upregulation may also occur in apparently normal breast tissues. Whatever the molecular pathways responsible for FasL expression are, this new phenotype is likely to result in being protected against T cell mediated killing, thus facilitating the accumulation of cell damages in benign lesions leading to malignant transformation.

COX2 expression
Besides a marked increase (11.4%) in COX2 expression in metastases versus primary tumors, significant correlation between COX2 expression and nodal status was found in our investigations. This finding may be explained with the data obtained by a study on murine model of metastatic breast cancer, where in contrast to the uniform in vitro COX2 expression, only tumors resulting from the transplantation of metastatic cell lines expressed COX2 in vivo suggesting that i) in the tumor milieu, COX2 expression may be regulated differently in non-metastatic versus metastatic lesions (30) and that ii) constitutive expression of COX2 may be required to maintain the altered phenotype of increased invasiveness.

COX2 and PGE_2S expression
Our samples showed a significant correlation between COX2 and PGE_2S expression in breast tumors. This finding is not unexpected, since human breast cancers were shown by others to contain high levels of PGE_2 provided by the breast fibroblasts under the influence of inflammatory mediators, and the ability of breast tumors to produce PGE_2 is also related to high COX2 expression and metastatic potential.
PGE_2S was also related to ER expression in our samples. These results can be explained by the findings that the aromatase enzyme complex, which catalyzes estrogen biosynthesis, is regulated by PGE_2 via four transmembrane receptors and via induction of interleukin-6, thus providing the increased estrogen level fundamental to hormone-dependent growth of breast cancer.

COX2 and EGFR expression
In contrast to the model system, COX2 expression was not associated to either EGFR or HER2 expression. Possible explanations, as offered by other authors, are as follows: (i) Tumor tissue specimens represent a different environment from those in *in vitro* models. The production of growth factors as EGFR as well as COX2 synthesis by endothelial and stromal cells contribute to realization of a regulatory microenvironment which might not fit the straight biochemical relationships found in cell culture models. (ii) The association between high EGFR expression and poor response to chemotherapy was reported in studies utilizing a

radioligand assay for EGFR determination, while no association between EGFR and response to chemotherapy or clinical outcome was reported in cases of immunohistochemical assessment of EGFR expression. This suggests that the methodological approach could heavily affect the evaluation of the prognostic value of the marker. (iii) COX2 expression is regulated by other signaling pathways in addition to those promoted by the erb-B family members. In this context it is worth noting that inhibition of HER2/HER3 complex formation in colon cancer cells failed to completely inhibit COX2 protein expression.

In conclusion, our results indicate that HER2 positive breast cancers may have underlying gene amplification not only in the BC itself but also in the morphologically normal-appearing adjacent parenchyma. We have also shown that p53 nuclear accumulation can be observed in a small percentage of PTT. Neither HER2 nor p53 showed a gradient of alterations starting from the closest to the farther PTT, suggesting that these two molecules in benign tissue of cancer-containing breast could reflect a genomic damage due to long-term carcinogenic exposure. In contrast, a gradient of expression was evident for FasL since the PTT closest to invasive cancer showed an upregulated FasL and this upregulation was lost in PTT farther from the invasive carcinoma. These data support the hypothesis that FasL, in combination with other biological parameters, may be a novel biomarker useful to identify patients at higher risk of developing BC.

Furthetmore, our data indicate that in high-risk breast cancer patients the immunohistochemical evaluation of COX2, together with PGE_2S, p53, Ki67, HER2, Fas and FasL, may be of clinical value in distinguishing different responses to adjuvant anthracycline-based chemotherapy. Furthermore, HER2 copy number determined by FISH not only in the tumor itself but also in the normal-appearing PTT may be of help in determining more accurate prognosis.

New statements

1. In 5 cases out of the 10 HER2 positive peritumoral tissue samples (1cm) investigated showed HER2 amplification, thus supporting the hypothesis that the morphologically normal-appearing breast tissue already harbours molecular changes which may predict malignant transformation.
2. A gradient of expression was evident for FasL since the peritumoral tissues closest to invasive cancer showed an upregulated FasL ($p=0.05$) and this upregulation was lost in PTT farther from the invasive carcinoma ($p=0.04$ at 2 cm, $p=0.02$ at 3 cm). Thus, FasL, in combination with other biological parameters, may be a novel biomarker useful to identify patients at higher risk of developing recurrent breast cancer.
3. Tumor size, nodal status, COX2, PGE_2S, Ki67, Fas and FasL expression were significant predictors of DFS ($p=0.005$, $p<0.001$, $p=0.004$, $p=0.004$, $p=0.009$, $p<0.001$ and $p<0.001$ respectively) while OS was significantly influenced by tumor size, nodal status, COX2, p53, Ki67, HER2, Fas and FasL expression ($p=0.013$, $p=0.001$, $p=0.05$, $p=0.02$, $p=0.009$, $p=0.007$, $p<0.001$ and $p<0.001$ respectively). Out of these investigated markers, COX2 and PGE2S may be used as new prognostic markers for breast cancer.
4. Concomitant overexpression of COX2 and p53 has significantly decreased 5 year disease free survival ($p=0.003$) and overall survival ($p=0.02$).
5. In high-risk breast cancer patients the immunohistochemical evaluation of COX2, together with PGE_2S, p53, Ki67, HER2, Fas and FasL, may be of clinical value in distinguishing different responses to adjuvant anthracycline-based chemotherapy.

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Publications related to the thesis:

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2. **Nádasi E,** Sándor J, Mottolese M, Ember I: Prognostic factors in breast cancer patients. *Anticancer Res* (accepted for publication)

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