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ORIGINAL RESEARCH

Correlation between ER, PR, HER-2, Bcl-2, p53, proliferative and apoptotic indexes with *HER-2* gene amplification and *TOP2A* gene amplification and deletion in four molecular subtypes of breast cancer

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Abstract The aim of our study was to investigate *HER-2* and *TOP2A* gene status and their correlation with Bcl-2, p53, Ki67, ssDNA, and clinicopathological parameters in four molecular subtypes of breast cancer. Seventy-four paraffin-embedded samples are immunohistochemically studied for the expression of estrogen receptor (ER), progesterone receptor (PR), HER-2, p53, Bcl-2, ssDNA, and Ki67, while *HER-2* and *TOP2A* gene status by fluorescence in situ hybridization was investigated in 60 samples. Luminal A and B subtypes were characterized with small tumor size, intermediate histological grade, negative lymph node, and metastatic status, while triple negative and HER-2 positive subtypes were associated with larger tumor size, poorly differentiated tumors, and positive lymph node status. p53, Ki67, and ssDNA expression was higher in triple negative and HER-2 positive than in luminal subtypes, while ER, PR, and Bcl-2 dominated in luminal subtypes. *HER-2* gene status was higher in luminal B and HER-2 positive than in luminal A and triple negative subtypes, while *TOP2A* gene status was similar. *HER-2* gene status positively correlated with *TOP2A* gene status, HER-2 receptor, and histological grade, while negative correlation characterized relationship between *HER-2* gene status and ER, PR, and Bcl-2. The shortened overall survival period characterized patients from triple negative breast cancer subtype (18.7 months). *HER-2* and *TOP2A* gene amplification showed a tendency to be associated with larger tumor size, positive lymph node status, high level of apoptotic and proliferative indexes, and low level of p53 and Bcl-2 expression, which all together indicate group of patients with similar outcome during the progression of the disease.

Keywords *HER-2* · *TOP2A* · FISH · Immunohistochemistry · Apoptosis · Molecular subtypes of breast cancer

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Introduction

According to immunohistochemical expression for estrogen receptor (ER), progesterone receptor (PR) and HER-2, it has been identified four different molecular subtypes of breast cancer: luminal A (ER^+ and/or PR^+, HER-2^-), luminal B (ER^+ and/or PR^+, HER-2^+), HER-2 positive (ER^- and/or PR^-, HER-2^+), and triple negative (ER^- and/or PR^-, HER-2^-) breast cancer [1].

Some of the most important biological markers associated with prediction and prognosis of breast cancer therapy are *HER-2* and *TOP2A* gene amplification and protein expression [2, 3]. *HER-2* gene amplification and protein overexpression are observed in 20–30 % of metastatic breast tumors [4].

HER-2 and *TOP2A* are the most frequent amplified oncogenes in breast cancer. Co-amplification of *HER-2* and *TOP2A* has been seen in approximately 12–38 % of patients with breast cancers [5, 6]. Gene amplification and protein overexpression of the HER-2 and TOP2A have been reported to be linked to the sensitivity to Herceptin and anthracycline-based therapy as well as prognosis for breast cancer [7–9]. A

few retrospective studies have reported that *TOP2A* gene amplification occurs almost exclusively in conjunction with *HER-2* gene amplification [10, 11].

The interaction between gene amplification and protein expression for *HER-2* and *TOP2A* is very complex and demands further investigation by the fact that the protein overexpression is not always a consequence of gene amplification especially for *TOP2A* [10]. In addition, proliferative index (Ki67) and its prognostic significance in breast cancers are very controversial. Few earlier studies have reported that Ki67 is absent in ER-positive cells of normal breast tissue, while human mammary tumors have a high proliferation of ER-positive cells [12]. Proliferative and apoptotic indexes are positively correlated in breast cancer tissue [13]. In high grade tumor, increased apoptotic index is positively correlated with proliferative index and poorer survival, as an independent prognostic factor in breast cancer. Previous study showed that large tumors in diameter have significantly higher apoptotic index than small tumors, and also positively correlated with lymph node infiltration [14]. Also, it has been shown that tumor suppressor protein p53 induces apoptosis, while p53 expression may occur early in breast cancer development and increases during progression [15]. In contrast, Bcl-2 suppresses apoptosis and gradually decreases during the development of breast cancer [15]. This antiapoptotic protein Bcl-2 is inversely correlated with apoptotic and proliferative indexes [15]. Bcl-2 protein expression in all types of early breast cancer is an independent indicator of favorable prognosis [16].

Clinically, breast cancer is categorized into three therapeutic groups according to level of expression of steroid receptors and HER-2 receptor. The most numerous therapeutic groups in ER-positive group were patients receiving endocrine therapy [17, 18]. Herceptin with chemotherapy has a great clinical success in *HER-2*-amplified therapy group, while patients in triple negative breast cancer receive only chemotherapy [19, 20].

According to our knowledge, little is known about *TOP2A* gene amplification and deletion and *HER-2* gene amplifications and their association with apoptotic and proliferative indexes, antiapoptotic protein Bcl-2, and their correlation with therapy effects in four molecular subtypes of breast cancer. This information may be important in therapy prediction, as *TOP2A* and *HER-2* gene status has been associated with dose-dependent sensitivity to anthracycline and Herceptin therapy. So, besides *TOP2A* and *HER-2* gene statuses and protein expression studied by fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) methods, we perform analysis of p53, Bcl-2, proliferative (Ki67), and apoptotic (ssDNA) indexes and their correlation with clinicopathological parameters and overall survival in four molecular subtypes of breast cancer.

According to our results, *HER-2* and *TOP2A* gene amplified tumors were associated with low differentiated tumors, large tumor size in diameter, positive lymph node status, no distant metastases, prolonged overall survival, low level of p53 and Bcl-2 protein expression, HER-2 overexpression, and high level of apoptotic and proliferative indexes. Antiapoptotic Bcl-2 protein expression is almost completely absent in HER-2-positive breast cancer subtype where *HER-2* and *TOP2A* genes were amplified in the most of cases, and largely increased in luminal A and luminal B subtypes of breast cancer. Proliferative index (Ki67) and tumor suppressor protein p53 had similar pattern of expression in triple negative and HER-2-positive subtypes of breast cancer.

Patients and methods

Patients

The study was performed according to the regulations of the local ethics committee. We included 74 patients with breast cancer immunohistochemically classified into four molecular subtypes: luminal A (24 cases, 32.4 %), luminal B (26 cases, 35.1 %), HER-2 positive (9 cases, 12.2 %), and triple negative (15 cases, 20.3 %).

Immunohistochemistry

The tumor tissues were fixed in 10 % buffered formalin solution and embedded in paraffin. The tissue sections were cut at 5 μm, heated at 56 °C for 60 min, then deparaffinized and rehydrated through a series of xylenes and alcohols followed by an epitope retrieval step. Tissue sections were treated with 3 % H_2O_2 solution in PBS to block endogenous peroxidase activity. The next step was incubation with the primary antibody (Table 1) in a humidity chamber for 60 min at room temperature. Immunostaining was performed using the streptavidin–biotin technique ($LSAB^+$/HRP Kit, DAKO). Immunoreactivity complex was visualized with DAKO Liquid DAB^+ Substrate/Chromogen System (code no. K3468), counterstained with Mayer's hematoxylin (Merck, Whitehouse Station, NJ), and evaluated under a light microscope.

Scoring system

The results of the HER-2 IHC tests were counted using the HercepTest scoring system (0–3^+). The tumor samples were immunohistochemically scored as 3^+ (strong complete membrane staining is observed in >30 % of tumor cells), 2^+ (weak to moderate complete membrane staining in >30 % of tumor cells), 1^+ (weak, incomplete membrane staining in >30 % of tumor cells), and 0 (no staining) for HER-2. Tissue samples for estrogen and progesterone receptors were scored on a scale representing the estimated proportion and intensity of positive-staining tumor cells (range, 0 to 8). The score was

Table 1 List of antibodies

Antibody	Mo/Po Antigen unmasking technique	Manufactured code no.	Dilution	Detection system
Mo mouse anti-hu Estrogen receptor Clone 6F11	Mo Pressure cooker, 2 min 0.01 M citrate retrieval solution pH 6.0	Novocastra Lab., UK (NCL-L-ER-6 F11)	1:100	LSAB+/HRP
Mo Progesterone receptor Clone 16	Mo Pressure cooker, 2 min 0.01 M citrate retrieval solution pH 6.0	Novocastra Lab., UK NCL-L- PGR-312	1:100	LSAB+/HRP
Po rabbit anti-hu HER-2 (c-erbB-2 oncoprotein)	Po Water bath, 40 min, 0.01 M citrate retrieval solution pH 6.0 or DAKO cytomation target retrieval solution No. S1700	DAKO Cytomation Denmark A0485	1:350	LSAB+/HRP
Mo mouse anti-hu p53 clone DO-7	Mo Microwave, 20 min, 0.01 M citrate retrieval solution pH 6.0 or DAKO cytomation target retrieval solution No. S1700	DAKO Cytomation Denmark Cat# M7001	1:200	LSAB+/HRP
Mo mouse anti-hu Bcl-2 Clone 124	Mo Microwave, 20 min, 0.01 M citrate retrieval solution pH 6.0 or DAKO cytomation target retrieval solution No. S1700	DAKO Cytomation Denmark Cat# M 0887	1:100	LSAB+/HRP
Mo mouse anti-hu Ki67 Clone MM1	Mo Microwave, 20 min, 0.01 M citrate retrieval solution pH 6.0 or DAKO cytomation target retrieval solution No. S1700	DAKO Cytomation Denmark Cat#M7187	1:50	LSAB+/HRP
Mo mouse anti-hu ssDNA Clone F7-26	Mo Microwave, 20 min, 0.01 M citrate retrieval solution pH 6.0 or DAKO cytomation target retrieval solution No. S1700	Abcam plc 332, UK ab79439	1:100	LSAB+/HRP

Mo monoclonal antibody, *Po* polyclonal antibody

determined by adding intensity for immunoreactivity staining of nucleus in tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong) and the average number of staining nucleus. The tumor samples were immunohistochemically scored as 3^+ (strong), 2^+ (moderate), 1^+ (weak), and 0 (not stained) for p53 and Bcl-2. The intensity of the cytoplasmic Bcl-2 and nucleus p53 immunostaining was evaluated by dividing the cytoplasmic and nucleus staining reactions in three score groups: 1—weak cytoplasmic/nucleus staining intensity in 1 to 25 % of tumor cells; 2—moderate cytoplasmic/nucleus staining intensity in 25–70 % of tumor cells; 3—strong cytoplasmic/nucleus staining intensity in >70 % of tumor cells. Ki67 score was counted in 1,000 malignant cells in five high powered fields in every tissue section. The apoptotic index was defined as the average number of apoptotic cells counted in 3,000 malignant cells selected in high power fields (×40 objective) in a single histological tumor section from each patient. Negative control staining was conducted by omission of the primary antibody. Paraffin slides of invasive breast carcinoma were used as a positive control.

Fluorescence in situ hybridization assay

HER-2 and *TOP2A* gene copy number were analyzed in 60 breast tumor samples by *HER-2* FISH pharmDxTM kit and *TOP2A* FISH pharmDx TM kit (DakoCytomation), respectively. The probe mix consisted of a mixture of Texas Red-labeled DNA cosmid clones, covering either the *HER-2* (approximately 220 kb) or the *TOP2A* (approximately 230 kb) amplicon, and fluorescein-labeled peptide nucleic acid (PNA) probes for the chromosome 17 centromeric region (CEN-17). After deparaffinization and rehydration, specimens were heated in pretreatment solution at 95 °C for 10 min. The next step involved a proteolytic digestion using pepsin incubated 10 min at 37 °C. It was followed with FISH probe mix incubation combining PNA and DNA technologies. The denaturation of probe and target DNA was performed on a heating block at 82 °C for 5 min and incubated overnight in a humidified hybridization chamber at 45 °C. After removal of the coverslips, the slides were washed in a stringent wash buffer at 65 °C for 10 min followed by buffer washes and dehydration. Fluorescence mounting media, including DAPI, was applied and the coverslipped specimens.

The results were analyzed using a 100-W fluorescence microscope with a Texas Red and fluorescein isothiocyanate double filter to locate the invasive tumor areas at low magnification and read the high magnification signals with ×/60 oil immersion objective. Then, the *HER-2*/CEN-17 and *TOP2A*/CEN-17 ratio was calculated. For each sample, gene copy level was assessed in four areas of 60 non-overlapping tumor

cell nuclei. The 60 nuclei were evaluated in each area, the CEN-17 copy numbers was counted for each cell, and the ratio of *HER-2* and *TOP2A* signals to CEN-17 signals was calculated. *HER-2* and *TOP2A* to CEN-17 ratios were defined as normal at less than 1.5, as low at ratio 1.5–2.0, and as high level of gene amplification at ratio greater than 2.0. Normal cells in the analyzed tissue section served as an internal positive control of pretreatment and hybridization efficiency.

Statistical analysis

Descriptive data were defined as frequencies±SD. The correlation between *HER-2* and *TOP2A* gene status defined by FISH methods and other biological markers (ER, PR, HER-2, p53, Ki67, Bcl-2 protein, and ssDNA) were done using the Pearson's correlation test and independent-samples t test. A p value of <0.05 were considered to be a statistically significant.

Results

Clinicopathological parameters and correlation with biological markers

The ages of patients ranged from 38 to 89 years (mean±SD, 65.59±10.17 years). The tumor size ranged between 0.4 and 5.5 cm, and the histological grades were GI, GII, and GIII in 6 (8.2 %), 28 (37.8 %), and 28 (37.8 %) patients, respectively, while for 12 patients we did not have information about histological grade (12/74, 16.2 %). In 30 patients (40.5 %), lymph node status was negative, while positive lymph node status was detected in 30 patients (40.5 %). In two patients, regional lymph nodes cannot be assessed (2/74, 2.8 %), while for 12 patients we did not have information about lymph node status (12/74, 16.2 %). Distant metastasis characterized 9 (12.2 %) patients and in 50 (67.6 %) patients we did not find a distant metastasis (Table 2). Significant differences in tumor size was found between luminal A and triple negative breast cancer subtype ($t=-2.515$, $p<0.01$), while histological grade was statistically different between luminal A and triple negative ($t=-3.374$, $p<0.01$), luminal B and triple negative ($t=-3.380$, $p<0.01$), and HER-2 positive and triple negative ($t=3.350$, $p<0.01$) breast cancer subtypes (Table 2).

ER, PR, and HER-2 receptor expression

ER, PR, and HER-2 receptor expression were used for the division of breast cancer on four different molecular subtypes. According to our results, positive expression for ER and PR was detected in patients from luminal A and B subtypes, while negative expression characterized patients from HER-2 positive and triple negative breast cancer subtypes. Overexpression of HER-2 protein was detected in 35 patients (35/74, 47.3 %) from luminal B and HER-2 positive breast cancer subtypes (Table 3). Significant differences in ER expression was found between luminal A and triple negative subtype ($t=42.813$, $p<0.01$), luminal A and HER-2 positive ($t=32.887$, $p<0.01$), luminal B and HER-2 positive ($t=12.249$, $p<0.01$), and luminal B and triple negative ($t=16.070$, $p<0.01$) molecular subtypes of breast cancer (Fig. 1). PR expression was significantly different between luminal A and triple negative subtype ($t=8.914$, $p<0.01$), luminal A and HER-2 positive ($t=8.074$, $p<0.01$), luminal B and HER-2 positive ($t=5.537$, $p<0.01$), and luminal B and triple negative ($t=6.196$, $p<0.01$) molecular subtypes of breast cancer (Fig. 1). In contrast to ER and PR, significant differences in HER-2 expression was found between luminal A and luminal B ($t=-12.439$, $p<0.01$), luminal A and triple negative subtype ($t=3.696$, $p<0.01$), luminal A and HER-2 positive ($t=-12.048$, $p<0.01$), triple negative and HER-2 positive ($t=-13.185$, $p<0.01$), and luminal B and triple negative ($t=13.635$, $p<0.01$) molecular subtypes of breast cancer (Fig. 1).

Bcl-2, p53, Ki67, and ssDNA immuneexpression

Positive p53 protein expression was detected in 58 patients (78.4 %) while positive expression for Bcl-2 oncoprotein was detected in 52 patients (52/74, 70.3 %) (Table 3). In our study group, low level (54/74, 72.9 %) of proliferative index dominated (Table 3). High (36/74, 48.6 %) and low (38/74, 51.4 %) level of apoptotic index was detected in similar number of patients (Table 3). Significant differences in Bcl-2 expression was found between luminal A and triple negative ($t=4.048$, $p<0.01$), luminal A and HER-2 positive ($t=7.141$, $p<0.01$), luminal B and HER-2 positive ($t=4.565$, $p<0.01$), HER-2 positive and triple negative ($t=2.314$, $p<0.05$), and luminal B and triple negative ($t=2.231$, $p<0.05$) molecular subtypes of breast cancer (Fig. 2). In triple negative ($t=-6.679$, $p<0.01$) and HER-2 positive ($t=-2.024$, $p<0.05$) breast cancer subtypes, proliferative index was significantly higher than in patients from luminal A subtype (Fig. 2). Also, significant differences in proliferative index was detected between luminal B and HER-2-positive subtype ($t=-3.104$, $p<0.01$), triple negative and HER-2 positive ($t=-3.104$, $p<0.01$), and luminal B and triple negative ($t=-9.997$, $p<0.01$) breast cancer subtypes (Fig. 2). Apoptotic index was significantly higher in triple negative than in HER-2-positive ($t=2.285$, $p<0.05$) and luminal A ($t=-1.976$, $p<0.05$) breast cancer subtypes (Fig. 2 and Fig. 3).

HER-2 and TOP2A gene status in four molecular subtypes of breast cancer

HER-2 gene amplification was detected in 17 patients (17/60, 28.3 %) (Fig. 3), while *TOP2A* gene was amplified in 24 patients (24/60, 40 %) (Fig. 4) and *TOP2A* gene deletion was detected in 5 patients (5/60, 8.4 %). *HER-2* and *TOP2A* gene

Table 2 Clinicopathological parameters and therapy

Clinicopathological parameters	Luminal A subtype	Luminal B subtype	Triple negative	HER-2 positive	∑
Age					
45 to 50	3 (12.5 %)	4 (15.4 %)	3 (20 %)	1 (11.2 %)	11 (14.9 %)
>50	21 (87.5 %)[a]	21 (80.8 %)	12 (80 %)[a]	8 (88.9 %)	62 (83.8 %)
Not available	0	1 (3.8 %)	0	0	1 (1.3 %)
Tumor size					
pTx	0	2 (7.7 %)	0	0	2 (2.7 %)
pT0	1 (4.2 %)	1 (3.8 %)	0	0	2 (2.7 %)
pT1	9 (37.5 %)	8 (30.8 %)	5 (33.3 %)	3 (33.3 %)	25 (33.8 %)
pT2	5 (20.8 %)	2 (7.7 %)	5 (33.3 %)	2 (22.3 %)	14 (18.9 %)
pT3	2 (8.3 %)	4 (15.4 %)	0	1 (11.1 %)	7 (9.5 %)
pT4a	0	0	0	1 (11.1 %)	1 (1.3 %)
pT4b	1 (4.2 %)	2 (7.7 %)	4 (26.7 %)	1 (11.1 %)	8 (10.8 %)
pT4c	0	2 (7.7 %)	1 (6.7 %)	0	3 (4.1 %)
Not available	6 (25 %)	5 (19.2 %)	0	1 (11.1 %)	12 (16.2 %)
Histological grade					
GI	3 (12.5 %)	3 (11.5 %)	0	0	6 (8.2 %)
GII	9 (37.5 %)	11 (42.3 %)	1 (6.7 %)	7 (77.7 %)	28 (37.8 %)
GIII	6 (25 %)[b]	6 (23.1 %)[c]	14(93.3 %) [b, c, d]	2 (22.3 %)[d]	28 (37.8 %)
Not assessable	6 (25 %)	6 (23.1 %)	0		12 (16.2 %)
Lymph node status					
Nx	0	2 (7.7 %)	0	0	2 (2.7 %)
N0	12 (50 %)	10 (38.6 %)	5 (33.3 %)	3 (33.3 %)	30 (40.6 %)
N1	4 (16.7 %)	3 (11.5 %)	4 (26.7 %)	3 (33.3 %)	14 (18.9 %)
N2	2 (8.3 %)	5 (19.2 %)	5 (33.3 %)	2 (22.3 %)	14 (18.9 %)
N3	0	1 (3.8 %)	1 (6.7 %)	0	2 (2.7 %)
Not available	6 (25 %)	5 (19.2 %)	0	1 (11.1 %)	12 (16.2 %)
Distant metastasis					
M0	17 (70.8 %)	16 (61.5 %)	12 (80 %)	5 (55.6 %)	50 (67.6 %)
M1	1 (4.2 %)	4 (15.4 %)	3 (20 %)	2 (22.2 %)	10 (13.5 %)
Not available	6 (25 %)	6 (23.1 %)	0	2 (22.2 %)	14 (18.9 %)
Chemotherapy					
Yes	17 (70.8 %)	18 (69.2 %)	5 (33.3 %)	9 (100 %)	49 (66.2 %)
No	4 (16.7 %)	4 (15.4 %)	10 (66.7 %)	0	18 (24.3 %)
Missing	3 (12.5 %)	4 (15.4 %)	0	0	7 (9.5 %)
Tamoxifen (Nolvadex)					
Yes	20 (83.4 %)	19 (73.1 %)	13 (86.7 %)	1 (11.1 %)	53 (71.6 %)
No	1 (4.2 %)	3 (11.5 %)	2 (13.3 %)	8 (88.9 %)	14 (18.9 %)
Missing	3 (12.5 %)	4 (15.4 %)	0	0	7 (9.5 %)
Radiotherapy					
Yes	5 (20.8 %)	3 (11.5 %)	9 (60 %)	1 (11.1 %)	18 (24.3 %)
No	16 (66.7 %)	19 (73.1 %)	6 (40 %)	8 (88.9 %)	49 (66.2 %)
Missing	3 (12.5 %)	4 (15.4 %)	0	0	7 (9.5 %)
Herceptin/Transtuzumab					
Yes	0	6 (23.1 %)	2 (13.3 %)	3 (33.3 %)	11 (14.9 %)
No	21 (87.5 %)	16 (61.5 %)	13 (86.7 %)	6 (66.7 %)	56 (75.6 %)
Missing	3 (12.5 %)	4 (15.4 %)	0	0	7 (9.5 %)

pTx primary tumor cannot be assessed, *pT0* no evidence of primary tumor, *pT1* tumor 2.0 cm or less in greatest dimension, *pT2* tumor more than 2.0 cm but not more than 5.0 cm in greatest dimension, *pT3* tumor more than 5.0 cm in greatest dimension, *pT4* tumor of any size with direct extension to (a) chest wall or (b) skin or both (c), *GI* low grade (well-differentiated), *GII* intermediate grade (moderately differentiated), *GIII* high grade (poorly differentiated), N_X regional lymph nodes cannot be assessed (previously removed), *N0* no regional lymph node metastasis, *N1* metastasis to movable ipsilateral axillary lymph node(s), *N2* metastasis to ipsilateral axillary lymph node(s) fixed to each other, *N3* metastasis to ipsilateral internal mammary lymph node(s), *M0* no distant metastases, *M1* distant metastases

[a] Luminal A and triple negative subtype—tumor size ($t=-2.515$, $p<0.01$)

[b] Luminal A and triple negative subtype—histological grade ($t=-3.374$, $p<0.01$)

[c] Luminal B and triple negative subtype—histological grade ($t=-3.380$, $p<0.01$)

[d] HER-2-positive and triple negative subtype—histological grade ($t=3.350$, $p<0.01$)

amplification was dominated in patients from luminal B (*HER-2*, 11/26, 42.3 % and *TOP2A*, 11/26, 42.3 %) and HER-2-positive breast cancer subtypes (*HER-2*, 5/6, 83.4 % and *TOP2A*, 4/6, 66.7 %) (Table 4).

Table 3 Expression of ER, PR, HER-2, p53, Bcl-2, Ki67, and ssDNA in four molecular subtypes of breast cancer

Level of expression	Luminal A subtype	Luminal B subtype	Triple negative	HER-2 positive	∑
P53 tumor suppressor protein					
0	5 (20.8 %)	5 (19.2 %)	3 (20 %)	3 (33.3 %)	16 (21.6 %)
1	6 (25 %)	3 (11.6 %)	1 (6.7 %)	0	10 (13.5 %)
2	12 (50 %)	13 (50 %)	5 (33.3 %)	2 (22.2 %)	32(43.3 %)
3	1 (4.2 %)	5 (19.2 %)	6 (40 %)	4 (44.5 %)	16 (21.6 %)
Bcl-2 oncoprotein					
0	2 (8.3 %)	5 (19.2 %)	8 (53.3 %)	7 (77.8 %)	22 (29.7 %)
1	2 (8.3 %)	4 (15.4 %)	1 (6.7 %)	0	7(9.5 %)
2	4 (16.7 %)	7 (26.9 %)	4 (26.7 %)	0	15 (20.3 %)
3	16 (66.7 %)	10 (38.5 %)	2 (13.3 %)	2 (22.2 %)	30 (40.5 %)
Proliferative index (Ki67 protein)					
≤0.5	21 (87.5 %)	24 (92.3 %)	1 (6.7 %)	8 (88.9 %)	54 (73 %)
>0.5	3 (12.5 %)	2 (7.7 %)	14 (93.3 %)	1 (11.1 %)	20 (27 %)
Apoptotic index (ssDNA)					
≤0.5	14 (58.3 %)	15 (57.7 %)	4 (26.7 %)	5 (55.5 %)	38 (51.4 %)
>0.5	10 (41.7 %)	11 (42.3 %)	11 (73.3 %)	4 (44.5 %)	36 (48.6 %)
Estrogen receptor					
Negative	0	0	15 (100 %)	9 (100 %)	24 (32.4 %)
Positive	24 (100 %)	26 (100 %)	0	0	50 (67.6 %)
Progesterone receptor					
Negative	2 (8.3 %)	6 (23 %)	15 (100 %)	9 (100 %)	32 (43.2 %)
Positive	22 (91.7 %)	20 (77 %)	0	0	42 (56.8 %)
HER-2 receptor					
Negative	24 (100 %)	0	15 (100 %)	0	39 (52.7 %)
Positive	0	26 (100 %)	0	9 (100 %)	35 (47.3 %)

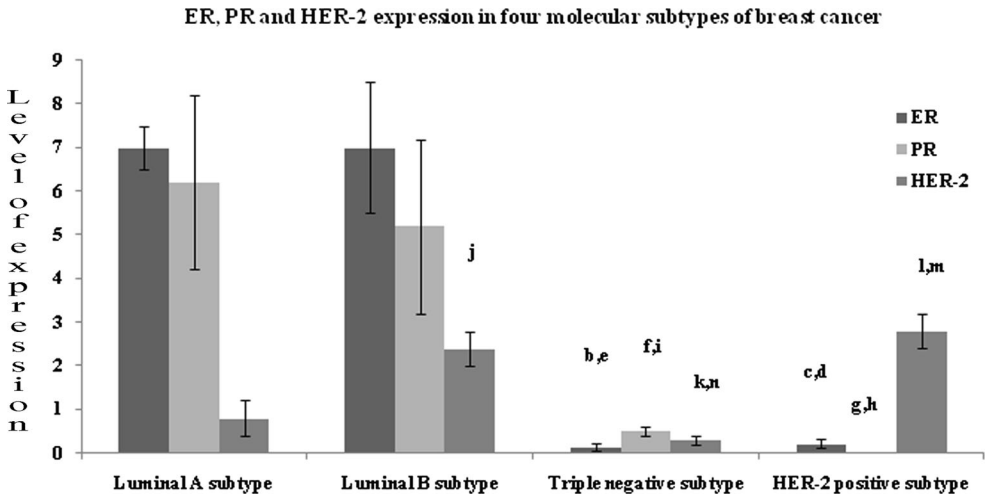

Fig. 1 ER, PR, and HER-2 immunoexpression in four molecular subtypes of breast cancer. ER: *b*—luminal A vs. triple negative subtype ($t=42.813$, $p<0.01$); *c*—luminal A vs. HER-2-positive subtype ($t=32.887$, $p<0.01$); *d*—luminal B vs. HER-2-positive subtype ($t=12.249$, $p<0.01$); *e*—luminal B vs. triple negative subtype ($t=16.070$, $p<0.01$). PR: *f*—luminal A vs. triple negative subtype ($t=8.914$, $p<0.01$); *g*—luminal A vs. HER-2-positive subtype ($t=8.074$, $p<0.01$); *h*—luminal B vs. HER-2-positive subtype ($t=5.537$, $p<0.01$); *i*—luminal B vs. triple negative subtype ($t=6.196$, $p<0.01$). HER-2 receptor: *j*—luminal A vs. luminal B subtype ($t=-12.439$, $p<0.01$); *k*—luminal A vs. triple negative subtype ($t=3.696$, $p<0.01$); *l*—luminal A vs. HER-2-positive subtype ($t=-12.048$, $p<0.01$); *m*—triple negative vs. HER-2-positive subtype ($t=-13.185$, $p<0.01$); *n*—luminal B vs. triple negative subtype ($t=13.635$, $p<0.01$)

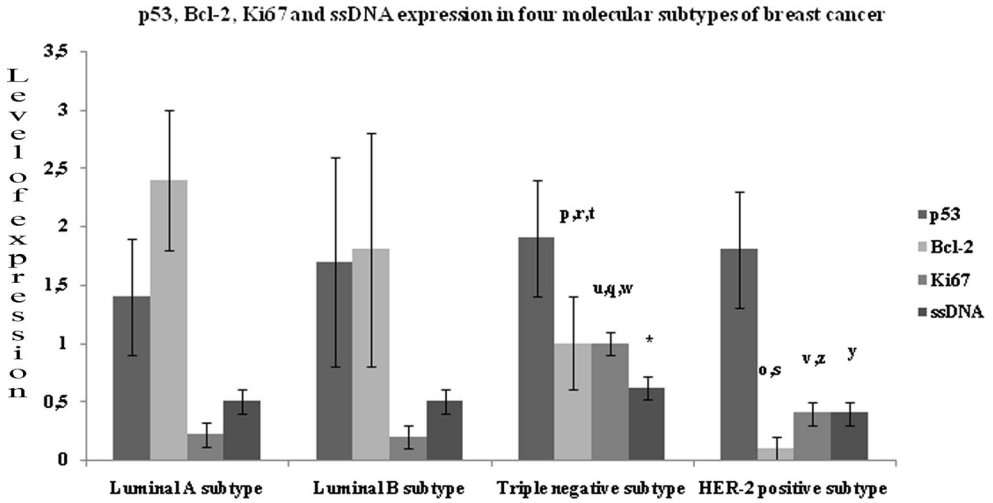

Fig. 2 p53, Bcl-2, Ki67, and ssDNA immunoexpression in four molecular subtypes of breast cancer. Bcl-2 protein: *p*—luminal A vs. triple negative subtype ($t=4.048$, $p<0.01$); *o*—luminal A vs. HER-2-positive subtype ($t=7.141$, $p<0.01$); *s*—luminal B vs. HER-2-positive subtype ($t=4.565$, $p<0.01$); *r*—HER-2-positive vs. triple negative subtype ($t=2.314$, $p<0.05$); *t*—luminal B vs. triple negative subtype ($t=2.231$, $p<0.05$). Ki67 protein: *u*—luminal A vs. triple negative subtype ($t=-6.679$, $p<0.01$); *v*—luminal A vs. HER-2-positive subtype ($t=-2.024$, $p<0.05$); *z*—luminal B vs. HER-2-positive subtype ($t=-3.104$, $p<0.01$); *q*—HER-2-positive vs. triple negative subtype ($t=-3.104$, $p<0.01$); *w*—luminal B vs. triple negative subtype ($t=-9.997$, $p<0.01$). ssDNA: *asterisk*—luminal A vs. triple negative subtype ($t=2.285$, $p<0.05$); *y*—luminal A vs. HER-2-positive subtype ($t=-1.976$, $p<0.05$)

HER-2 gene status was significantly higher in luminal B ($t=-3.300$, $p<0.01$) and HER-2 positive ($t=-4.244$, $p<0.01$) than in luminal A breast cancer subtype (Fig. 5). Also, significant differences in *HER-2* gene status was detected between

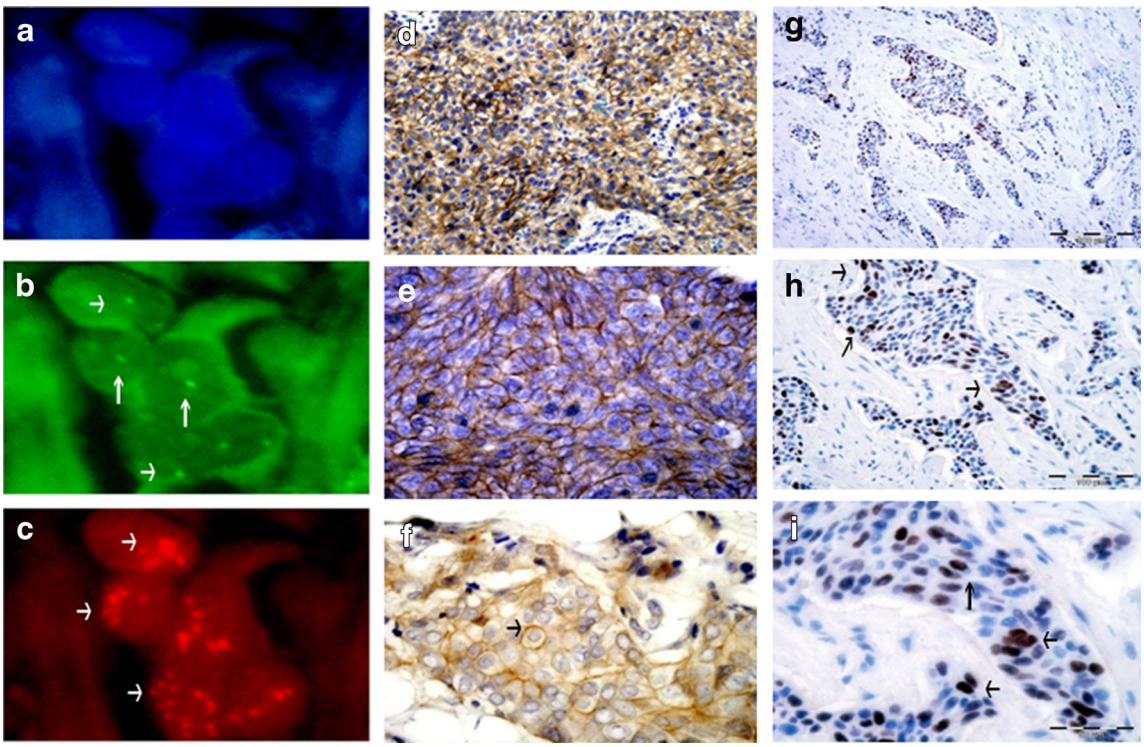

Fig. 3 High level of *HER-2* gene amplification (**a**–**c**) by FISH and HER-2 receptor (**d**–**f**), ssDNA (apoptotosis, **g**–**i**) expression by immunohistochemistry in patients from luminal B breast cancer molecular subtype. High level of *HER-2*-amplified breast cancer cells with 5 to 12 HER-2 gene copies per cells (red signals) and an *HER-2*/CEP17 ratio of ≥2 (**c**). Completely, strong circumferential HER-2 membrane staining in more than 30 % of tumor cells (**d**, **e**) and partial moderate intensity HER-2 membrane staining in more than 30 % of breast cancer cells with no complete circumferential staining (**f**). Strong nuclear immunostaining for ssDNA in breast cancer cells (**g**–**i**). Magnification ×60 (**a**–**c**), ×40 (**e**, **f**, **i**), ×20 (**d**, **h**), ×10 (**g**)

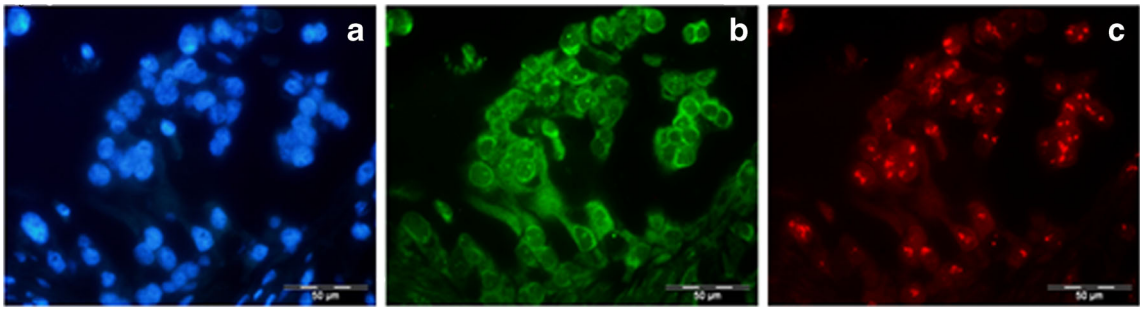

Fig. 4 High level of *TOP2A* gene amplification in breast cancer cells in patients from luminal B breast cancer subtype. Dual-color FISH with specific gene probe (red signals for *TOP2A*) and green CEP17 signals (**b**) on formalin-fixed, paraffin-embedded sections of breast carcinomas. Miniclusters of red signals found in nucleus of breast cancer cells (**c**) indicate high level of *TOP2A* gene amplification. Magnification ×40 (**a**-**c**)

HER-2-positive and luminal B ($t=-2.550$, $p<0.01$) and HER-2-positive and triple negative ($t=-2.149$, $p<0.05$) breast cancer subtypes (Fig. 5). *TOP2A* gene status was not significantly different between four molecular breast cancer subtypes (Fig. 5).

Correlation between HER-2 and TOP2A gene status with ER, PR, HER-2, p53, Bcl-2, Ki67, and ssDNA immunoexpression in four molecular subtypes of breast cancer

Statistically significant positive correlation characterized relationship between *HER-2* gene status and HER-2 receptor ($r=0.555$, $p<0.05$) expression, *TOP2A* gene status ($r=0.362$, $p<0.01$), and histological grade ($r=0.362$, $p<0.01$) in all patients from our study. Negative correlation characterized relationship between *HER-2* gene status and ER ($r=-0.283$, $p<0.05$), PR ($r=-0.302$, $p<0.05$), and Bcl-2 ($r=-0.280$, $p<0.05$) protein expression (Table 5). In luminal A breast cancer subtype significant positive correlation was detected between *HER-2* gene status and HER-2 receptor expression ($r=0.698$, $p<0.01$). In luminal B subtype, positive correlation characterized relationship between *HER-2* gene status and *TOP2A* gene status ($r=0.517$, $p<0.05$), lymph node status ($r=0.562$, $p<0.05$), and metastases ($r=0.528$, $p<0.05$), while negative correlation was detected between *HER-2* gene status and PR expression ($r=-0.429$, $p<0.05$) as well as *HER-2* gene status and tumor size ($r=-0.469$, $p<0.05$) (Table 5). Significant positive correlation characterized relationship between *HER-2* gene status and p53 protein expression in triple negative breast cancer subtype ($r=1$, $p<0.01$), while significant negative correlation was detected between *HER-2* gene status and metastatic status in HER-2-positive ($r=-0.884$, $p<0.05$) breast cancer subtype (Table 5).

Survival analysis and therapy

Treatment was nonrandomized and consistent with endocrine therapy, chemotherapy, radiotherapy, Herceptin, and their different combinations (Table 6). A combination of chemotherapy and endocrine therapies was given to 33 patients (44.6 %), while a combination of those two therapies with radiotherapy was given to 8 patients (10.8 %) (Table 6).

Follow-up of the patients extended from January 2009 to January 2013. Two patients (2.7 %) died of breast carcinoma and 62 patients (83.8 %) did not have disease symptoms. Four years of overall survival (OS) was detected in 62 patients (83.8 %). The median disease-free survival (DFS) and OS were 35.7 months. We found that DFS and OS were significantly longer (46 months) in patients from HER-2-positive molecular subtype. Those tumors were also characterized with intermediate histological grade (GII), overexpression of HER-2 receptor, p53 protein, *HER-2* and *TOP2A* gene amplification, and negative expression for Bcl-2 in all patients. The shortened OS characterized patients from triple negative breast cancer subtype (18.7 months) associated with negative expression for HER-2, ER, PR, and high proliferative index. In luminal B breast cancer subtype, increased Bcl-2 protein expression was significantly associated with more prolonged period of OS ($p<0.05$).

Discussion

According to our study, *HER-2* gene amplification is registered in 28.3 %, while *TOP2A* gene amplification is detected in 40 % of patients with breast cancer. *HER-2* gene status positively correlated with HER-2 receptor, *TOP2A* gene status, and histological grade, while negative correlation characterized relationship between *HER-2* gene status and ER, PR, Bcl-2 protein expression, and metastatic status. The most significant correlation *HER-2* gene status was detected in luminal B breast cancer subtype where *HER-2* gene status positively correlated with *TOP2A* gene status, metastatic status, lymph node status, and also negatively correlated with PR expression and tumor size. Generally, *HER-2* and *TOP2A* gene amplified tumors were associated with low differentiated tumors, large tumor size in diameter, positive lymph node status, no distant metastases, prolonged overall survival, low level of p53 and Bcl-2 protein expression, HER-2 overexpression, and high level of apoptotic and proliferative indexes.

Table 4 *HER-2* and *TOP2A* gene status and their correlation with HER-2 protein expression in four molecular subtypes of breast cancer

Luminal A breast cancer subtype							
HER-2 IHC	*HER-2* non-amplified	*HER-2* low amplified	*HER-2* high amplified	*TOP2A* non-amplified	*TOP2A* low amplified	*TOP2A* high amplified	*TOP2A* deletion
0	5 (20.8 %)	0	0	4 (16.7 %)	1 (4.2 %)	0	0
1	18 (75 %)	1 (4.2 %)	0	11 (45.8 %)	4 (16.7 %)	3 (12.5 %)	1 (4.2 %)
∑	23 (95.8 %)	1 (4.2 %)	0	15 (62.5 %)	5 (20.8 %)	3 (12.5 %)	1 (4.2 %)
Luminal B breast cancer subtype							
HER-2 IHC	*HER-2* non-amplified	*HER-2* low amplified	*HER-2* high amplified	*TOP2A* non-amplified	*TOP2A* low amplified	*TOP2A* high amplified	*TOP2A* deletion
2	10 (38.5 %)	1 (3.8 %)	4 (15.4 %)	6 (23.1 %)	1 (3.8 %)	5 (19.2 %)	3 (11.5 %)
3	5 (19.2 %)	0	6 (23.1 %)	4 (15.4 %)	2 (7.7 %)	3 (11.5 %)	1 (3.8 %)
∑	15 (57.7 %)	1 (3.8 %)	10 (38.5 %)	10 (38.5 %)	3 (11.5 %)	8 (30.8 %)	4 (15.4 %)
Triple negative breast cancer subtype							
HER-2 IHC	*HER-2* non-amplified	*HER-2* low amplified	*HER-2* high amplified	*TOP2A* non-amplified	*TOP2A* low amplified	*TOP2A* high amplified	*TOP2A* deletion
0	1 (25 %)	0	0	1 (25 %)	0	0	0
1	3 (75 %)	0	0	2 (50 %)	1 (25 %)	0	0
∑	4 (100 %)	0	0	3 (75 %)	1 (25 %)	0	0
HER-2 positive breast cancer subtype							
HER-2 IHC	*HER-2* non-amplified	*HER-2* low amplified	*HER-2* high amplified	*TOP2A* non-amplified	*TOP2A* low amplified	*TOP2A* high amplified	*TOP2A* deletion
2	1 (16.7 %)	0	0	1 (16.7 %)	0	0	0
3	0	1 (16.7 %)	4 (66.6 %)	1 (16.7 %)	1 (16.7 %)	3 (50 %)	0
∑	1 (16.7 %)	1 (16.7 %)	4 (66.6 %)	2 (33.3 %)	1 (16.7 %)	3 (50 %)	0

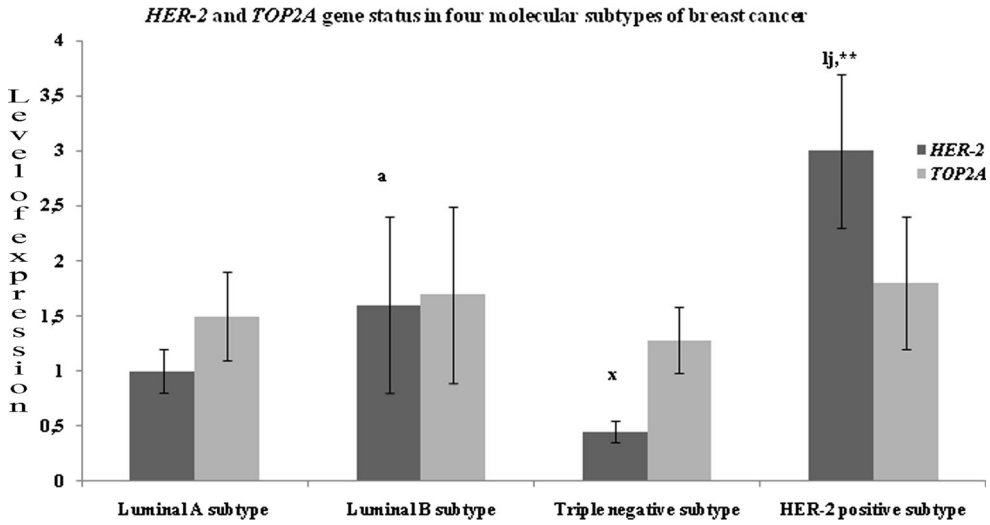

Fig. 5 *HER-2* and *TOP2A* gene status in four molecular subtypes of breast cancer. *HER-2* gene status: *a*—luminal A and luminal B subtype ($t=-3.300$, $p<0.01$); *lj*—luminal A and HER-2-positive subtype ($t=-4.244$, $p<0.01$); *double asterisks*—luminal B and HER-2-positive subtype ($t=-2.550$, $p<0.01$); *x*—HER-2 positive and triple negative subtype ($t=-2.149$, $p<0.05$)

Anti-apoptotic Bcl-2 protein expression is almost completely absent in HER-2-positive subtype and largely increased in luminal A and B subtypes of breast cancer. Similar pattern of expression, for proliferative index (Ki67) and tumor suppressor protein p53, is registered in triple negative and HER-2-positive subtypes of breast cancer. The combination of endocrine and chemotherapy was used in the majority cases of luminal A breast cancer subtype, while in luminal B and HER-2 positive breast cancer subtypes patients were treated with chemotherapy, endocrine therapy, and Herceptin.

HER-2 and *TOP2A* gene statuses and protein overexpression have important prognostic and therapeutic value in breast cancer patients, related to a poor prognosis, high frequency of disease recurrence, and reduced overall survival [9]. Our results showed that overall survival was the most reduced in triple negative breast cancer subtype associated with no gene amplification for *HER-2* and *TOP2A* and negative expression for HER-2 protein, but for some general conclusion we should mentioned that this could be consequence of small sample size. When analyzing overall survival in breast cancer subtypes, we found that majority of HER-2-positive tumors had the longest overall survival period and also disease-free period in relation to other subtypes. This is also could be a consequence of small sample size in HER-2-positive molecular subgroup of patients.

According to literature data, *HER-2* and *TOP2A* genes are usually co-amplified in the same breast cancer cases [10, 11], but our results showed that *TOP2A* gene amplification is not completely restricted to the cases with *HER-2* gene amplification. A study, conducted by Manna et al., showed that regulation of *TOP2A* gene status and protein expression is more complex and involves processes such as apoptosis and cell proliferation [21].

In this study, we also evaluated expression of different biological markers, including Bcl-2 protein, in four molecular subtypes of breast cancer. The expression of Bcl-2 protein was detected in luminal cells from normal breast tissue, gradually decreasing during the development of breast cancer [22]. In our study, all luminal tumors were Bcl-2 and steroid receptors positive with a good prognosis in agreement with previous reports [14, 23]. In contrast to luminal breast cancer subtypes, poorly differentiated HER-2-positive tumors were usually negative for Bcl-2 protein, with a poorer prognosis than luminal breast cancer subtypes. Daidone et al. showed that Bcl-2 overexpression correlates with low level of proliferative index, absence of p53 protein expression and HER-2 overexpression, and generally was associated with a favorable outcome [24]. Reduction in expression of Bcl-2 protein was associated with progression and aggressive form of disease [25], and in our study, most patients (seven of nine) from HER-2-positive subtype of breast cancer were characterized with no expression of Bcl-2 protein.

Our results showed that triple negative breast cancer had the highest level of proliferative index in relation to luminal A and B subtypes and this was in agreement with previous findings [26]. p53 tumor suppressor protein is mutated in approximately 30 % of breast cancers and seems to be more frequent in HER-2-positive and triple negative breast cancer subtype, while is not so numerous in luminal tumors [27]. Our results confirmed the findings related to a low level of p53 protein expression in luminal breast cancer subtypes [15, 27].

Apoptosis plays a critical role in breast tumorogenesis controlled by Bcl-2 and tumor suppressor protein p53 [13, 15]. To our knowledge, we were the first who investigated a correlation between apoptotic index and *HER-2* and *TOP2A* gene statuses in four different molecular subtypes of breast cancer. Only in luminal B and HER-2-positive breast cancer subtypes that apoptotic index positively correlated with *HER-2* gene status, while in triple negative and luminal A breast cancer subtypes, the *HER-2* gene status negatively correlated with apoptotic index. However, this correlation was not statistically significant.

Table 5 Correlations between HER-2 and TOP2A gene status with ER, PR, HER-2, p53, Bcl-2, Ki67, ssDNA, and clinicopathological parameters in four molecular subtypes of breast cancer

HER-2/*TOP2A* gene status	All 60 patients ($n=60$)												
	ER	PR	HER-2	p53	Bcl-2	Ki67	ssDNA	*HER-2*	*TOP2A*	Metastases	Lymph node status	Histological grade	Tumor size
HER-2 gene status	−0.283*	−0.302*	0.555*	0.198	**−0.280***	−0.014	−0.025	1	**0.362****	**−0.128**	−0.060	**0.362****	−0.257
TOP2A gene status	0.075	−0.107	0.160	−0.078	0.053	−0.201	−0.016	**0.362****	1	0.030	−0.054	0.099	0.035
Luminal A subtype													
	ER	PR	HER-2	p53	Bcl-2	Ki67	ssDNA	*HER-2*	*TOP2A*	Metastases	Lymph node status	Histological grade	Tumor size
HER-2 gene status	−0.311	0.323	**0.698****	0.063	0.148	0.031	−0.176	1	0.197	0.041	0.034	0.016	0.030
TOP2A gene status	0.075	0.104	0.231	0.082	−0.317	−0.078	0.011	0.197	1	0.062	−0.290	0.232	0.384
Luminal B subtype													
	ER	PR	HER-2	p53	Bcl-2	Ki67	ssDNA	*HER-2*	*TOP2A*	Metastases	Lymph node status	Histological grade	Tumor size
HER-2 gene status	−0.236	**−0.429***	0.048	0.114	−0.147	0.132	0.058	1	**0.517***	**0.528***	**0.562***	−0.242	**−0.469***
TOP2A gene status	0.230	−0.264	0.321	−0.194	0.364	−0.256	−0.052	**0.517***	1	0.012	0.224	0.281	0.083
Triple negative subtype													
	ER	PR	HER-2	p53	Bcl-2	Ki67	ssDNA	*HER-2*	*TOP2A*	Metastases	Lymph node status	Histological grade	Tumor size
HER-2 gene status	/	−0.577	0.577	**1****	0.192	0.784	−0.600	1	0.412	−0.577	−0.577	/	0.577
TOP2A gene status	/	−0.714	−0.238	0.412	−0.175	−0.226	−0.842	0.412	1	0.333	−0.714	/	-0.238
HER-2 positive subtype													
	ER	PR	HER-2	p53	Bcl-2	Ki67	ssDNA	*HER-2*	*TOP2A*	Metastases	Lymph node status	Histological grade	Tumor size
HER-2 gene status	/	/	0.386	0.325	/	−0.199	0.579	1	0.483	**−0.884***	−0.598	−0.732	−0.678
TOP2A gene status	/	/	0.540	/	/	−0.605	0.543	0.483	1	0	−0.258	0.577	−0.732

Bold indicate significant level of correlations

* $p<0.05$; ** $p<0.01$

Table 6 Therapy combination in patients from four molecular subtypes of breast cancer

Therapy/molecular subtype	Luminal A subtype	Luminal B subtype	Triple negative subtype	HER-2-positive subtype
Endocrine therapy	3 (12.5 %)	3 (11.5 %)	0	0
Chemotherapy	0	2 (7.7 %)	2 (13.3 %)	2 (22.2 %)
Chemo/endocrine therapy	11 (45.8 %)	9 (34.6 %)	11 (73.4 %)	2 (22.2 %)
Chemo/endocrine/radiotherapy	6 (25 %)	2 (7.7 %)	0	0
Chemo/endocrine/Herceptin	0	2 (7.7 %)	0	0
Herceptin	0	0	2 (13.3 %)	3 (33.4 %)
Chemo/radiotherapy	0	2 (7.7 %)	0	0
Chemo/Herceptin	0	0	0	2 (22.2 %)
Chemo/endocrine/ Radiotherapy/Herceptin	0	2 (7.7 %)	0	0
Not available	4 (16.7 %)	4 (15.4 %)	0	0

p53 protein expression can be detected early in breast cancer development and increases during progression [15]. Previous studies have showed an inverse relationship between Bcl-2 and p53 protein expression in invasive breast cancer [15]. The study conducted by Bottini et al. showed that Bcl-2 protein has inverse correlation with proliferative index, also positive correlation with p53 protein and steroid receptors expression in agreement with our results [28]. This is confirmed by the fact that the highest level of Bcl-2 protein expression was detected in luminal A and B breast cancer subtypes in correlation with triple negative and HER-2-positive subtypes. This positive association supports a previously posted hypothesis that positive estrogen and progesterone receptor status up-regulated Bcl-2 expression which could decrease apoptosis and proliferation [29].

Luminal breast cancer subtypes, in our study, share some similar patterns of expression for different biological markers including steroid receptors, low level of expression for p53 and apoptotic index, proliferative index, and high level of expression for Bcl-2 protein. Those tumors also have small size in diameter, negative lymph node status, no metastasis, combination of endocrine and chemotherapy, and similar period of overall survival (36 months in luminal A and 32 months in luminal B subtype). In luminal B breast cancer subtype, *HER-2* gene status positively correlated with *TOP2A* gene status, metastatic status, and lymph node status and also negatively correlated with PR expression and tumor size. In contrast to luminal breast cancer subtypes, triple negative and HER-2-positive breast cancers were characterized with increased expression for p53 protein, Ki67 protein, and decreased Bcl-2 protein expression. Basic pathological characteristics of tumors in triple negative and HER-2-positive breast cancer subtypes were larger tumor size, positive lymph node status, no metastasis, and low level of Bcl-2 protein expression. In contrast to luminal breast cancer subtypes, in triple negative breast cancer subtype, *HER-2* gene status positively correlated with p53 protein expression, while in HER-2-positive subtype, *HER-2* gene status negatively correlated with metastatic status. Overall survival period was prolonged in HER-2 positive, but reduced in triple negative breast cancer.

The presented results showed that the genetic modification of *TOP2A* gene was significantly more individual and independent of the modification of *HER-2* gene, so *TOP2A* gene status should be treated as a separate predictive and prognostic marker in breast cancer. *HER-2* gene status positively correlated with *TOP2A* gene status and HER-2 receptor expression, positive lymph node status, and metastatic status, while negative correlation characterized relationship between *HER-2* gene status and ER, PR, and Bcl-2 expression and tumor size, which all together indicate a group of patients with similar outcome during cancer progression.

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Conflict of interest The authors declare that they have no conflict of interest.

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