

BRCA1 AND TOP2A GENE AMPLIFICATION AND PROTEIN EXPRESSION IN FOUR MOLECULAR SUBTYPES OF BREAST CANCER

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Abstract - We studied *TOP2A* amplification (using FISH methods), and *TOP2A* and *BRCA1* protein overexpression (immunohistochemistry) in four molecular subtypes of breast cancer. Of 53 patients, 32 showed *TOP2A* and 38 showed *BRCA1* overexpression. The highest percentage of *TOP2A* amplification (47.8%) and deletion (13%) was detected in Luminal B subtypes. Of 11 Luminal B tumors with *TOP2A* amplification, 9 (81.8%) overexpressed *TOP2A*. *BRCA1* protein overexpression showed significant positive correlation with *TOP2A* protein expression. *BRCA1* and *TOP2A* proteins exhibited similar patterns of expression in Luminal B and triple-negative breast cancer, suggesting the same prognosis in those patients.

Key words: *BRCA1*, *TOP2A*, FISH, immunohistochemistry, molecular subtypes of breast cancer

Abbreviations: *TOP2A* – Topoisomerase II A; *BRCA1* – Breast Cancer Type 1 Susceptibility Protein; CEF – cyclophosphamide, epirubicin and 5-fluorouracil; ER – estrogen receptor; PR – progesterone receptor.

INTRODUCTION

According to immunohistochemical identification of three biological markers, there are four different subtypes of breast cancer: Luminal A (ER⁺ and/or PR⁺, HER2⁻), Luminal B (ER⁺ and/or PR⁺, HER2⁺), HER-2 positive (ER⁻ and/or PR⁻, HER2⁺) and triple-negative breast cancer (ER⁻ and/or PR⁻, HER2⁻) (Bertolo et al., 2008, Marchio et al., 2008).

Some of the most important biological markers associated with the prediction and prognosis for breast cancer therapy are *BRCA1* and *TOP2A* (Arriola et al., 2008, Miyoshi et al., 2008). The *TOP2A* gene is located on the long arm of chromosome 17 and plays an important role in DNA replication, transcription and recombination (Arriola et al.,

2008). The *BRCA1* gene is located on chromosome 17q12-21. *BRCA1* gene mutations are responsible for about 40-45% of hereditary breast cancers, while in sporadic breast cancer those mutations are rare. Expression of *BRCA1* protein is frequently reduced in sporadic breast cancer, suggesting a very important and wider role in mammary carcinogenesis (Miyoshi et al., 2008). Previously it was observed that the *BRCA1* protein has different biological functions, including roles in cell cycle progression, DNA repair processes, DNA damage (responsive cell cycle check points), regulation of specific transcriptional pathways and in apoptosis. Breast cancer with *BRCA1* gene mutations has a high frequency of p53 gene mutations, usually linked to a negative estrogen and progesterone receptor status (Miyoshi et al., 2008).

Recently, it has been suggested that the *TOP2A* gene could play a critical role in response to therapy of breast cancer cells (Coon et al., 2002). Doxorubicin, epirubicin and other anthracyclines bind to *TOP2A*, resulting in cell cycle arrest and apoptosis (Schindlbeck et al., 2010, Brase et al., 2010). Previous findings also suggested that *TOP2A* gene amplification and protein overexpression was found in 70% of breast cancer cases (Bhargava et al., 2005).

Little is known about *TOP2A* gene amplification and protein status and their association with BRCA1 protein expression in four different molecular subtypes of sporadic breast cancer. This information may be of importance for predicting the effect of therapy, as the amplification of *TOP2A* has been associated with a dose-dependent sensitivity to anthracycline therapy in breast cancer. In addition, results from this study will contribute to completing the knowledge about the similar pattern of expression of *TOP2A* and BRCA1 in four different molecular subtypes of breast carcinoma and their prognostic significance.

MATERIALS AND METHODS

Subjects

The study was performed according to the regulations of the local ethics committee. We included 53 patients with breast cancer who underwent surgery from 2009 through 2010. The tissue samples of the breast cancer patients were immunohistochemically classified into 4 molecular subtypes: Luminal A (20 cases, 37.7%), Luminal B (23 cases, 43.4%), HER-2 positive (6 cases, 11.4%) and triple-negative (4 cases, 7.5%).

Immunohistochemistry

The material was fixed in 10% buffered formalin solution and embedded in paraffin. The tissue sections were cut at a thickness of 5 μ m, heated at 56°C for 60 min, and then deparaffinized and rehydrated through a series of xylenes and alcohols, followed by an epitope retrieval step in which the tissue sam-

ples were heated in microwave oven (at 680 W, in 10 mmol/L citrate buffer pH 6.0, for 21 min). Tissue sections were treated with 3% H₂O₂ solution in PBS to block endogenous peroxidase activity. The next step was incubation with the specific antibody in a humidity chamber for 60 min at room temperature (Table 1). Immunostaining was performed using the streptavidin biotin technique (LSAB⁺/HRP Kit, Peroxidase Labeling, K0690, DAKO Cytomation, Denmark). The immunoreactivity complex was visualized with DAKO Liquid DAB⁺ Substrate/Chromogen System (Code No. K3468) and counterstained with Mayer's hematoxylin (Merck) and examined under a light microscope. For *TOP2A*, the nuclei of 50 tumor cells were counted under a microscope by two independent examiners, and the proportion of stained cells was recorded. A cut-off value of 15% separated the negative ($\leq 15\%$) and positive cases ($>15\%$).

Overexpression of the BRCA1 protein was identified when nuclear staining was found in more than 25% of tumor cells.

The results of the HER-2 IHC tests were scored 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 is observed in $>30\%$ of tumor cells), 1⁺ (weak, incomplete membrane staining in $>30\%$ of tumor cells) and 0 (no staining) for HER-2. Determinations of estrogen and progesterone receptors were based on both the intensity and the proportion of nuclear staining (0 to 8). Negative control staining was conducted by omission of the primary antibody. Paraffin slides of invasive breast carcinoma were used as a positive control.

FISH methods

The *TOP2A* FISH pharmDx TM Kit (DakoCytomation, Glostrup, Denmark) was used to investigate *TOP2A* gene copy number. The probe mix consisted of a mixture of Texas Red-labeled DNA cosmid clones covering the *TOP2A* (approximately 230 kb) amplicon and fluorescein-labeled peptide nucleic

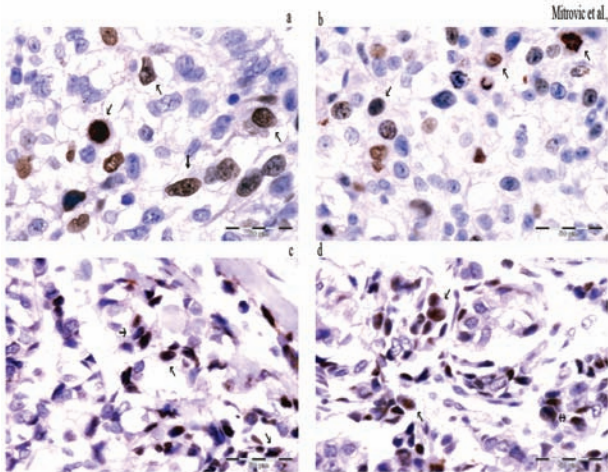


Fig. 1. Immunohistochemical staining of TOP2A and BRCA1 proteins in the Luminal B breast cancer subtype. Representative results of immunohistochemical staining for TOP2A (a, b) and BRCA1 (c, d) in nuclei of breast cancer cells of the Luminal B molecular subtype. Immunoreactive cells – arrow.

acid (PNA) probes for the chromosome 17 centromeric region. After deparaffinization and rehydration, specimens are heated in pre-treatment solution at 95°C for 10 min. The next step involved a proteolytic digestion using ready-to-use pepsin for 10 min at 37°C. Following the heating and proteolytic pre-treatment steps, the samples were incubated with ready-to-use FISH probe mix based on a combination of PNA (peptide nucleic acid) and DNA technology. The denaturation of probe and target DNA was performed on a heating block at a temperature of 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 stringent wash buffer at 65°C for 10 min followed by buffer washes and dehydration. Fluorescence mounting media including DAPI was applied and the specimens coverslipped.

The results were analyzed using a 100-W fluorescence microscope fitted with the Texas Red and fluorescein isothiocyanate (FITC) double filter for locating the invasive tumor areas at low magnification and reading the signals at high magnification with a x/60 oil-immersion objective. Then the TOP2A/CEN-17 ratio was calculated. For each specimen, the gene

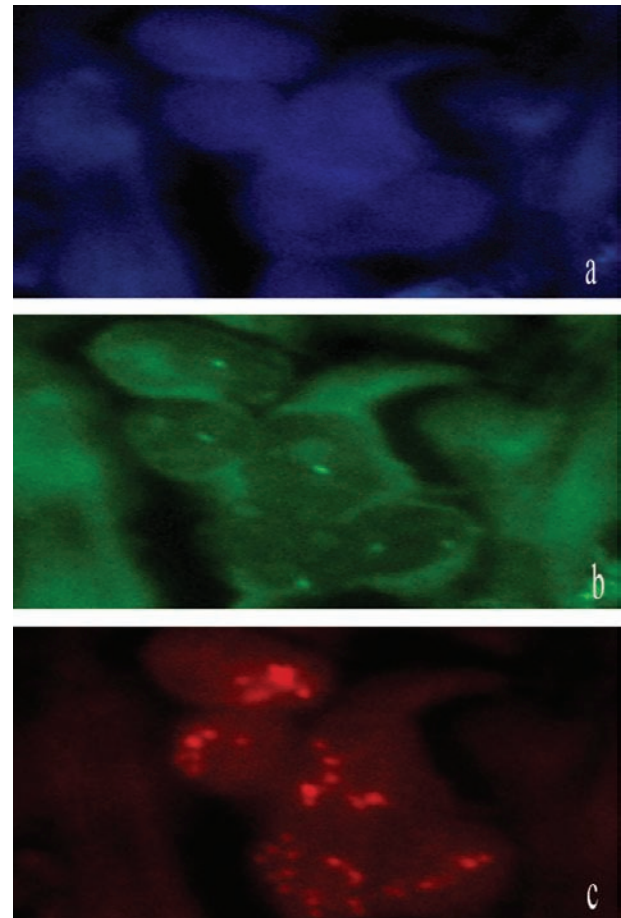


Fig. 2. High level of TOP2A gene amplification in nucleus of infiltrative breast cancer cells Luminal B breast cancer subtype. Note high level of TOP2A gene amplification in nucleus of breast cancer cells (c). FISH analysis shows more than 10 signals per cell, indicating a high level of TOP2A gene amplification. Centromeres of chromosome 17 were detected by FITC green signals in single nucleus (b). Counterstained DAPI (a). Bar = 50 μ m (a - c).

copy level was assessed in four areas in 60 non-overlapping tumor cell nuclei. Sixty nuclei were assessed in each area, the chromosome 17 copy numbers were counted for each cell, and the ratio of TOP2A signals to chromosome 17 signals was calculated. The normal mean TOP2A to chromosome 17 ratios was defined as less than 1.5; a ratio 1.5-2.0 was interpreted as a low level of amplification; greater than 2 was interpreted as high level of gene amplification. Normal cells in the analyzed tissue section served as

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 -2min 0.01M citrate retrieval solution pH6.0	Novocastra Lab., UK (NCL-L-ER-6F11)	1:100	LSAB+ /HRP
Mo Progesterone Receptor Clone 16	Mo Pressure cooker -2min 0.01M citrate retrieval solution pH6.0	Novocastra Lab., UK NCL-L- PGR-312	1:100	LSAB+ /HRP
Po rabbit anti-hu HER-2 (c-erbB-2 oncoprotein)	Po Water bath 40min, 0.01M citrate retrieval solution pH6.0 or DAKO cytometry target retrieval solution No. S1700	DAKO Cytomation Denmark A0485	1:350	LSAB+ /HRP
Mo mouse anti-hu TOP2A clone 3F6	Mo Microwave-20min, 0.01M citrate retrieval solution pH6.0 or DAKO cytometry target retrieval solution No. S1700	Novocastra Lab., UK NCL-TOPOIIA	1:100	LSAB+ /HRP
Mo mouse anti-hu BRCA1 Clone Ms110	Mo Microwave-20min, 0.01M citrate retrieval solution pH6.0 or DAKO cytometry target retrieval solution No. S1700	Oncogene Research Products, Cat# OP92	1:100	LSAB+ /HRP

an internal positive control of pre-treatment and hybridization efficiency.

Statistical Analysis

Descriptive data were defined as frequencies \pm SD. The correlation between the BRCA1 protein expression and TOP2A protein and gene status defined by FISH methods was done using the Mann-Whitney test, χ^2 test and Pearson's correlation test. Calculations were performed using SPSS 11.0. A P value of <0.05 was considered to be statistically significant.

RESULTS

TOP2A gene and protein expression

Overexpression of the TOP2A protein was detected in 32 patients (32/53, 60.4%) (Fig. 1a, b). Significant differences in TOP2A protein expression was detected between Luminal A and Luminal B breast

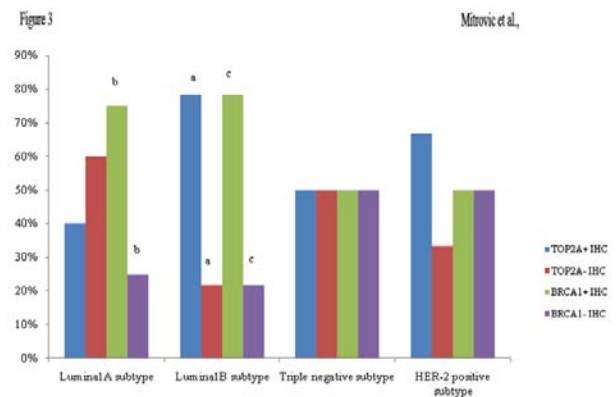


Fig. 3. The number of TOP2A⁺ and TOP2A⁻, BRCA1⁺ and BRCA1⁻ breast cancer cells in four molecular breast cancer subtypes. A – Luminal B subtype: TOP2A⁺ vs. TOP2A⁻ ($\chi^2=7.348$, $p=0.01$); b - Luminal A subtype: BRCA1⁺ vs. BRCA1⁻ ($\chi^2=5$, $p=0.05$); c - Luminal B subtype: BRCA1⁺ vs. BRCA1⁻ ($\chi^2=7.348$, $p=0.05$)).

cancer molecular subtypes (Table 2). Of the 53, 22 (41.5%) patients showed TOP2A gene amplification

Table 2. Expression of TOP2A and BRCA1 in four molecular subtypes of breast cancer

Level of expression	Molecular subtypes of breast cancer				Σ
	Luminal A (n=20)	Luminal B (n=23)	Triple negative (n=4)	HER-2 positive (n=6)	
TOP2A IHC ⁺	8 (40 %) ^a	18(78.3%) ^{a,b}	2 (50 %) ^{**}	4 (66.7 %)	32 (60.4%)
TOP2A IHC ⁻	12 (60%) ^a	5 (21.7%) ^b	2 (50 %) ^{**}	2 (33.3%)	21 (39.6%)
TOP2A FISH-gene amplification	6 (30 %)	11 (47.8 %)	1 (25%)	4 (66.7 %)	22 (41.5%)
TOP2A FISH-gene deletion	1 (5 %)	3 (13 %)	0	0	4 (7.6%)
TOP2A no amplification	13 (65%)	9 (39.2%)	3 (75%)	2 (33.3%)	27 (50.9%)
BRCA1 IHC ⁺	15 (75%) ^a	18 (78.3%) ^b	2 (50%) ^{**}	3 (50%)	38 (73.4%)
BRCA1 IHC ⁻	5 (25%) ^a	5 (21.7%) ^b	2 (50%) ^{**}	3 (50%)	15 (26.6%)

*- TOP2A - Luminal A and Luminal B subtypes (U=142, p=0.01); ** - triple-negative subtype: TOP2A and BRCA1 (r=1, p=0.01); a - Luminal A subtype: TOP2A and BRCA1 (r=0.471, p=0.05); b - Luminal B subtype: TOP2A and BRCA1 (r=0.489, p=0.05).

Table 3. Correlation between TOP2A gene amplification and deletion with TOP2A protein overexpression in four molecular subtypes of breast cancer

	Molecular subtypes of breast cancer			
	Luminal A	Luminal B	Triple negative	HER-2 positive
TOP2A ⁺ /TOP2A amplification	4 (20%)	9 (39.2%)	0	2 (33.3%)
TOP2A ⁻ /TOP2A amplification	2 (10%)	2 (8.7%)	1 (25%)	2 (33.3%)
TOP2A ⁺ /TOP2A deletion	1 (5%)	3 (13%)	0	0
TOP2A ⁻ /TOP2A deletion	0	0	0	0
TOP2A ⁺ /TOP2A no amplification	3 (15%)	6 (26.1%)	2 (50%)	2 (33.4%)
TOP2A ⁻ /TOP2A no amplification	10 (50%)	3 (13%)	1 (25%)	0

and 4 (7.4%) patients showed TOP2A gene deletion (Table 2). Amplification of the TOP2A gene (Fig. 2) was detected in 6 (30%) patients Luminal A, 11 (47.8%) patients Luminal B, 4 (66.7%) patients in HER-2 positive and 1 (25%) patients in triple-negative breast cancer subtypes. The amplification ratios in these 22 tumors ranged from 2.2 to 8.2. No TOP2A gene amplification was found in 27 (50.9%) tumors (Table 2). Deletion of TOP2A was seen in 1 (5%) out of 20 Luminal A tumors and in 3 (13%) out of 23 Luminal B tumors. Of 22 tumors with TOP2A amplification, 15 (68.2%) overexpressed TOP2A (4 cases in Luminal A, 9 cases in Luminal B, 2 cases in the HER-2 positive subgroup) (Table 3). Only 13

(48.1%) of 27 tumors without TOP2A amplification overexpressed TOP2A: 3 in Luminal A, 6 in Luminal B, 2 in triple-negative and 2 patients in HER-2 positive breast cancer subtypes (Table 3). The number of TOP2A positive breast cancer cases was statistically significantly higher in Luminal B breast cancer subtypes in comparison to TOP2A negative cases (Fig. 3). Correlation between the TOP2A gene and protein expression was positive but not statistically significant (r=0.094, p=0.502) (Table 2).

BRCA1 protein expression

The overexpression of BRCA1 protein was detected

in 38 patients (38/53, 71.7%): 15 cases in Luminal A, 18 cases in Luminal B, 3 cases in HER-2 positive and 2 cases in triple-negative molecular subtypes (Table 2).

In Luminal A ($\chi^2=5$, $p=0.05$) and B ($\chi^2=7.348$, $p=0.05$) breast cancer subtypes, statistically significantly more BRCA1 protein positive cases were observed in comparison to BRCA1 negative breast cancer cases (Fig. 3). BRCA1 protein overexpression showed significant positive correlation with TOP2A protein expression in Luminal A ($r=0.471$, $p=0.05$), Luminal B ($r=0.489$, $p=0.05$) and triple-negative ($r=1$, $p=0.01$) breast cancer subtypes (Table 2).

DISCUSSION

Most of the studies that have investigated genetic aberrations in breast cancer cells have focused on *HER-2* gene amplification and its relationship with the *TOP2A* gene aberration (Arriola et al., 2008). According to our knowledge, little is known about the relationship of BRCA1 protein expression with the *TOP2A* gene and protein aberration in four molecular subtypes of breast cancer.

The genes *BRCA1* and *TOP2A* are located on chromosome 17 (Järvinen et al., 1999; Järvinen et al., 2003; Rosen et al., 2005). Owing to *TOP2A* proximity to the *BRCA1* gene on chromosome 17 we originally thought that gene and protein aberration are present in the same cancer cells.

BRCA1 protein overexpression is linked to familial breast cancers in patients with a positive family history (Rosen et al., 2005). Overexpression of BRCA1 protein has been detected in 71.7% of patients, slightly higher than the overexpression of TOP2A protein, which was detected in 60.4% of breast cancer patients. A completely positive agreement was established between the overexpression of BRCA1 and TOP2A proteins in Luminal B and triple-negative breast cancer subtypes.

The results of previous studies have shown that BRCA1 protein overexpression is associated with the

negative expression of estrogen and progesterone receptors, as well as with the positive overexpression of the HER-2 receptor (Rosen et al., 2005). Our results agree, in part, with this statement, bearing in mind that the overexpression of the BRCA1 protein was detected in 2/3 of patients with Luminal B subtypes of breast cancer where the steroid receptors were positive.

In contrast to previous results (Miyoshi et al., 2008), our results showed that TOP2A protein overexpression is higher in tumors with ER (78.3%) and PR (78.3%) receptor positivity, and HER-2 positivity (78.3 %) in Luminal B subtype of breast cancer. The same relationship was detected for BRCA1 protein and those three receptors in Luminal B breast cancer subtype. The highest number of patients with positive expression of BRCA1 and TOP2A proteins was detected in the Luminal B breast cancer subtype. This finding is supported by the fact that all patients with Luminal B breast carcinoma displayed positive expression of the HER-2 receptor. The frequency of TOP2A and BRCA1 protein expression was identical in the subtypes of Luminal B and triple-negative breast cancer.

The *TOP2A* gene is amplified in a significant number of breast cancer cases and might be the target for TOP2A inhibitors during the therapy. We identified *TOP2A* gene deletion in 13% (3/23) of Luminal B breast cancer subtype. We also identified one tumor with *TOP2A* deletion in Luminal A breast cancer without HER-2 protein overexpression. Our data demonstrated that TOP2A overexpression rarely occurs in the absence of gene amplification, and it is also in correlation with previous results (Miyoshi et al., 2008).

Our study showed that more than 31.8% of *TOP2A* gene amplification cases showed an absence of protein overexpression. Negative immunohistochemical results in tumors with *TOP2A* gene amplification could be the consequence of several factors (Olsen et al., 2004; Miyoshi et al., 2008). One of the factors could be a loss of antigenicity during tissue processing. Another reason for this discrepancy be-

tween gene amplification and protein overexpression might be the fact that TOP2A is a cell cycle regulated protein. The level of TOP2A protein expression is linked to the rate of cell proliferation. Its protein levels change during the cell cycle without contribution of gene amplification. According to this finding, it is suggested that factors, other than gene amplification, also participate in the regulation of TOP2A protein expression (Miyoshi et al., 2008).

A recent study has shown the importance of TOP2A as a predictive factor for epirubicin-based therapy in breast cancer. Miyoshi et al. (2008) reported that patients with TOP2A gene amplification and BRCA1 negative protein expression show an enhanced recurrence-free survival when treated with specific CEF therapy. A similar finding has been reported by Tanner et al. (2006) who also reported better survival for patients with TOP2A gene amplification. Several studies showed that both immunohistochemically determined TOP2A protein expression and TOP2A gene amplification have been associated with response to epirubicin-based therapy. Cardoso et al. (2004) analyzed the expression of both TOP2A proteins as determined by immunohistochemistry and TOP2A gene amplification as determined by FISH, and compared their association with response to epirubicin therapy. The results of this study showed a stronger prognostic association between TOP2A protein expression and epirubicin therapy (Cardoso et al., 2004). According to the literature, the association between TOP2A overexpression and TOP2A gene amplification is not so strong since only 33% of breast tumors with gene amplification show TOP2A protein overexpression (Miyoshi et al., 2008). In contrast to this statement our results showed a higher level of correlation between TOP2A gene amplification and protein overexpression, especially in the Luminal B molecular subgroup (9/11, 81.8 %).

TOP2A expression is of considerable clinical significance. As far as we know, we were the first to investigate the correlation between BRCA1 protein and TOP2A protein expression in four molecular subtypes of breast cancer. In conclusion, we dem-

onstrated that TOP2A and BRCA1 positive phenotypes are in significant correlation in Luminal B and triple-negative breast cancer subtypes. On the basis of this result we suggest that BRCA1 and TOP2A protein expression have similar patterns in these two molecular subgroups, and that patients from these groups could benefit from epirubicin-based therapy.

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