Original paper

Topoisomerase 2α status in invasive breast carcinoma – comparison of its clinical value according to immunohistochemical and fluorescence *in situ* hybridization methods of evaluation

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The main purpose of the study was to compare topoisomerase 2α (TOP2A) status in invasive breast carcinomas to the outcome of a therapy containing neoadjuvant treatment with anthracyclines (a combination chemotherapy treatment for breast cancer, namely AC [cyclophosphamide, doxorubicin]).
To achieve these goals we created a method of evaluation with criteria based on two methods used in the present study (immunohistochemical [IHC] and fluorescence *in situ* hybridization [FISH]). The threshold for positive immunohistochemically evaluated status was set for all cases with: nuclear stain intensity score 3+ in 10% or more nuclei and nuclear stain intensity score 2+ in 50% or more nuclei.
Our results suggest that TOP2A status may be used as a predictive factor for patient selection for protocols which include anthracyclines as one of the chemotherapeutics. Both methods, IHC and FISH, are suitable for implementation for diagnostic purposes, but IHC positive status measured according to the criteria presented above is the best predictor of longer disease-free survival (DFS) according to our study. Immunohistochemical also gave satisfactory results in all analyzed cases in comparison to only 60% of cases analyzed by FISH.

Key words: TOP2A, IHC, FISH, breast carcinoma, anthracyclines.

Introduction

In the era of targeted therapy there is a need to establish predictive factors which will allow treatment to be personalized. In breast carcinoma, according to the St Gallen recommendation, in each case the following markers should be tested: estrogen receptor (ER), progesterone receptor (PgR), HER2, as well as the proliferation index by Ki67 protein before systemic treatment is introduced [1-5].

There are a number of potentially valuable predictive markers both for new therapeutic agents and for those known for decades such as anthracyclines. Topoisomerase 2α (TOP2A) for years has been considered a potential predictive factor for anthracycline-containing therapies, although the results are still inconclusive [6-11].

Topoisomerase 2α is a nuclear enzyme that changes the way the DNA is organized in nuclei. Therefore TOP2A is required in almost all processes involving

changes and untangling of DNA. This enzyme is encoded by the *TOP2A* gene located in the 17q12-21 region. This region is also responsible for human epidermal growth factor receptor 2 (HER2). HER2 is one of the most frequently amplified genes in breast cancer. The exact mechanism of rearrangement of this region is not completely understood; however, some data suggest that even though high copy numbers of TOP2A and HER2 are common findings, these genes belong to separate amplicons. HER2 status has often been used to select patients for analysis of TOP2A alterations. Clinical relevance has usually been investigated in the context of HER2 overexpression or HER2 amplification. This is also the case in the present study. It is possible because at the time of treatment of patients from our study (2002-2005) group, anti-HER2 therapy were given in Poland after dissemination of neoplastic disease. Both TOP2A amplifications and deletions were shown to confer an adverse prognosis but also a greater benefit from anthracycline-containing therapy. Many authors have suggested the possibility of guiding therapy based on TOP2A status [9, 12-15]. However, a recent meta-analysis did not confirm the predictive value of TOP2A alterations [9]. Information on the predictive role of TOP2A status, and even the percentage of cases of breast carcinoma which are TOP2A positive, since more than a decade have been surprisingly incoherent, partly because of different diagnostic methods (immunohistochemistry – IHC, fluorescent *in situ* hybridization – FISH, other molecular biology methods) and different thresholds for positive status [10, 16-18]. On the other hand, particularly in recent years, treatment of breast carcinoma is almost always a combination of different chemotherapeutics, radiotherapy, and different surgical techniques (e.g.: mastectomy, breast-conserving therapy, sentinel lymph node procedure, lymphadenectomy) [19, 20]. Recently introduced intrinsic subtypes for breast carcinoma make constructing a homogeneous group for such a study almost impossible [1,2]. The group of patients in the present study was selected mainly on the basis of the same neoadjuvant therapy which was introduced according to the stage of the disease.

Table I. Material

Total number of cases	148
Number of cases with positive HER2 status	60 (43%)
Number of cases with negative HER2 status	88 (56%)
Number of cases with TOPA2a result by IHC method	148
Number of cases with TOPA2a result by FISH method	98

Table II. 5-year disease-free survival (DFS) among analyzed cases

Total number of cases with 5-year DFS	88/148 (59.5%)
Number of cases with 5-year DFS in HER2 positive group	38/60 (63.3%)
Number of cases with 5-year DFS in HER2 negative group	50/88 (56.9%)

The main purpose of the study was to compare TOP2A status in invasive breast carcinomas to outcome of a therapy containing neoadjuvant treatment with anthracyclines (a combination chemotherapy treatment for breast cancer, namely AC (cyclophosphamide, doxorubicin)), in analyzed cases. Because of the published information on a correlation between the status of HER2 and TOP2A with the outcome of the therapy, the study group was subdivided into two tiers: HER2 positive and HER2 negative. We also wanted to find out if there is any influence of these factors on outcome of the therapy.

To achieve these goals we wanted to create an easy method of evaluation with criteria based on two methods used in the present study (IHC and FISH).

Material and methods

All 150 cases selected for the study were clinically locally advanced (clinical stage III) breast carcinoma. Neoadjuvant therapy containing anthracyclines (AC) was introduced in all cases. All patients were treated in the Maria Sklodowska-Curie Cancer Center, Warsaw, Poland, between 2002 and 2005. Primary diagnosis took place in the years 2002-2004. In that period in the Maria Sklodowska-Curie Cancer Center anti-HER2 therapy was given only to patients after recurrence. Therefore, in our group both HER2 positive and HER2 negative tumors were treated similarly in the time of follow-up. HER2 status was positive in 62 of the analyzed cases. In 88 cases HER2 status was negative. At least 5-year observation was available for the study. Evaluation of TOP2A was performed retrospectively. Finally 148 cases (60 HER2 positive cases, 88 HER2 negative cases) were analyzed due to lack of valuable tissue block material in two cases. We divided our cases according to HER2 status because of published information on the correlation of HER2 status and TOP2A status in invasive breast carcinoma (Tables I and II).

A list of 150 consecutive patients with invasive breast carcinoma, stage III according to the AJCC/UICC classification, treated with chemotherapy (AC), was prepared in the Breast Cancer Clinic of the Maria Sklodowska-Curie Cancer Center (Table II). In all but two cases, paraffin blocs containing tumor tissue were selected for IHC and FISH evaluation of TOP2A status. Both methods were used in all 148 cases, but, due to technical reasons, FISH re-

sults were obtained in 92 cases in contrast to IHC, in which satisfactory results were obtained in 148 cases. In all cases we decided to use only diagnostic material for evaluation of TOP2A status. In 138 cases material came from a core biopsy (CB) and in 10 cases from surgical biopsy. Histologic slides from all cases were reviewed. In the period of interest (2002-2004) histologic grade and histologic type were seldom part of the pathology report for CB material. In reviewed slides two pathologists evaluated histologic types of tumors (130 cases of ductal carcinoma (no special type according to the 2012 WHO Breast Tumor Histologic Classification), 10 cases of classical lobular carcinoma and 8 cases of metaplastic carcinoma), tumor grades (100 cases of grade 2 tumors, 38 cases of grade 3 tumors, and 10 cases of grade 1 tumors – grade 1 cases were all lobular carcinoma). Because of known discordance between CB evaluation of grade and histologic type, these data were not analyzed statistically. Data on HER2 status originated from medical documentation. Slides for the study were prepared from routinely made paraffin blocs for diagnostic purposes. Both IHC and FISH methods of evaluation were applied in each case. The results were then compared to clinical response (DFS). We decided to use DFS because of relatively long overall survival (OS), which makes 5-year observation invalid statistically as there were only a few breast cancer related deaths in the studied group.

Immunohistochemical method

For TOP2A immunocytochemistry, 4 μm sections were deparaffinized in xylene for 30 min and rinsed in ethanol. Sections were then subjected to antigen retrieval by immersion in citrate buffer (pH 6.0) preheated to 99°C for 40 min. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 in methanol for 30 min, followed by rinsing in Tris-buffered saline containing Tween 20. Immunohistochemical staining was performed with the EnVision+ System Kit (DakoCytomation, Glostrup, Denmark). Afterwards, the sections were incubated overnight in a humidity chamber with a monoclonal primary antibody against TOP2A (DakoCytomation; dilution 1 : 100) followed by incubation for 30 min with a dextran polymer conjugated with horseradish peroxidase enzyme and with goat anti-rabbit antibody. The antigen–antibody immunoreaction was revealed with 3,3'-diaminobenzidine tetrahydrochloride as the chromogen, and the slides were counterstained with hematoxylin.

For analysis we implemented an original system of IHC scoring partially based on the literature and partially on comparison of both our IHC and FISH results. Both a semi-quantitative intensity four-tier system (0, 1+, 2+, 3+) (Figs. 1-3) and the percentage value of stained cells (nuclei) were taken into account for IHC evaluation of TOP2A in the present study [16, 17]. The threshold for positive status was set for all cases with:

- nuclear stain intensity score 3+ in 10% or more nuclei,
- nuclear stain intensity score 2+ in 50% or more nuclei.

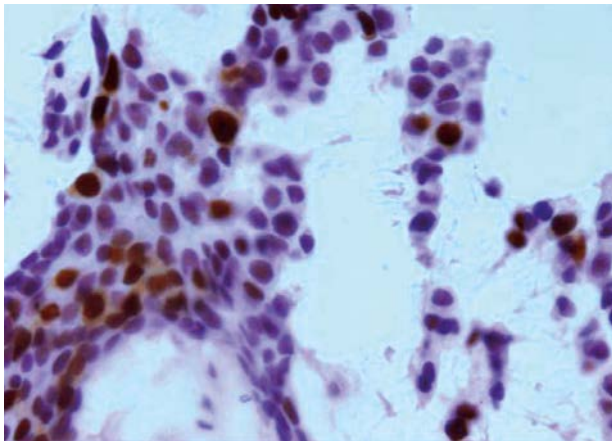

Fig. 1. Topoisomerase 2α IHC 1+

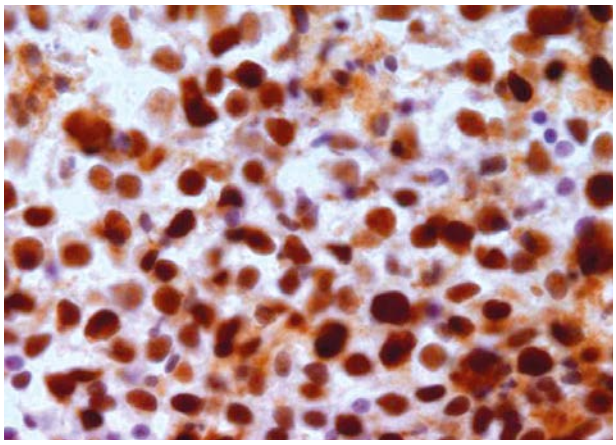

Fig. 2. Topoisomerase 2α IHC 2+

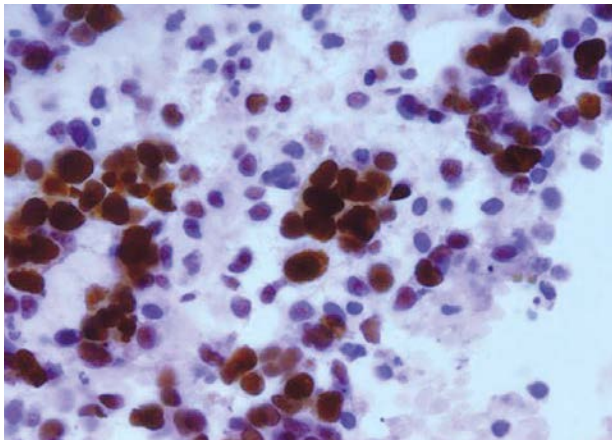

Fig. 3. Topoisomerase 2α IHC 3+

Table III. Topoisomerase 2α criteria of interpretation of immunohistological stains

Score	Criteria	Interpretation
0	No nuclear stain	Overexpression absent Score 0 or 1+ is present in majority of cancer cases (often in more than 10% of cells) as well as cases of benign breast lesions and benign cells.
1+	Weak nuclear stain (brown-blue) in carcinoma cells visible only in high magnification (> 200×). Nuclear structure (nucleoli and chromatin) visible	
2+	Intermediate nuclear stain (brown) in carcinoma cells visible already in low magnification (40×). Nuclear structure (nucleoli and chromatin) visible	Overexpression absent < 50% Overexpression ≥ 50% 2+
3+	Strong nuclear stain (very dark brown, almost black) in carcinoma cells visible already in low magnification (40×). Nuclear structure (nucleoli and chromatin) in visible	Overexpression ≥ 10% 3+

The precise criteria of our original IHC scoring system which was designed for this study is presented in Table III.

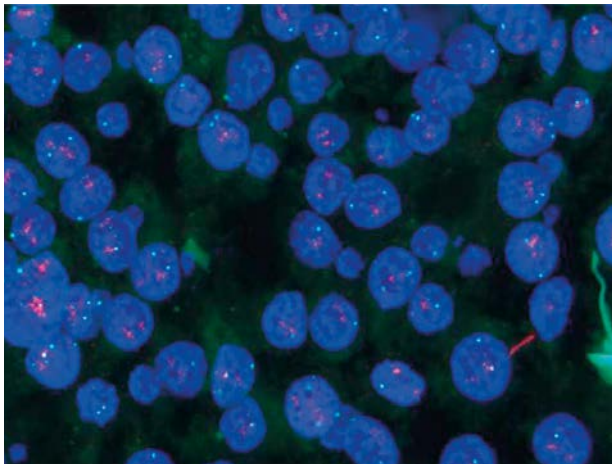

Fig. 4. Topoisomerase 2α FISH, no amplification

FISH method

In the study the *TOP2A* gene was evaluated using Path Vysion Abbott probes according to the instructions of the producer. FISH results suitable for interpretation in the fluorescent microscope were obtained in 98 cases. In those cases it was possible to calculate the ratio R = TOP2A/CEP17 – both types of signals were acceptable for interpretation. In 26 cases the average number of gene *TOP2A* signals per nucleus were calculated only – CEP17 signals were unsatisfactory. We did not include those cases in the study group. The remaining 24 cases were classified as non-diagnostic for TOP2A evaluation because:

- there were no visible signals for the *TOP2A* gene,
- too few carcinoma cells were found in material for objective evaluation.

For FISH evaluation we selected a method similar to that published by Mano *et al.* [18]. Cases with a ratio of 1.5 or more were considered amplified. Ad-

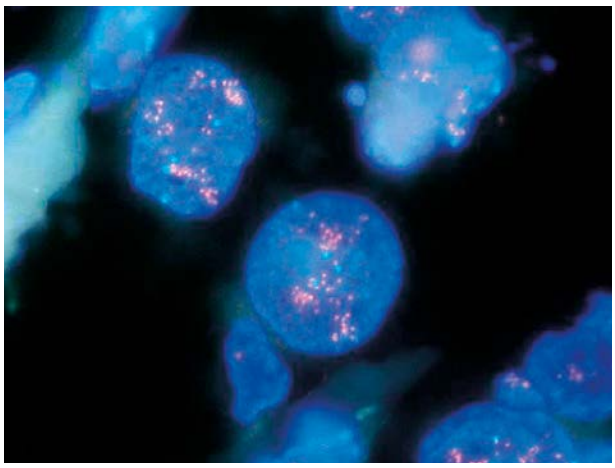

Fig. 5. Topoisomerase 2α FISH, low amplification

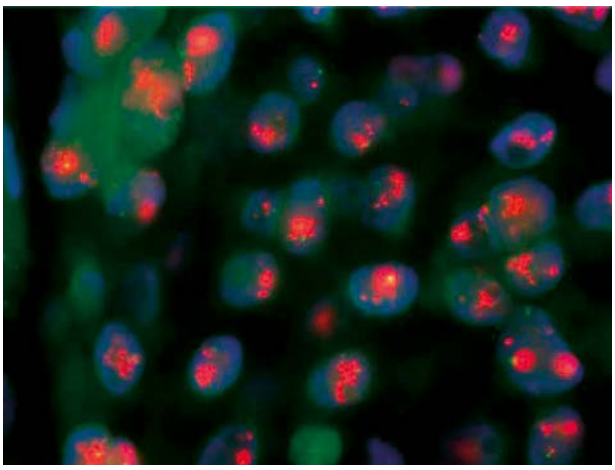

Fig. 6. Topoisomerase 2α FISH, high amplification

Table IV. Topoisomerase 2α results by IHC method

	Total	HER2 negative group	HER2 positive group
No overexpression	79 (53.4%)	48 (54.5%)	31 (51.7%)
Overexpression	69 (46.6%)	40 (45.5%)	29 (48.3%)

Table V. Topoisomerase 2α results by FISH method

		Total (n = 98)	HER2 negative group (n = 60)	HER2 positive group (n = 38)
No amplification		63 (64.3%)	39 (65%)	24 + 2del (68.4%)
Amplification (any)	Low amplification	24 (24.5%)	13 (21.7%)	10 (26.3%)
	High amplification	11 (11.2%)	8 (13.3%)	2 (5.3%)

ditionally, cases with a ratio of 10 or more were considered as highly amplified (HA) (Figs. 4-6).

Results

The IHC method for TOP2A gave visually acceptable result in 100% (148) of cases. The FISH method gave a visually acceptable result in 60% (98) of cases. Cases in which results from both methods were available and were used for "calibration" of the IHC scoring system are presented in Table III. We selected cases with amplification and compared them with intensity and proportions of nucleus staining. The final correlation of TOP2A status and DFS in studied cases was counted for IHC and FISH results separately, but statistically significant data were obtained for the IHC method. The scoring system based on the IHC method allowed clinical analysis of 138 cases in comparison to 92 available by FISH.

Positive status of TOP2A was found in 35.7% of cases by FISH and 46.6% by IHC. In our material it was found that there is a strong positive correlation between amplification of TOP2A and IHC results with positive status defined as presented in Table III (87.4%). In the HER2 negative group we found 54.5% of cases with negative TOP2A status and 45.5% with positive TOP2A status. In the HER2 positive group we found 51.7% of cases with negative TOP2A status and 48.3% with positive TOP2A status.

Results of TOP2A status measured by both methods (IHC and FISH) are presented in Table IV and V.

Results from TOP2A status obtained by IHC and FISH were compared with DFS in both HER2 positive and HER2 negative groups. Results for those groups are shown in Tables VI and VII for the FISH method and Tables VIII and IX for the IHC method.

Table X presents statistical significance of obtained data (confidence interval CI 95%) for the whole study group and for subgroups (TOP2A status measured by FISH, TOP2A status measured by IHC, and for HER2 status according to clinical data). According

Table VI. Correlation of topoisomerase 2α status by FISH and DFS

	TOPA2a (%)	5-year DFS (%)
NA (no amplification)	39 (65)	23 (59)
LA (low amplification)	13 (21.7)	9 (69)
HA (high amplification)	8 (13.3)	8 (100)

Table VII. Correlation of topoisomerase 2α status by FISH and DFS

	TOPA2a (%)	5 year DFS (%)
NA (no amplification)	26 (68.4)	14 (54)
LA (low amplification)	10 (26.3)	6 (60)
HA (high amplification)	2 (5.3)	2 (100)

Table VIII. Correlation of topoisomerase 2a status by IHC and DFS

	TOPA2a (%)	5-year DFS (%)
No overexpression	48 (54.5)	15 (31)
Overexpression	40 (45.5)	35 (88)

Table IX. Correlation of topoisomerase 2a status by IHC and DFS

	TOPA2a (%)	5-year DFS (%)
No overexpression	31 (51.7)	15 (48)
Overexpression	29 (48.3)	23 (94)

Table X. Five-year disease-free survival (95% PU)

	DFS (95% PU)	p (log-rank)
Whole study group	0.60 (0.52; 0.68)	
TOPA2 FISH		0.091
NA	0.63 (0.51; 0.75)	
LA	0.61 (0.41; 0.81)	
HA	1.00	
TOPA2 IHC		**< 001**
NEG	0.38 (0.27; 0.49)	
POS	0.84 (0.75; 0.93)	
HER2		0.494
NEG	0.57 (0.46; 0.68)	
POS	0.63 (0.51; 0.75)	

NA – no amplification; LA – low amplification; HA – high amplification; NEG – negative; POS – positive

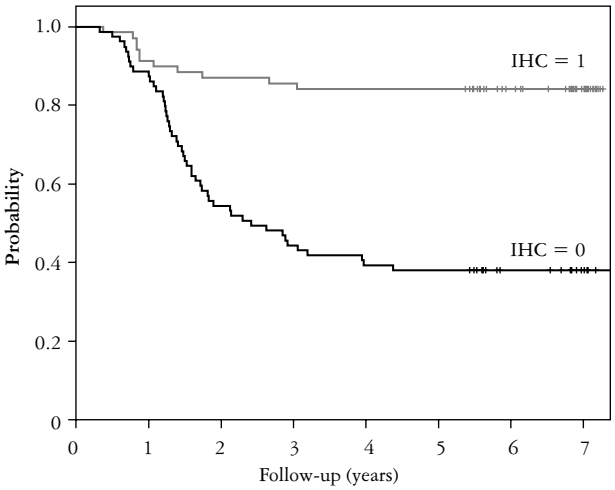

Fig. 7. Disease-free survival according to topoisomerase 2α status measured by immunohistochemistry. 1 = positive status, 0 = negative status; $p < 0.001$

to the statistical analysis TOP2A positive status measured by IHC and using the scoring system introduced for this study correlates with longer DFS regardless of HER2 status (p (log-rank) < 0.001; Fig. 7). Statistical data are shown in detail in Table XI.

Discussion

Analyzing data from the literature on TOP2A status and its role as a predictive factor in invasive breast carcinoma is a confusing experience [18-23]. Outcomes of results both at the level of evaluation of TOP2A as well as at the level of interpretation are extremely different [24-33]. If biology of breast carcinoma is similar around the world, then methods of TOP2A evaluation, criteria of interpretation and selection of patients are responsible for the differences [34-41]. We put emphasis on criteria of interpretation, and decided to create out own system of interpretation based on comparison of the results of two methods used in the same material.

Table XI. Distribution of TOP2A status in HER2 negative and HER2 positive groups

	HER2 NEG	HER2 POS
TOPA2 FISH		
NA	39 (65%)	26 (69%)
LA	13 (22%)	10 (26%)
HA	8 (13%)	2 (5%)
Total	60 (100%)	38 (100%)
TOPA2 IHC		
NEG	48 (55%)	31 (52%)
POS	40 (45%)	29 (48%)
Total	88 (100%)	60 (100%)

NA – no amplification; LA – low amplification; HA – high amplification; NEG – negative; POS – positive

We found in our study that cases with positive TOP2A status measured by the IHC method for TOP2A protein presented a statistically significantly better response to therapy which included anthracyclines as one of the chemotherapeutics (AC program in our study) in neoadjuvant therapy measured by relapses (DFS). In our study, we had a possibility to evaluate TOP2A in breast cancer cells using two methods (IHC and FISH) in all cases.

Using IHC and FISH methods, we compared the percentage of stained cells, strength of nuclear stain, presence of co-expressed cytoplasmic and membranous stain, amplification of *TOP2A* gene and also response to anthracycline-containing chemotherapy from clinical data. As the result of analysis of obtained data, we set our own cutoff points. We assumed that the best correlation with genomic aberrations is when both the percentage and strength of the stain are taken into account. In our study, a cutoff point for positive TOP2A status evaluated by the IHC method is at least 10% of carcinoma cells with strong (3+) nuclear staining or at least 50% of carcinoma with intermediate (2+) nuclear staining. The presented criteria as defined above correlate with amplification (ratio ≥ 1.5) and response to anthracycline-based neoadjuvant therapy (measured by DFS).

For the IHC method different criteria of scoring and different thresholds of positivity found in the literature make it impossible to perform valuable comparison of results achieved in numerous publications [16-21]. In the case of FISH, criteria of scoring are more objective – counting dots theoretically generates less interobserver variation than classifying strength of the stain in IHC, especially when measured without an image analyzing system [17]. Im-

portant technical problems – there were acceptable results in only 98 from 148 cases – derive in our opinion mainly from quality of tissue from paraffin blocks. In the years 2002-2004, when the paraffin blocks were prepared, the standards of tissue fixation in formalin (e.g.: formalin pH, time of fixation, processing of tissue, quality of slides) in our laboratory were suboptimal. We found uneven signal distribution in tumor tissue. This may be an important reason for different outcomes of FISH and IHC results, particularly since we worked only on limited diagnostic tissue material, mostly CB. Heterogeneity for TOP2A may be an important factor for uneven distribution seen in the fluorescence microscope. On the other hand, IHC results obtained in all 148 studied cases were acceptable, though in the case of negative results (0 according to our scoring system) the lack of a proper control system makes it uncertain if some cases are truly negative or just nondiagnostic.

In our study we found a similar frequency of *TOP2A* amplifications in both HER2 negative and HER2 positive breast cancers, which challenges the common opinion that TOP2A alteration is mostly restricted to HER2 positive tumors. On the other hand, we have only locally advanced cases in our study group, which is overrepresented by histologically high grade tumors. This factor alone can influence the proportion of TOP2A positive and negative cases [22, 23].

In our study immunohistochemically evaluated TOP2A status statistically significantly correlates with DFS during 5-year follow-up in breast carcinoma stage III patients treated with anthracyclines-containing chemotherapy. Also in all cases with high amplification of the TOP2A gene there was 5-year DFS. However, the small number of those cases in the studied group makes this observation statistically irrelevant. Inconclusive results on the role of TOP2A in breast carcinoma biology and therapy are partially due to numerous thresholds of positivity measured by immunohistochemical analysis [16-18].

Analyzing methodical data, we believe that FISH should not be used in routine diagnostic circumstances, at least alone. In around 40% of cases the results are below the quality that allows calculation of the TOP2A/CEP17 ratio.

Our results suggest that TOP2A status may be used as a predictive factor for patient selection for protocols which include anthracyclines as one of the chemotherapeutics. Both methods are suitable for implementation for diagnostic purposes, but IHC status measured according to criteria presented in Table III is the best predictor of DFS according to our study. IHC also gave satisfactory results in all analyzed cases in comparison to only 60% of cases analyzed by FISH.

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