

RELATIONSHIP OF FLOW CYTOMETRY WITH OTHER CLINICAL AND HISTOPATHOLOGICAL PARAMETERS IN PATIENTS WITH NEUROENDOCRINE DUCTAL CARCINOMA OF THE BREAST

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Summary

Aims. The aim of the study was to determine cellular ploidy of invasive ductal breast carcinoma with neuroendocrine (NE) differentiation and its share in certain phases of the cell cycle. It was also aimed at assessing the relationship of the cell cycle profile with other clinical and histopathological features.

Methods. The study was carried out in 80 patients with invasive ductal breast cancer, and classified as breast carcinoma with NE differentiation according to their histopathological parameters. The patients underwent treatment at the University Hospital for Tumors, Zagreb, Croatia during the period January 1 to December 31, 1992. Data about patients' age, estrogen and progesterone receptor concentration, cancer size, and treatment modality were retrospectively collected from their case histories. Paraffin blocks were used for immunohistochemical and histochemical analysis and flow cytometric analysis of the tumor cell cycle. Neuroendocrine tumor diagnosis was made using Grimelius and immunohistochemical staining including neuron-specific enolase (NSE), chromogranin A and synaptophysin.

Results. Analysis by flow cytometry detected 27 tumors (33.8%) with DNA diploidy showing proliferative activity lower than 20%, and 53 tumors (66.2%) with DNA aneuploidy, tetraploidy and/or DNA diploidy with proliferative activity over 20%. Progesterone receptor concentration in DNA-diploid tumors was significantly higher than in DNA-aneuploid, tetraploid tumors and tumors with proliferative activity of $\geq 20\%$ ($p < 0.001$). Concentration of estrogen receptors, age, histological grade, tumor size, Grimelius staining and immunohistochemical markers did not significantly differ between the groups.

Conclusion. Data collected in our study show a higher mean concentration of progesterone receptors in the group of diploid tumors and tumors with low proliferative activity. In consideration with the above criterium, other pathological and clinical parameters did not show any significant difference relative to both tumor ploidy and its proliferative activity. For final conclusion on the clinical significance of neuroendocrine differentiation in breast cancer further studies that would include monitoring of the course and outcome of the disease are required.

KEYWORDS: *neuroendocrine ductal carcinoma of the breast, flow cytometry, clinical and histopathological parameters*

POVEZANOST PROTOČNE CITOMETRIJE S DRUGIM KLINIČKIM I PATOHISTOLOŠKIM PARAMETRIMA U BOLESNICA S NEUROENDOKRINIM DUKTALNIM KARCINOMOM DOJKE

Sažetak

Ciljevi. Cilj našeg istraživanja bio je odrediti ploidnost stanica kod invazivnih duktalnih karcinoma dojke s neuroendokrinom (NE) diferencijacijom i njihov udio u pojedinim fazama staničnog ciklusa. Također se htjelo utvrditi povezanost profila staničnog ciklusa s drugim kliničkim i patohistološkim značajkama.

Metode. Istraživanje je provedeno na 80 bolesnica s duktalnim invazivnim karcinomom dojke koji su prema patohistološkim parametrima svrstani u karcinome dojke s NE diferencijacijom. Bolesnice su liječene u razdoblju od 01.01. do 31.12.1992. godine na Klinici za tumore u Zagrebu. Podaci o dobi bolesnica, koncentraciji estrogenskih i progesteronskih receptora, veličini karcinoma, te načinu liječenja dobiveni su retrospektivno iz povijesti bolesti. Parafinski blokovi upotrijebljeni su za imunohistokemijsku i histokemijsku analizu te analizu staničnog ciklusa tumorskih stanica protočnom citometrijom. Dijagnoze neuroendokrinih tumora postavljene su na temelju bojenja po Grimeliusu te imunohistokemijskih bojenja uključujući neuron-specifičnu enolazu (NSE), kromogranin A i sinaptofizin.

Rezultati. Protočnom citometrijom kod 27 tumora (33.8%) nađena je DNA diploidija i visina proliferativne aktivnosti manja od 20%, dok su DNA aneuploidija, tetraploidija i/ili DNA diploidija s proliferativnom aktivnošću većom od 20% nađene kod 53 tumora (66.2%). Koncentracija progesteronskih receptora u DNA-diploidnim tumorima bila je značajno viša nego kod DNA-aneuploidnih, tetraploidnih i tumora s proliferativnom aktivnošću $\geq 20\%$ tumora ($p < 0.001$). Koncentracija estrogenskih receptora, dob, histološki stupanj, veličina tumora, bojanje po Grimeliusu te imunohistokemijski markeri nisu se značajno razlikovali u ove dvije skupine.

Zaključak. Podaci iz našeg istraživanja upućuju na veću prosječnu koncentraciju progesteronskih receptora u skupini diploidnih tumora i tumora s niskom proliferativnom aktivnošću. Prema navedenom kriteriju drugi patološki i klinički parametri nisu pokazali značajnu razliku u odnosu na ploidnost i proliferativnu aktivnost. Za donošenje konačnog zaključka o kliničkoj važnosti neuroendokrine diferencijacije kod karcinoma dojke potrebno je provesti istraživanja koja bi uključila praćenje tijeka i ishoda bolesti.

KLJUČNE RIJEČI: *neuroendokrini karcinom dojke, protočna citometrija, klinički i histopatološki parametri*

INTRODUCTION

Breast carcinoma is the most common female malignancy (1-3) that rarely occurs before 25 and after 80 years of age (2).

Breast carcinoma that may develop from ductal (90%) or lobular epithelium (10%) is divided to those having (infiltrating) and having not (noninfiltrating) break the basal membrane. Infiltrating (invasive) tumors are divided to ductal, lobular, mucinous, medullary, papillary, tubular, cribriform, adenoid cystic, secretory, apocrine carcinoma, carcinoma with metaplasia and some other more uncommon histological types (3).

The most significant prognostic factors for invasive carcinoma of the breast are metastases into lymph nodes, tumor size, age, histological type, tumor differentiation grade and steroid receptor (estrogen and progesterone) status. The literature also quotes some other prognostic factors such as the presence and differentiation grade of an intraductal component, vascular invasion, growth factors, oncogenes and chromosome abnormalities (4-7).

Breast carcinomas with neuroendocrine (NE) differentiation show individual morphological and histochemical features similar to neuroendocrine-differentiated gastrointestinal or lung carcinoma. The identification of these tumors relies on morphological analysis and histochemical and im-

munohistochemical methods. According to some authors, argirophylia (by Grimelius' approach), presence of chromogranin A and B, synaptophysin and neuron-specific enolase (NSE) are the most reliable histochemical, or immunohistochemical methods (8, 9). They correlate with an ultrastructural finding of dense secretory granules and light-colored vacuoles, or vesicles of the synaptic type that are characteristic for neuroendocrine cells (8-10). These tumors are rarely hormone-active. The most commonly excreted hormones include ACTH, leukoencephalin, gastrin, pancreatic polypeptide, bombesin, serotonin, HCG, prolactin, vasoactive intestinal polypeptide and luteinizing hormone releasing factor.

Flow cytometry showed to be a valuable tool in determining prognosis and therapy of tumor diseases of other localizations (1, 2, 11-13). The method also gives information about DNA ploidy and the percentage of cells in G0/G1, S-phase fraction (SPF) and G2/M phase of the cell cycle. DNA ploidy and high SPF, separately or combined, can be linked with a higher risk of recurrence and death of patients with primary carcinoma of the breast (7, 14-16).

The study is aimed to determine the cellular ploidy of invasive ductal carcinoma with neuroendocrine (NE) differentiation, their share in certain phases of the cell cycle, and to assess the relationship of the cell cycle profile with other clinical and histopathological features.

PATIENTS AND METHODS

In the period January 1 - December 31, 1992, 444 patients underwent treatment for primary breast carcinoma at the University Hospital for Tumors, Zagreb, Croatia; 394 of them had invasive ductal carcinoma, and 50 had medullary, mucinous or papillary carcinoma of the breast. Data about patients' age, steroid receptor (estrogen and progesterone) concentration, cancer size, and treatment modality were retrospectively collected from their case histories. Tumor grade was assessed using the **Elston modification** of the Bloom-Richardson grading system (17). Patients underwent either mastectomy or segmentectomy, in both cases with axillary lymph node dissection. Stupanj je određivan prema Elstonovoj modifikaciji Bloom i Richardsonove klasifikacije (17).

A retrospective review of histopathological parameters (histologic grade of neoplastic differentiation, histologic type, number of mitoses, vascular and lymph node metastases) was performed by two pathologists. Neuroendocrine tumor diagnosis was made using Grimelius and immunohistochemical staining including monoclonal antibodies to neuron-specific enolase and chromogranin A (Mo; DAKO, Glostrup, Denmark), and polyclonal antibody to synaptophysin (Rb; DAKO, Glostrup, Denmark). As proposed by Papotti et al (18), tumors were considered to be neuroendocrine in case they met histologic criteria and showed a diffuse cytoplasmic positive reaction with the Grimelius stain, or positive reaction for chromogranin A, or if at least 10% of tumor cells showed positivity for synaptophysin.

Final analysis included 80 (20.3%) patients with invasive ductal carcinoma of the breast, which were classified into neuroendocrine tumors according to their histopathological parameters. Paraffin blocks were also used for flow cytometric analysis of the tumor cell cycle. As a biological standard, normal breast tissue was employed. For the purposes of flow cytometry the Hedley's and Vindelov's methods were used to isolate nuclei from paraffin-embedded tumor sections (19) and to stain the isolated nuclei with propidium iodide (20), respectively. Three 40 μm thick sections were cut from paraffin blocks. In addition, two 4 μm thick sections, one from the beginning (the first section) and another from the end (the fifth sec-

tion), were cut and stained using standard hemalaun and eosin procedures for histopathological analysis to demonstrate the presence of tumor tissue. The thick sections were then dewaxed in xylene and rehydrated by sequential immersing in 100, 95, 70, and 50% ethanol. After washing the specimen two times in distilled water, an enzymatic reaction was carried out with 0.5% pepsin (Sigma, St. Louis) in 0.9% NaCl (pH 1.5) in water bath for 30 minutes with constant stirring at 37°C. The specimen was centrifuged, the supernatant was removed by a vacuum pump, 2 ml RPMI medium added, and filtered through a nylon mesh. The specimen was then recentrifuged at 800 G for 5 minutes to add 1 ml trypsin in citrate buffer (10 min in water bath with constant stirring at 37°C). After centrifugation, the sediment of obtained tumor cell nuclei was processed by ribonuclease S, and then stained with propidium iodide for 30 minutes at room temperature. The nuclear DNA content was measured on a FACScan flow cytometer (Becton Dickinson, California, USA) at a wavelength of 488 nm using a red filter at a laser power of 500 mW. The flow cytometer allowed 10,000 nuclei per specimen pass through, and the obtained histograms of fluorescence intensity were analyzed intenziteta fluorescencije analizirani su programom CONSORT 30.

The histograms of fluorescence intensity show, graphically and numerically, the proportion of cells in certain phases of the cell cycle, i.e. in the G₀/G₁, S and G₂/M phases. A histogram is interpreted as diploid when there is one cell in the G₀/G₁; in case there are more, it is interpreted as DNA aneuploidy, or as a larger number of cells in the G₂/M phase of the cell cycle (>10% cells - DNA tetraploidy). All tumors with DNA tetraploidy are classified into the group of DNA aneuploid tumors. In our study, the proliferative activity is described by the sum of cells in the S and G₂/M phase of the cell cycle. As a quantitative indicator of analysis of the ploidy state, the term of DNA index has been introduced. This reflects a ratio between a relative DNA content in the G₀/G₁ cell population and a relative DNA content of cell standards (12, 23). For assessing the quality of sample preparation the coefficient of variation (CV) was employed. When handling paraffin-embedded samples CV should not exceed 10% (12, 21, 22). If the CV was greater than 10%, the procedure would be repeated.

In this analysis, χ^2 test was used, including the Yates's continuity correction factor and both T-test and U-test with the security level p of less than 0.05. Statistical analysis was performed using the SPSS computer program.

RESULTS

The mean age of patients was 59 years (range: 31-87 years). A tumor in the right breast was found in 38 (47.5%) patients, and 42 (52.5%) patients had a tumor in their left breast. The tumor size ranged between 1.0 and 8.5 cm. The histological grade I, II and III was found in 12 (15.0%), 44 (55.0%), and 24 (30.0%) patients, respectively (Table 1).

Immunohistochemical and histochemical analysis showed positive reaction to Grimelius stain, chromogranin, synaptophysin and NSE in 70 (87.5%), 34 (42.5%), 33 (41.3%) and 72 (90.0%) tumors, respectively (Table 1). The average concentration of estrogen and progesterone receptors was 30 fmol/mg (range: 0-424 fmol/mg protein)

Table 1.

CLINICOPATHOLOGICAL FEATURES AND FLOW CYTOMETRY RESULTS IN 80 PATIENTS WITH NE BREAST CARCINOMA

Age (mean, age)	59 (31-87)
Tumor size (cm) (mean, range)	2.7 (1.0-8.5)
Histological grade (N, %):	
I	12 (15%)
II	44 (55%)
III	24 (30%)
Histochemical and immunohistochemical positivity (N, %):	
Grimelius	70 (87.5%)
Chromogranin	34 (42.5%)
Synaptophysin	33 (41.3%)
NSE*	72 (90.0%)
Steroid receptor concentration (fmol/mg; mean, range):	
ER**	30 (0-424)
PR***	35 (0-767)
Flow cytometry results (N, %):	
DNA diploidy and prolif. activity < 20%	27 (33.8%)
DNA aneuploidy and/or prolif. activity ≥ 20%	53 (66.2%)
DNA index (median, range)	1.7 (1.1-2.8)
Coefficient of variation (median, range)	5.0 (2.9-9.2)

* NSE – neuron-specific enolase
 ** ER - estrogen receptors
 *** PR - progesterone receptors

Table 2.

RESULTS OF STATISTICAL ANALYSIS OF CLINICOPATHOLOGICAL FEATURES AND CELL CYCLE IN 80 PATIENTS WITH NE BREAST CARCINOMA ADJUSTED DNA PLOIDY

	DNA diploidy and prolif. activity <20% (N=27)	DNA aneuploidy and/or prolif. activity ≥20% (N=53)	p
Mean age (yrs)	58.2	59.0	0.765
Mean tumor size (cm)	2.9	2.6	0.736
Histological grade:			
I	7	5	0.126
II	14	30	
III	6	18	
Grimelius	26	44	0.18
Synaptophysin	7	27	0.057
Chromogranin	8	26	0.155
NSE*	25	47	0.875
ER** (mean)	31.6	29.3	0.091
PR*** (mean)	75.6	16.0	<0.001

* NSE – neuron-specific enolase
 ** ER estrogen receptors
 *** PR progesterone receptors

and 35 fmol/mg protein (range: 0-767 fmol/mg) (Table 1).

Flow cytometry revealed DNA diploidy and proliferative activity less than 20% in 27 (33.8%), while DNA aneuploidy, tetraploidy and/or DNA diploidy with proliferative activity higher than 20% were found in 53 (66.2%) tumors (Table 1). The average SPF value in diploid and aneuploid tumors with proliferative activity ≥20% was $4.2 \pm 3.2\%$ (median 4.1%, range: 0.9-11.3%) and $7.2 \pm 4.3\%$ (median 7.0%, range: 0.0-13.7%; $p < 0.05$), respectively.

The concentration of progesterone receptors was statistically significantly higher in DNA-diploid tumors than in DNA-aneuploid and tetraploid tumors and tumors with proliferative activity ≥20% ($p < 0.001$, Table 2). Concentration of estrogen receptors, age, histological grade, tumor size, Grimelius staining and immunohistochemical markers did not significantly differ between the groups.

DISCUSSION

Based on histochemical analysis using the Grimelius stain and immunohistochemical assess-

ment of the expression of neuroendocrine markers – chromogranin, synaptophysin and neuron-specific enolase 20.3% invasive ductal carcinoma of the breast diagnosed in our study were classified into the group of tumors with neuroendocrine differentiation. In the literature, the percentage of breast carcinoma with NE differentiation ranges from 2 to 25% (3, 8, 11, 18, 23). Such large differences can be attributed to the absence of unique criteria for diagnosis of carcinoma with neuroendocrine components. The newest WHO classification therefore suggests the term of „mixed endocrine-exocrine carcinoma“ (MEEC) for carcinomas with an neuroendocrine component originating from the pancreas, stomach and appendix (24). For carcinomas with NE elements originating from the breast, prostate and colon, the suggested term is „adenocarcinoma with NE differentiation“ (3, 11). Recent studies suggest the term „pure“ (neuro)endocrine tumors for all tumors with 0 to 30% of a nonendocrine component. The term „nonendocrine carcinoma with focal NE component“ would include all cases with 1 to 30% of endocrine component. For tumors with at least 30% of endocrine and nonendocrine component the term MEEC has been suggested. In MEECs, the most aggressive cell population drives the clinical behavior (11). In breast carcinoma with NE differentiation, the classic grading criteria are used as in other types of breast cancer.

In the literature, there is a scant number of papers on the proportion of tumor cells in certain phases of the cell cycles and their ploidy in patients with neuroendocrine carcinoma of the breast (8, 25, 26). In our study, the majority of breast cancer with NE differentiation was in the group of carcinoma with enaploidy and/or proliferative activity $\geq 20\%$ (N=53, 66%). In the study of Wilander et al. (26), positive Grimelius staining was shown in 4 out of 60 (6.7%) samples of breast carcinoma. On the other hand, Nesland et al. (8) analysed 61 breast cancer samples of which there were 23 positive to NSE. In NSE positive tumors, there were 61% of aneuploid tumors unlike 45% in NSE negative tumors, which complies with the results of our study. The results of the study analysing the relationship between the histological grade and ploidy in invasive ductal carcinoma showed the correlation between these parameters, especially with regard to tumor cell polymorphism and mitotic count (27). In our study, ploidy and

proliferative activity did not correlate with the histological grade.

In our study, we found a larger average concentration of progesterone receptors in the group of diploid tumors and tumors with low proliferative activity ($p < 0.001$). According to our knowledge, the relationship of hormone receptor concentration and proliferative activity in breast cancer with NE differentiation has not yet been explored. The results of our study mostly comply with the results of other studies obtained on samples from breast cancer patients, however, they did not investigate neuroendocrine breast carcinoma (7). In these studies, the high SPF correlates with hormone receptor negativity. It is known that the level of estrogen and progesterone receptors has been proved predictive of clinical response to therapy and adjuvant hormonal treatment (28). Progesterone receptor content rather than the content of estrogen receptors is said to be a more sensitive parameter for predicting response to hormonal therapy (29). In our study, the difference in the average concentration of estrogen receptors was not statistically significant. The study carried out on a larger breast cancer sample showed a better effect of adjuvant tamoxifen treatment in the high SPF group of patients with progesterone receptor positivity. A relatively poorer response to tamoxifen therapy found in progesterone-positive tumors with low SPF the authors explain by otherwise favorable prognosis in thus treated group (30).

In our study, the average median SPF in diploid and aneuploid tumors was 4.1% and 7.0% ($p < 0.05$), respectively. These values are equal (16, 31) or somewhat higher (32) than the values obtained by analyses carried out on fresh and frozen materials, which requires caution in their interpretation.

CONCLUSION

The study demonstrated a higher average concentration of progesterone receptors in the group of diploid tumors and tumors with the low proliferative activity. According to the above criteria, other pathological and clinical parameters did not show any significant difference related to tumor ploidy and its proliferative activity. For final conclusion on the clinical significance of neu-

roendocrine differentiation in breast cancer further studies that would also include monitoring of the course and outcome of the disease are required.

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