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Source / Izvornik: **Collegium Antropologicum, 2007, 31, 1043 - 1047**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:736449

Coll. Antropol. **31** (2007) 4: 1043–1047
Original scientific paper

Tumor Growth Fraction, Expression of Estrogen and Progesterone Receptors, p53, Bcl-2 and Cathepsin D Activity in Primary Ductal Invasive Breast Carcinoma and their Axillary Lymph Node Metastases

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A B S T R A C T

The aim of this paper is to determine similarities and differences between tumor cell subclones in cases of ductal invasive breast carcinoma, and which occupy primary tumor and local axillary lymph metastases. The tumor growth fraction evaluated by Ki-67 was analyzed along with the expression level of estrogen and progesterone receptors, protein p53, proto-oncogene protein bcl-2 and cathepsin D in 60 patients. Metastatic lymph node in axilla has a higher growth fraction of the tumor cells than the primary tumor (p=0.045), as well as the higher level of bcl-2 overexpression (p=0.014). No statistically significant difference was found in the presence of immunohistochemically identified estrogen receptors (p=0.161) and progesterone receptors (p=0.081) between the primary tumor and the metastatic lymph node in axilla. Likewise, no difference was found between the immunohistochemical evaluation of p53 (p=0.356) and cathepsin D activity (p=0.928). A higher growth fraction of the tumor cells and the higher level of bcl-2 overexpression in metastatic tumor cells indicate the more aggressive cell subclones. This study does not support the routine testing of both primary tumor and locoregional metastasis to evaluate the breast cancer hormone receptor status.

Key words: *carcinoma, ductal, breast, receptors, estrogen, progesterone, Ki-67, bcl-2, p53, cathepsin D*

Introduction

It has been postulated that the equilibrium of different clones within a tumor is eventually overcome by a biologically dominant one with enhanced metastatic potential[1,2]. There are no sufficient proofs as to whether regional lymph metastases develop out of an individual malignant cell (monoclonal metastases), or whether lymph node is successively populated by different primary tumor subclones (polyclonal metastases)[3]. The metastatic tumor could be composed of more aggressive tumor cell clones.

We were interested in finding out about existing differences, if any, in proliferational activity of the tumor, the expression levels of estrogen (ER), progesterone (PrR), p53, bcl-2, and cathepsin D activity in tumor cells of primary tumor and secondary lymphogenic metastasis. Since during this study several publications appeared claiming that there are no differences in the expression levels of HER-2 receptors of primary tumor and axillary node metastases or distant hematogenic metastasis[4–7],

Received for publication June 8, 2006

we excluded the determination of expression level of that marker from our study.

Material and Methods

Patients

In this study 60 consecutive patients of ductal invasive breast carcinoma with regional (axillary) lymph node metastases were included. The age of patients was between 40 and 65 (mean 55). Tumor size was up to 5 cm (T1 and T2). All patients were diagnosed and treated with modified radical mastectomy and axillary node dissection in Clinical Hospital Osijek. Tumor grade was determined using Bloom and Richardson sheme[8]. For all patients number of positive lymph nodes was determined histologicaly.

Immunohistochemistry

Immunohistochemical staining was performed by standard avidin-biotin method (DAKO LSAB®2 System, HRP) using 4 μm sections from representative paraffin blocks of primary and metastatic tumor[9]. Following antibodies were used:

- Ki-67 nuclear antigen (Dako, Glostrup, Denmark, Ki-67 Antigen, cat. no. N 1574);
- estrogen (ER) and progesterone (PrR) receptors (Dako, Glostrup, Denmark, Estrogen Receptor/Progesterone Receptor Kit, cat. no. K 1900);
- protein p53 (Dako, Glostrup, Denmark, p53 Protein, cat. no. N 1581);
- proto-oncogene protein bcl-2 (Dako, Glostrup, Denmark, BCL2 Oncoprotein, cat. no. N 1587);
- cathepsin D – lysosomal protease (Dako, Glostrup, Denmark, Cathepsin D, cat. no. N 1625).

As positive control samples of breast carcinomas positive for selected antibodies were used. Negative controls were breast carcinoma samples stained by omitting primary antibody.

All samples were analyzed by light microscope by one pathologist. Staining with anti-ER, PR, Ki-67 and p53 antibodies were nuclear, while bcl-2 and Cathepsin D staining were located on membrane and intracytoplasmatic. For nuclear straining the percentage of positive cells per 500 tumor cells was calculated, while p53 and bcl-2 staining was shown as positive-negative, and the percentage of positive cells per 500 tumor cells was calculated [10].

Statistical analysis

Analysis was carried out using data analysis software package Statistica ver. 6.0 (StatSoft, 2001). The existence of differences in the values of individual pathohistological indicators in the primary tumor and the axillary lymph node was tested by t-test for paired samples and by the application of Student's distribution. Scatter plots of individual data were used to determine more precisely the relationship between parameter value in the primary breast tumor and in the axillary node.

Results

Statistically significant difference was observed between Ki-67 expression in primary (21.08±20.05) and metastatic (25.43±22.84) tumor. The metastatic tumor tissue has a greater number of cycling cells ($p=0.045$, Table 1). Likewise, the statistically significant difference ($p=0.014$) was determined in the overexpression of the bcl-2 oncoprotein of the primary tumor (58.08±36.77) and the corresponding axillary lymph node metastasis (64.25 ± 34.92, Table 1).

The difference between ER in primary and metastatic tumor was not found ($p=0.161$, Table 1). A similar observation can be made for the values of progesterone receptors ($p=0.081$), protein p53 ($p=0.356$) and cathepsin D activity ($p=0.928$) in the cells of the primary tumor and axillary lymph node metastasis (Table 1).

Figure 1a shows the scatter plot of Ki-67 distribution in the primary tumor and the metastatic tissue of the lymph node. For majority of our patients with high Ki-67 expression in primary tumor the same high level of expression was found also in metastatic tumor. Figure 1b shows that ER has similar distribution in primary and metastatic tumor, while on Figure 1c is evident that for PrR generally this uniformity does not exist.

TABLE 1
DIFFERENCES BETWEEN THE PRIMARY AND METASTATIC TUMOR CELLS IN 60 BREAST CANCER PATIENTS
(T-TEST FOR PAIRED SAMPLES)

Parameters	Findings (mean ± SD)			
	Tumor	t	p	Lymph node
Tumor growth fraction	21.08±20.05	–2.04	0.045	25.43±22.84
Estrogen receptors	51.81±41.95	1.41	0.161	50.48±40.77
Progesterone receptors	39.16±39.33	1.77	0.081	34.73±35.26
p53	17.66±17.77	0.93	0.356	16.18±24.54
Bcl-2	58.08±36.77	–2.52	0.014	64.25±34.92
Cathepsin D	92.01±13.78	–0.08	0.928	92.23±11.74

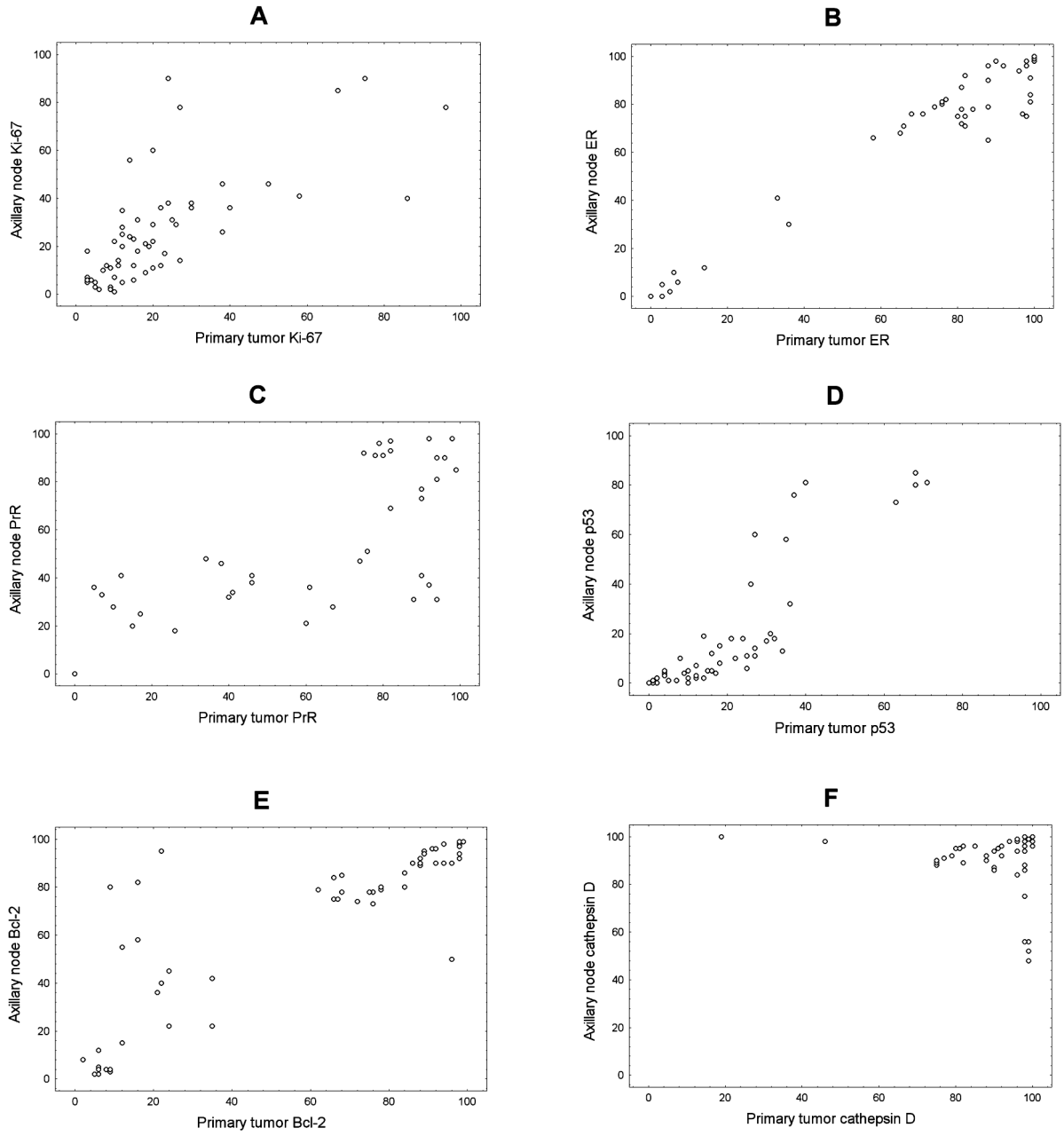

Fig. 1. a) Tumor growth fraction (evaluated by Ki-67) in primary and metastatic tumor. b) ER receptors expression in primary and metastatic tumor. c) PrR receptors expression in primary and metastatic tumor. d) p53 expression in primary and metastatic tumor. e) Bcl-2 expression in primary and metastatic tumor. f) Cathepsin D expression in in primary and metastatic tumor.

The scatter plots of p53 and of bcl-2 (Figures 1d and e) distribution in the primary tumor and the metastatic lymph node showed that in a few of our patients p53 and especially bcl-2 was increased in the metastatic node in comparison to the primary tumor. Bcl-2 scatter plot shows that high expression of bcl-2 in the primary tumor remained high in the axillary site, but there is a fraction of patients with bcl-2 expression much higher in the metastatic site. Figure 1f shows the scatter plot of cathepsin D distribution in the primary tumor and the metastatic tissue of the lymph node. In most of our patients cathepsin D values did not differ between the primary tumor and metastatic node and just in few of them cathepsin D was reduced in the metastatic node.

Discussion

Breast cancer is a heterogeneous disease[11]. Instead of a linear progression from the primary tumor to the metastasis, an early stem-line clone might evolve independ-

ently in both sites[3]. The resulting heterogeneity of metastatic breast cancer may explain why biomarkers of prognosis or therapy responsiveness measured exclusively from primary tumors may not entirely reveal all the biological properties of breast cancer. The main target of any therapy in metastatic breast cancer are the metastases. However, in a great majority of cases, the mitotic index, ER, PrR, and HER-2/neu overexpression are determined on the primary tumor.

The proliferational activity is an important prognostic factor, being an important part of tumor histologic grade determination[8]. Mitotic index can be determined by mitosis count in the tumor tissue, and by the application of antibodies on Ki-67 nuclear antigen which characterizes cycling cells[12]. The analysis of the Ki-67 antigen expression, points at a statistically significant difference in the number of cycling cells in the primary tumor and the metastatic lymph node. The finding of a more mitotic active tumor tissue in the axillary lymph node metastasis could imply that the locoregional metastases are composed of more aggressive tumor cell clones than the primary breast carcinoma, as already mentioned in some research[13].

It is a well known fact that an increased concentration of estrogen in circulation raises the number of estrogen receptors, but also induces a heightened activity of progesterone receptors. Progesterone decreases the number of estrogen and progesterone receptors in cells[9–12,14–21]. Hormone receptors (ER and PrR) are involved in the control of cell growth and influence the development and the success of treatment of patients with breast carcinoma. Their prognostic value has been tested in numerous clinical studies which show that patients with a certain level of expression are more likely to survive than others. In recent times, the presence of hormone receptors is important for the evaluation of response to adjuvant therapy[14,15]. Those with one positive receptor ER+/PrR– or ER–/PrR+ respond positively to hormonal therapy in 34–45% of the cases. Patients with both negative receptors ER–/PrR– have a therapeutic response in 5–10% of the cases[22–24].

We have not found a significant difference in the ER and PrR levels between the primary breast cancer and the metastatic axillary lymph node metastasis. However, subgrouping is evident for the expression of PrR. Small groups of patients showed that PrR content in the axillary node can differ from the primary tumor. In some patients PrR expression in the primary tumor is associated with reduced and in some with increased PrR levels in the metastatic node. Knowing that the PrR expression depends on functional estrogen receptors[25], the distributions of ER and PrR observed in primary tumors and axillary nodes taken together might indicate that in some of subclones ER might have become dysfunctional and thus alter their PrR expression. The scatter plot in Fig. 1. C shows that among our patients the subclone with dysfunctional ER might be dominant in the primary tumor (patients above the diagonal) while in others the metastasizing subclone seems to be ER dysfunctional (patients bellow the diagonal). There are some data published which indicate no correlation in 21% of cases with regard to the estrogen receptors in primary tumor and axillary lymph node. The ER were mainly positive in primary tumor and negative in metastatic tissue. The disagreement between the results can be explained by the loss of receptors in the metastatic cells or by tumor heterogeneity. This also explains the failure of endocrine therapy with some patients who have positive estrogen receptors of primary tumors[26]. The progression to an ER negative, estrogen-independent, antiestrogen-resistant, EGFR (epidermal growth factor receptor) positive and highly metastatic phenotype is often considered to be a common course of the breast cancer[27]. Since the ER content is often highly varying in different cells of the same tumor[28,29], the occurrence of ER negative tumor cells might be a consequence of the clonal selection among tumor cells.

Bcl-2 onkoprotein is coded by a gene affected by chromosome translocation, and it participates in cell death programming (apoptosis) as an inhibitor factor of apoptosis process[30,31]. In a few of our patients bcl-2 was increased in the metastatic node in comparison to the primary tumor, suggesting that among them the metastasizing subclone overexpressed bcl-2. Its increased expression is a prognostic indicator in patients with breast cancer. By inhibiting apoptosis in tumor tissue it is given an additional vitality which makes patients' prognosis worse.

Gene p53 belongs to a group of tumor gene suppressors. Family members with Li-Fraumeni syndrome who inherit the defect in gene p53 suffer 50% higher risk of developing breast cancer before the age of 30[32,33]. In our study, the expression of p53 protein determined immunohistochemically was equal in the primary tumor and its metastasis. There are studies which suggest that p53 protein expression in some patients with breast carcinoma need not be linked to metastatic disease[33].

Cathepsin D is a lysosomal acid protease. The majority of the neoplasms have an increased production and an increased action of the enzyme when compared to normal tissue[34,35]. According to some authors, the increased activity of cathepsin D can mark a tumor population with high proliferation activity and invasive potential related to a more aggressive tumor phenotype[36]. However, others found no significant correspondence between the presence of cathepsin D and the tumor size, the number of metastatic lymph nodes, the histological carcinoma type, and the status of steroid receptors[37].

No significant difference was found in our study with respect to the cathepsin D activity in the cells of primary neoplasm and its locoregional metastasis. Nevertheless, the majority of patients with high cathepsin D expression in the primary tumor (70–100% cells) had a different, lower as a rule, expression of cathepsin D in metastatic lymph node, which could point at the conclusion that the activity of cathepsin D is rather characteristic of primary breast cancer than of metastatic tumor. This is an unexpected finding, contrary to the common belief that meta-

static cell clones are characterized with higher cathepsin D activity.

In conclusion, this study does not support the routine testing of both primary tumor and locoregional metastasis to evaluate the breast cancer hormone receptor status. Among our patients we have found no consistent metastasizing phenotype. Although it seems that in individual patients overexpression of some cellular markers might be linked to the process of metastasizing, we need more data on cellular mechanisms of metastasizing in order to answer remaining clinical dilemmas regarding the size of primary tumor, its grade and malignant potential.

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USPOREDBA DIOBENE AKTIVNOSTI, STUPNJA IZRAŽENOSTI ESTROGENSKIH I PROGESTERONSKIH BILJEGA I AKTIVNOSTI KATEPSINA D U STANICAMA INVAZIVNOG DUKTALNOG KARCINOMA DOJKE I U STANICAMA PAZUŠNIH LIMFNIH METASTAZA

SAŽETAK

Cilj rada je utvrditi sličnosti i razlike između subklonova tumorskih stanica duktalnog invazivnog karcinoma dojke koji naseljavaju primarni tumor i lokalne pazušne limfne metastaze. Analizirana je diobena aktivnost tumorskih stanica izražajem Ki-67, stupanj izraženosti estrogenskih i progesteronskih biljega, proteina p53, proto-onkogena bcl-2 i aktivnost katepsina D u 60 bolesnica. Metastatski limfni čvor u pazuhu ima izraženiju diobenu aktivnost od primarnog tumora (p=0,018) i jači izražaj bcl-2 (p=0,014). Nije utvrđena statistički značajna razlika u nazočnosti imunohistokemijski određenih estrogenskih (p=0,16) i progesteronskih biljega (p=0,08) između primarnog i metastatskog tumorskog tkiva. Također nije bilo razlike između imunohistokemijske procjene p53 (p=0,356) i aktivnosti katepsina D (p=0,92). Veća diobena aktivnost i jači izražaj bcl-2 u metastatskom tkivu upućuje na agresivnije metastatske subklonove tumorskih stanica. Rezultati ne ukazuju na potrebu istovremene evaluacije hormonskih biljega u primarnom tumoru i pazušnim metastazama.