# ONCOLOGY

# Expression of Biomolecular Markers (Ki-67, PCNA, Bcl-2, BAX, BclX, VEGF) in Breast Tumors

**N. E. Kushlinskii, M. B. Orinovskii, L. E. Gurevich, I. A. Kazantseva, Sh. Zh. Talaeva, V. P. Shirokii, V. D. Ermilova, E. K. Dvorova, and A. S. Ozherel'ev**

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We carried out a retrospective immunohistochemical study of Ki-67, PCNA, Bcl-2, BAX, BclX, and VEGF expression in tumors of two groups of breast cancer patients with favorable and unfavorable course of the disease. Considerably enhanced VEGF expression was detected in tumors of patients with early relapses of breast cancer. High VEGF expression was paralleled by high level of Ki-67 and PCNA expression in tumors. It can be hypothesized that expression of VEGF, Ki-67, and PCNA in primary tumor can be used for predicting the course of breast cancer or detecting the patients at a high risk of early relapses.

**Key Words:** *breast cancer; Ki-67, PCNA, Bcl-2, BAX, BclX, VEGF expression; prediction*

The incidence of breast cancer (BC) ranks first among oncological diseases in Russia. According to statistical data the prevalence of breast cancer increases very rapidly compared to other malignant tumors [3].

It is well known that early detection of BC is associated with better delayed outcome of therapy. However, even at the early stages prediction of the disease course and outcome should be individual. The study of the expression of biomolecular markers in malignant tumors of the mammary gland helps to detect BC patients at a high risk of early relapses [1,2, 6,13]. One of the most important biological characteristics of the tumor for the disease prediction is evaluation of the potential of its proliferative activity, invasive capacity, angiogenesis, and apoptosis [4,7,8]. The level of Ki-67 antigen expression is closely associated with biologically aggressive behavior of the tumor [14]; a relationship between the expression of PCNA in the tumor and time of the disease progress was revealed [11]. A relationship between the level of Bcl-2 molecular marker expression in the tumor and its sensitivity to adjuvant therapy was noted.

It was proven that the formation of vascular network is an obligatory condition for tumor growth. The dynamic balance between numerous angiogenic and antiangiogenic factors regulates neoangiogenesis in the tumor [9,10]. Many known growth factors and cytokines are involved in the regulation of angiogenesis, but vascular endothelium growth factor (VEGF) is, no doubt, the most important positive regulator of angiogenesis [5,9, 12]. In contrast to other growth factors VEGF acts as active mitogen for micro- and macrovascular cells of blood and lymph vessels, but not for other cell types.

This paper presents the data of retrospective analysis of the expression of biomolecular markers Ki-67, PCNA, Bcl-2, BAX, BclX, and VEGF in tumors of 21 patients with BC and their value prognostic.

## MATERIALS AND METHODS

Patients with BC were treated and observed for 5 years since 1997 at the Cancer Research Center. The diag-

N. N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Moscow; M. F. Vladimirskii Moscow Regional Research and Clinical Institute

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nosis was confirmed by the results of histological analysis of removed tumor.

Immunohistochemical study of the expression of biomolecular markers in tumors from 2 groups of BC patients was carried out using mono- and polyclonal antibodies. Immunohistochemical staining of BC cell nuclei with antibodies to PCNA and Ki-67 was evaluated by a quantitative method. The labeling index or number of marker-stained tumor cell nuclei per 100 tumor cells was determined in representative visual fields. This parameter was calculated by analyzing at least 500-1000 cells. The following antibodies were used: to VEGF (working dilution 1:100, clone VEGF (C-1)sc-7269; Santa Cruz Biotechnology Inc.), BAX (1:50; polyclonal; DAKO), BclX (1:50; polyclonal; DAKO), Bcl-2 (1:80; Bcl-2/100/D5; NOVOCASTRA), Ki-67 (1:50; Ki-S5; DAKO), and PCNA (clone PC10; DAKO). Immunohistochemical staining of tumor cell cytoplasm with VEGF, Bcl-2, BclX, and BAX antibodies was analyzed by a semiquantitative method using a 4-point scale: no staining (0); >20% cells with weakly stained cytoplasm (1); moderately stained cytoplasm of cancer cells (2); intensive staining in >50% cells; intensive staining in >80% cells (4). The mean score was then calculated. Estrogen receptors (ER) were detected by radioligand method [1].

Statistical analysis was carried out using SPSS 9.0 and STATISTICA 6.0 software.

The examinees were divided into 2 groups. Group 1 consisted of 9 BC patients with relapses within 5-year follow-up. The mean term of relapse in this group was 454.8 [95% CI=0-915.2] days from the start of therapy. The patients aged 37-69 years (1 in the reproductive and 8 in the postmenopausal age) with the following disease stages: 3 with $T_2N_0M_0$, 1 with $T_2N_1M_0$, 1 with $T_2N_2M_0$, 2 with $T_3N_1M_0$, 1 with $T_4N_1M_0$, and 1 with $T_4N_2M_1$. Lobular infiltrative BC was detected in 3 and ductal infiltrative cancer in 6 patients. The following operations were performed: radical mastectomy without pectoral muscle excision in 8 patients and radical resection in 1 (the patients were operated in 1997). ER were detected in 3 tumors (33.3% — 12, 31, and 40 fmol/mg total protein). After surgery 4 patients received a course of radiotherapy in a total focal dose (TFD) 46 Gy (single focal dose (SFD) 4 Gy). Seven patients received (also after surgery) courses of adjuvant chemotherapy according to the following protocols: CMF (3 patients), FAP, CAF, taxoter+adriablastin, taxoter+doxorubicin (1 patient each protocol). All patients received tamoxifen in a daily dose of 20-40 mg. Relapses or remote metastases manifested 5-30 months after the start of treatment. Metastases in bones were detected in 4 patients, in the liver in 2, in the lungs in 2, and in one patient a tumor relapse was found in the postoperative cicatrix 7 months after the start of treatment, in parallel with metastases in the liver and regional (clavicular area) lymph nodes.

Group 2 consisted of 12 BC patients aged 35-68 years (6 of reproductive and 6 of postmenopausal age) without relapses for 5 years postoperation with the following disease stages: 3 with $T_2N_0M_0$, 4 with $T_2N_1M_0$, 3 with $T_3N_1M_0$ and 2 with $T_4N_1M_0$. Lobular infiltrative cancer was detected in 4, ductal infiltrative in 7, and poorly differentiated squamous-cell carcinoma in 1 patient. Ten patients received no specific therapy before surgery. Only 1 of 11 patients (with stage $T_4N_1M_0$) received neoadjuvant treatment: radiotherapy (TFD 32 Gy, SFD 2 Gy) and chemotherapy according to Couper's protocol (CMF+vincristine). The following

**TABLE 1.** Characteristics of VEGF, BAX, BclX, Bcl-2, Ki-67, and PCNA in Patients with BC

| Parameter | | Mean value | 95% CI | Median |
|---|---|---|---|---|
| Group 1 | VEGF (*n*=9) | 3.08 (2-4)* | 2.51-3.64 | 3 |
| | BAX (*n*=9) | 1.89 (1-3) | 1.5-2.3 | 2 |
| | BclX (*n*=9) | 2.56 (1.5-3.0)* | 2.15-2.96 | 2.5 |
| | Bcl-2 (*n*=8) | 1.31 (0-3) | 0.34-2.28 | 1.75 |
| | Ki-67 (*n*=9) | 26.99 (12.2-38.5)* | 20.8-33.2 | 26.5 |
| | PCNA (*n*=9) | 42.93 (29.8-55.0)* | 36.0-49.9 | 47 |
| Group 2 | VEGF (*n*=12) | 1.38 (0.5-2.0)* | 1.01-1.74 | 1.5 |
| | BAX (*n*=12) | 2.33 (1-3) | 1.86-2.81 | 2.5 |
| | BclX (*n*=12) | 1.96 (0.5-2.5)* | 1.61-2.30 | 2 |
| | Bcl-2 (*n*=11) | 1.91 (0.0-3.5) | 1.09-2.73 | 2 |
| | Ki-67 (*n*=12) | 14.87 (11.0-21.8)* | 12.8-16.9 | 14 |
| | PCNA (*n*=12) | 23.08 (14.5-32.0)* | 19.8-26.4 | 24.5 |

**Note.** *$p$<0.001 compared to group 2. The minimum and maximum values are shown in parentheses.

**TABLE 2.** Coefficients Reflecting the Ratio of Molecular Biological Markers in Breast Tumors of Patients with Favorable and Unfavorable Course of the Disease

| Coefficients | Group 1 | Group 2 | *p* |
|---|---|---|---|
| BAX/BclX | 0.76±0.07 | 1.46±0.35 | i. d. |
| VEGF/BAX | 1.73±0.21* | 0.76±0.18 | 0.002 |
| VEGF/BclX | 1.26±0.15* | 0.80±0.14 | 0.039 |
| Ki-67/VEGF | 9.39±1.33 | 13.29±2.16 | i. d. |
| PCNA/VEGF | 14.80±1.74 | 21.32±4.02 | i. d. |
| Ki-67/BAX | 15.25±1.97* | 7.42±1.21 | 0.002 |
| PCNA/BAX | 24.37±3.15* | 11.16±1.62 | 0.0007 |
| Ki-67/BclX | 11.10±1.36 | 9.19±1.88 | i. d. |
| PCNA/BclX | 17.31±1.43 | 14.03±2.73 | i. d. |

**Note.** * compared to group 2; i. d.: insignificant differences.

operations were performed: radical mastectomy without pectoral muscle excision in 10 and radical resection in 2 cases. ER were detected in 6 tumors (50.5%), their levels varied from 12 to 21 fmol/mg total protein. The following adjuvant therapy was carried out: radiotherapy (5 patients), polychemotherapy (7 patients; CMF protocol in 5, CAP in 1, and CAF in 1 case), radiotherapy+polychemotherapy (4 patients). Two patients received tamoxifen in a daily dose of 20-40 mg.

## RESULTS

Analysis of molecular biological markers in the primary tumor showed significant differences in the expression of VEGF, BclX, Ki-67, and PCNA in tumors of patients with early relapses and without relapses (Table 1).

Expression of VEGF, Ki-67, and PCNA in group 1 surpassed the corresponding parameters by 2.23, 1.81, and 1.86 times, respectively. On the other hand, expression of proapoptotic indicator BAX in group 2 surpassed that in group 1 by 1.23 times. Bcl-2 expression was the same (Table 1).

Estimation of the coefficients reflecting the ratios of the studied biomolecular markers in the studied groups showed the most significant differences in the VEGF/BAX, MCNA/BAX, and Ki-67/BAX coefficients (Table 2).

A high positive correlation ($r$=0.89, [95% CI= 0.74-0.95], $p$<0.05) between Ki-67 and PCNA expression was detected in both groups of patients with BC. A linear positive relationship between the expression of VEGF and PCNA was detected. PCNA and BclX expression also correlated ($r$=0.43, $p$=0.047).

Higher levels of VEGF expression in the tumor were associated with shorter remission. The same, but less pronounced relationship was characteristic of Ki-67 ($r$=-0.57, $p$>0.05) and PCNA ($r$=-0.63, $p$>0.05). The expression of BclX and Bcl-2 was not linearly related to the duration of relapse-free period.

The prognostic potential of the studied markers was evaluated for certain values of the markers in the tumor using multiregression analysis for estimating the probability of a relapse within 5 years from the start of therapy. The level of the model significance $p$=0.0001 confirms strict relationship between the studied factors and probability of relapse in BC patients during the studied period. The regression coefficients, indicating the validity of this relationship, were as follows: $\beta_{VEGF}$=-0.46±0.11 ($p$=0.001), $\beta_{Ki\text{-}67}$=-0.43±0.17 ($p$=0.027), and $\beta_{PCNA}$=-0.44±0.19 ($p$=0.0475); the regression coefficients of other markers differed negligibly from the zero ($p$>0.05).

In order to predict a relapse within 5-year follow-up in BC patients on the basis of the studied biomolecular markers, the threshold levels were calculated by the method of binary logistic regression with estimation of the sensitivity and representativeness of the tested threshold levels of the markers and their evaluation (Table 3).

Multifactorial analysis of survival (Proportional hazard (Cox) model) using the levels of all markers and TNM characteristics showed that VEGF was the only independent prognostic factor ($p$=0.0023).

**TABLE 3.** Threshold Levels of Parameters Determined by Binary Logistic Regression and Their Evaluation

| Parameter | VEGF | BAX | BclX | Bcl-2 | Ki-67 | PCNA |
|---|---|---|---|---|---|---|
| Threshold value, points (labeling index) | 2 | 2 | 2 | 1.5 | 24 | 30 |
| Sensitivity | 0.89 (0.68-1.00) | 0.89 (0.58-1.00) | 0.78 (0.43-0.98) | 0.44 (0.18-0.82) | 0.78 (0.51-1.00) | 0.89 (0.68-1.00) |
| Repres entativeness | 1.0 (0.76-1.00), corrected 0.97 | 0.58 (0.26-0.88) | 0.75 (0.38-0.96) | 0.58 (0.26-0.88) | 1.0 (0.76-1.00), corrected 0.97 | 1.0 (0.76-1.00), corrected 0.97 |

**Note.** 95% confidence interval is given in parentheses.

Hence, high expression of VEGF positively stimulating neoangiogenesis in the primary tumor is characteristic of mammary tumors with unfavorable course and hence, unfavorable prognosis (all patients had relapses soon after the start of treatment). High levels of VEGF in tumors of these patients were paralleled by elevated index of proliferative activity of Ki-67 and PCNA antigens. The levels of these markers can also be used for disease prognosis.

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