

Expression of Steroid Receptors and Cytokeratin 18 in Breast Cancer after Neoadjuvant Chemotherapy

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Abstract

Nowadays treatment of breast cancer (BC) is based on the tumor receptor status measured by the expression of ERs, PR, Her2/neu receptors. Our objective was to determine the gene expression of steroid receptors (ER α , ER β , PR), cytokeratin 18 (KRT18) in malignant breast tumors of patients untreated and treated with neoadjuvant chemotherapy (NAC). BC samples were obtained after surgery of patients who were treated in the State Regional Oncology Center (Novosibirsk) from 2011 to 2014 (n = 122). We have found that the expression of ER α in patients after NAT decreased almost three times compared to the untreated group (p<0,05). To evaluate receptor status of BC, we used the semi-quantitative real-time RT-PCR method. It should be noted that mRNA level for ER might not always correlate with the protein level. In this regard, one of the objectives of this study was to conduct comparative analysis of immunohistochemical (IHC) results and ER α mRNA level obtained by RT-PCR. We have found that the results for ER α receptor expression measured by IHC and RTPCR match in 85.9% of cases. Besides, we determined the expression of KRT18, a marker of tumor cell proliferation, in patients under study with various tumor phenotypes. Our results also showed that the expression level of KRT18 decreased 3.2 times in tumor tissue of patients treated with NAC compared with an untreated group (p <0,05). We also evaluated the expression of KRT18 depending on the tumor phenotype. We have found that patients with ER+PR+ tumors had an increased level of KRT18 in both groups under study 2.7 times on average in comparison with ERPR+-phenotype (p<0,05). Thus, selected markers can be appropriate candidates for personalized treatment of BC patients.

Introduction

Breast cancer (BC) is the most common malignancy in women, and the second most frequent among all cancers in the general population. Over the last few years, significant progress has been made in the treatment and diagnosis of breast cancer due to intensive development of modern molecular genetic techniques. In many cases, the treatment of this disease is based on the knowledge of receptor status of the tumors, as measured by the expression of ERs, PGR, and Her2/neu receptors.

Today, an imbalance in steroid receptors ER α / ER β is one of the leading hypothesized mechanisms behind the development of breast cancer. It was shown that in normal tissue, ratio of ER α / ER β is about 1 : 8; for example, of all estrogen receptors expressed on normal mammary epithelial cells, 7-10% is ER α and 80-85% is ER β [1]. In tumor cells, ER α expression is several times higher than normal, and ER β receptor levels are reduced compared to normal tissue. The degree to which ER β expression is reduced depends on the degree to which transformed tissue proliferates and differentiates [1,2].

At present, neoadjuvant chemotherapy (NAC) is in widespread use as a preoperative treatment of breast cancer. This therapy is used for patients with locally advanced cancer (conventionally not operable) to reduce the cancer into an operable form, allowing for organ-saving surgery. The ratio of ER α to ER β , after a course of chemotherapy, is a criterion for determining whether breast cancer tumors are susceptible to NAC treatment.

Also, in recent years a lot of new molecular markers of breast cancer have been discovered, including cytokeratin 18, a filament protein of the cytoskeleton of epithelial cells, is one indicator of survival rate [3]. Cytokeratin 18 is expressed in single-layer epithelial tissues, predominantly in the cytoplasm and perinuclear regions. High levels of expression of cytokeratin 18 are found in patients with epithelial cell carcinomas, e.g., breast and prostate cancer, ovarian and gastrointestinal carcinoma [4]. It has been shown that having high levels of cytokeratin

18 after three cycles of chemotherapy was significantly correlated with low one- and two-year survival rates. Furthermore, this protein is detected in high concentrations in patients with rapidly progressing metastatic cancers [3-5].

Objective: To determine the effect of neoadjuvant chemotherapy on the genetic expression of select steroid receptors (ER α , ER β , PGR) and the KRT18 gene in malignant tumors of the breast cancer patients.

Materials and Methods

Breast tumor samples were obtained from patients who underwent

| Age of the patients | The number of patients, n | Percentage ratio (%) |
|---------------------|---------------------------|----------------------|
| Under 40 years | 4 | 8,2 |
| From 41 to 50 years | 11 | 22,4 |
| Over 50 years | 34 | 69,4 |

Table 1: Distribution of patients by age in the first group.

| BC stage | The number of patients, n | Percentage ratio (%) |
|--------------|---------------------------|----------------------|
| II A, II B | 26 | 53,1 |
| III A, III B | 19 | 38,8 |
| IV | 4 | 8,1 |

Table 2: Distribution of patients by stage in the first group.

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| Age of the patients | The number of patients, n | Percentage ratio (%) |
|---------------------|---------------------------|----------------------|
| Under 40 years | 9 | 12,3 |
| From 41 to 50 years | 24 | 32,9 |
| Over 50 years | 40 | 54,8 |

Table 3: Distribution of patients by age in the first group.

| BC stage | The number of patients, n | Percentage ratio (%) |
|------------|---------------------------|----------------------|
| I | 20 | 27,4 |
| II A, II B | 53 | 72,6 |

Table 4: Distribution of patients by stage in the first group.

| Gene | Primer sequence | |
|---------|-----------------|----------------------------------|
| β-actin | Forward | 5'-GCCGAGGACTTTGATTGC-3' |
| | Reverse | 5'-GTGTGGACTTGGGAGAGGA-3' |
| RPL | Forward | 5'-CATTTCCGAAGCGAGTGTCT-3' |
| | Reverse | 5'-GAGCGATTCCGACTACCTT-3' |
| Pol II | Forward | 5'-GCACCACGTCCAATGACAT -3' |
| | Reverse | 5'-GTGCGGCTGCTTCCATAA -3' |
| ERα | Forward | 5'-ATGATGAAAGGTGGGATACGA-3' |
| | Reverse | 5'-ACTCTCATGTCTCCAGCAGA -3' |
| ERβ | Forward | 5'-TTGGATGGAGGTGTTAATGATG-3' |
| | Reverse | 5'-GAAGTAGTTGCCAGGAGCATGT-3' |
| PGR | Forward | 5'-TCATTCTATTTCATTATCCCTTACCA-3' |
| | Reverse | 5'-GACTTCGTAGCCCTTCCAAAG -3' |
| KRT 18 | Forward | 5'-CTTGCTGCTGATGACTTTAG-3' |
| | Reverse | 5'-TTACTTCTCTTCGTGGTTC-3' |

Table 5: Sequences for primers that used in study.

surgical treatment in the thoracic department of the State Regional Oncology Center (Novosibirsk) from 2011 to 2014 (n = 122). Adjacent untransformed breast tissue served as a control. Biopsy specimens was carried out with the consent of the patients and complied with the standards set by the Ethics Committee of the Russian Federation. The medical records of patients provided data for clinical-anamnestic analysis. These data were obtained through patient questioning that was recorded on specially designed individual questionnaires, with the analysis of causes and conditions of the pathological process, age, menstrual and reproductive functions, constitutional features, and comorbidities.

All patients were divided into 2 groups.

The first group (cases): 49 samples of tumor tissue were taken from breast cancer patients aged 34 to 78 years old (mean age 56 ± 10.4 years), all of whom had a breast cancer diagnosis of stage T₁₋₄N₀₋₁M₀₋₁ and were treated with neoadjuvant chemotherapy (from two to four cycles with scheme «FAC» or «CAF»). Dosages for the «FAC» chemotherapy scheme are as follows: 5-fluorouracil 500 mg/m², doxorubicin 50 mg/m², cyclophosphamide 500 mg/m². All drugs were injected intravenously on the first day. Dosages for the «CAF» chemotherapy scheme are as follows: cyclophosphamide 100 mg/m² intramuscularly from day 1 to day 14, doxorubicin 30 mg/m² intravenously on days 1 and 8, 5-fluorouracil 500 mg/m² delivered intravenously on days 1 and 8. Surgery was performed 7-14 days after the end of neoadjuvant chemotherapy. Tables 1 and 2 show age and tumor stage of patients in first group.

Tissue samples extracted from patients were placed in liquid nitrogen and then stored at -70°C.

A second group of samples were taken from 73 patients' tumors as a control group; all patients were diagnosed with a breast cancer of stage

T₁₋₃N₀₋₁M₀, and all were aged between 35 and 74 years. These patients did not receive neoadjuvant chemotherapy. Their mean age was 52.5 ± 10.5 years. The technique of collection and storage of the tissue samples was similar to that for the patients of the first group. Tables 3 and 4 show age and tumor stage of patients in the second group.

RNA was isolated from the tissue samples using the "RNeasy Lipid Tissue Mini Kit" (Qiagen, USA). Reverse transcription was performed using an RNA-dependent DNA polymerase "SmartTaq" (Medigen, Russia). To determine the level of gene expression, real-time PCR was performed on a thermal cycler CFX96 (Bio-Rad, USA) in the presence of SYBR Green I ("Molecular Probes", USA). To normalize the sample concentration "normalization coefficient" was used. It was calculated as the arithmetic mean of the relative amount of cDNA of RPL32, b-actin, Pol-II genes. Calculation of the relative levels of mRNA expression of the genes in question was carried out using the ΔCt method. Statistical data processing was carried out using non-parametric statistical methods

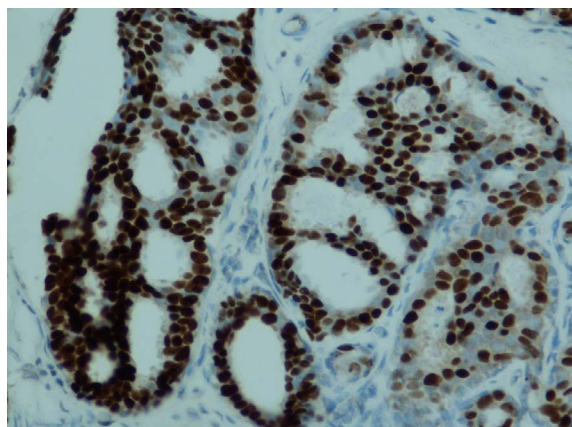


Figure 1: Expression of ER with an estimate of 7-8 points.

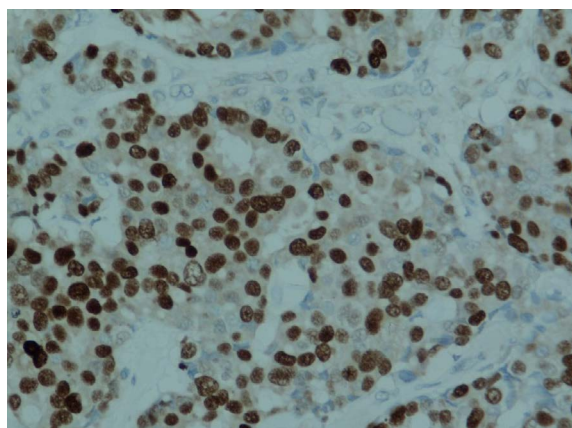


Figure 2: Expression of PGR with an estimate of 7 points.

| Receptor status | First group, n=49 | | Second group, n=73 | |
|-----------------|-------------------|----|--------------------|----|
| | Abs | % | Abs | % |
| ERα+/ERβ+ | 14 | 29 | 1 | 1 |
| ERα+/ERβ- | 17 | 34 | 48 | 66 |
| ERα-/ERβ+ | 2 | 4 | 6 | 8 |
| ERα-/ERβ- | 16 | 33 | 18 | 25 |

Abs – the absolute number of patients.

Table 6: The expression of receptors ERα and ERβ in the study and control groups of patients.

(Mann-Whitney test, Wilcoxon test). Oligonucleotide primers for the genes in question were selected using the Primer-BLAST program. Sequences for primers used in study are provided in Table 5.

Determination of ER, PGR expression in samples of malignant breast tumors by IHC analysis was performed according to standard protocols. For this study the tumor tissue was fixed in 10% buffered formalin and embedded in paraffin blocks from which histological sections of 3-4 microns thick were made. All steps of the IHC analysis (dewaxing, unmasking, incubation with primary antibodies) was performed automatically in the Bench Mark HT (Ventana) using the Rabbit monoclonal primary antibodies: Estrogen receptor (SP1 clone), Progesteron receptor (1E2 clone).

Finished preparations were studied on light microscopes NIKON - Eclipse - 50i (Japan). To determine the level of ER and PGR expression the percentage of positive cells and intensity of staining nuclei was evaluated and scored on a scale from 1+ to 3+ points. Points summarized according to clinical guidelines. Figures 1 and 2 shows a typical picture of ER-positive and PGR-positive breast cancer with an estimate of 7-8 points.

Results and Discussion

Expression of steroid receptors

In our work to assess receptor status of breast cancer, the qPCR method was used. Table 6 shows the results of expression of ERα and ERβ receptors in the both groups of patients. Cutoff value for estrogen receptor expression was defined as 1. All samples with expression values greater than the threshold were considered as estrogen-positive. All samples with expression values below the threshold were considered as estrogen-negative.

Twice-positive estrogen-receptor status was observed in 29% of patients in the first group, while in the control group there were only 1% of patients with ERα + / ERβ +. The presence of ERα + / ERβ + status can be seen as more favorable prognostic sign, as it is characterized as a hormone-dependent tumor, which allows us to use

| mRNA level | After NAC | Without NAC |
|------------|--------------|-------------|
| ERα | 0,43 ± 0,17* | 1,2 ± 0,35* |
| ERβ | 0,54 ± 0,39 | 0,47 ± 0,28 |

Mean values ± SD. * p<0.05

Table 7: The relative mRNA levels of ERα and ERβ in the control and study groups in CU.

| mRNA level | After NAC | Without NAC |
|------------|--------------|--------------|
| PGR | 0,83 ± 0,37* | 1,05 ± 0,25* |

Mean values ± SD. * p=0.138

Table 8: The relative mRNA levels of PGR in the control and study groups in CU.

| Parameter 1 | Parameter 2 | Matching percentage | N (number of patients) |
|-------------|-------------|---------------------|------------------------|
| ER (IHC) | ERα (PCR) | 85,9% | 122 |
| PGR (IHC) | PGR (PCR) | 61,5 % | 122 |

Table 9: Verification data between qPCR and immunohistochemical analysis.

| Parameter 1 | Parameter 2 | Matching percentage | N (number of patients) |
|-------------|-------------|---------------------|------------------------|
| ER (IHC) | ERα (PCR) | 85,9% | 122 |
| PGR (IHC) | PGR (PCR) | 61,5 % | 122 |

Mean values ± SD. * p<0.05

Table 10: Cytokeratin 18 gene expression profile comparisons in CU.

hormone therapy. The low expression of ERβ prevailed in both groups, while ERα + / ERβ- phenotype, reflecting the “classic” imbalance of these types of receptors in the malignant “transformation of breast tissue in patients receiving therapy was significantly lower in the first group (34% compared with 66% in the control group). These figures confirm the current literature data [6,7]. ERα- / ERβ- tumor phenotype was observed in 33% of the treatment group and 25% in the control. This phenotype may be indicative of the worst prognosis: patients with such tumors should be treated with chemotherapy only. The next step of our work was the calculation of the relative mRNA levels of steroid receptors, both in normal and in tumor samples based on data from the qPCR reaction. This was done using the 2^{-ΔCt} formula, where ΔCt = (Ct_{steroid.recept.} - Ct_{householdgene}) tumor sample - (Ct_{steroid Receptor.} - Ct_{householdgene}) conventional norm. As the conventional norm sample was taken from the control group with the smallest difference of Ct_{steroid.recept.} - Ct_{actin}. Thus, the conventional level of mRNA for this sample is equal to 1. Regarding it mRNA levels were counted in conventional units (CU) for the remaining samples. Table 7 shows the relative mRNA levels of ERα and ERβ in the control and study groups in CU.

We found that the expression of ERα in the study group decreased almost three times compared to the control group. This is probably associated with an increase in the degree of inflammatory infiltration and fibrosis in the tumors as a sign of therapeutic pathomorphism. The average value of the relative level of mRNA in the group of patients treated with neoadjuvant therapy was 0,43 ± 0,17 CU; in the group of patients who did not receive treatment, it was 1,2 ± 0,35 CU (p <0.05). The expression level of ERβ in both groups did not differ; the values of the relative level of mRNA in the study group was 0,54 ± 0,39 CU, and in the control group was 0,47 ± 0,28 CU. In the literature, it is shown that the use of neoadjuvant chemotherapy reduces mRNA expression of both types of receptors [6,7].

In most cases, along with changes in estrogen receptor gene expression, there is also a decrease in progesterone gene expression after chemotherapy. In our study, we did not find any significant differences in the gene expression profile ratios between ERα and PGR in both groups (Table 8).

Comparison of the expression of steroid receptors

At present, immunohistochemical testing is the “gold standard” in characterizing ER and PGR expression in breast tissues, and it is widely used in clinical practice. To evaluate the receptor status of breast cancers, we used the qPCR method. It should be noted that the determination of receptor levels by mRNA levels (i.e. through qPCR) may not always be accurate due to post transcriptional regulation mechanisms. In this regard, one of the objectives of the study was to conduct comparative analysis of IGH1 results from tumor samples with mRNA levels obtained by qPCR. Mathematical analysis was performed to determine the percentage of agreement between IHC and qPCR.

For each tumor sample threshold is selected : an ER (IHC) level greater than 3 is denoted as “1”, ER (IHC) less than 3, as “0”. An ERα (PCR) level > 1 is denoted as “1”, ERα (PCR) <1, as “0”. Then there are two vectors V and W with components 0 or 1. Percentage match is calculated by the formula:

$$n \sum_{i=1}^n C(V_i, W_i) \times 100$$

| Phenotype of the tumor | mRNA level | |
|------------------------|--------------------|--------------------|
| | After NAC | Without NAC |
| ERα+ | 3,16 ± 1,22 (n=31) | 4,8 ± 1,41 (n=49) |
| ERα- | 0,9 ± 0,47 (n=18) | 2,17 ± 0,90 (n=24) |

Mean values ± SD. * p<0.05

Table 11: The expression profile of cytokeratin 18 based on the phenotype of the tumor in CU.

n = 122, where C, a function of two arguments, is defined as follows: $C(V_i W_i) = 1$, if $V_i = W_i$, and $C(V_i W_i) = 0$, if $V_i \neq W_i$

Thus, we obtained verification data between qPCR and immunohistochemical analysis (Table 9).

From Table 9, we see that the results between IHC and qPCR match in 85.9% of all cases. The discrepancy between the results in the remaining 14.1% of cases may be due to a lack of specificity in the IHC method, which determines the total content of all types of estrogen receptors (ERα and ERβ). However, in most cases, qPCR was shown to match IHC. These results also speak in favor of the transcriptional machinery, which means that an increase in mRNA largely correlated with an increase in protein levels. A large percentage of mismatch results (38.5%) for the PGR may indicate a more complex mechanism of regulation of the expression of this receptor. However, elucidation of this mechanism is a complex task and not the purpose of our work.

It is important to note that the proposed methodological approach allows us to discriminate between ERα and ERβ, which are difficult to differentiate using immunohistochemical analysis alone. According to the latest data, IHC may miss up to 10% of ERα+ tumors, some of which could be caught by qPCR method [8].

Recent studies have shown that the use of qPCR is more accurate than IHC in determining the expression of steroid receptors in breast cancer cells, allowing for more accurate prognoses and the application of individual chemotherapy [9].

Expression of cytokeratin18

In addition to examining gene expression levels of steroid receptors, we also looked at the expression of cytokeratin 18, a marker of tumor cell proliferation in patients with epithelial cell carcinoma. We found that, in both groups, there is an increase in the expression levels of the mRNA for cytokeratin 18 as the disease progresses. In the first group, mRNA expression increased from 0.41 to 0.59 as breast cancer stage increased from two to four. A similar pattern was observed for the control group: KRT 18 gene expression was increased from 0.60 to 1.14 for the 1st and 2nd stages, respectively (Table 10).

These findings are consistent with the literature, which show that an increase in KRT 18 expression levels is correlated with increasing tumor size and lymph node involvement [4]. Our results also show that the expression levels of the gene KRT 18 are 3.2 times less in the tumor tissues of patients treated with preoperative therapy (reference mRNA level - 0.41), compared with the control group (reference mRNA level - 1,14) (p<0,05). These results can be explained by the fact that the expression of KRT 18 is specific to epithelial cells. As the tumors were exposed to chemotherapy, the primary tumor reached a complete morphological regression with the growth of connective tissue as fibrous component formed. This was correlated with a reduction in the expression of cytokeratins at the tumor site. Thus, KRT 18 can be considered as a surrogate marker for not only good responses to the proposed type of therapy, but also improved long-term results, such as 5-and 10-year survival rates.

We also evaluated how the expression of cytokeratin 18 depends on

the tumor subtypes. We found that, in both groups, patients with ER+ breast tumors had increased levels of expression of cytokeratin (Table 11).

On average, the level of expression of cytokeratin in ER+ tumors was 2.7 times higher in comparison with ER- tumors. This is most certainly due to the fact that estrogens have a stimulatory effect on epithelial growth. Our data are consistent with the current literature that suggest that the luminal type of mammary tumors has increased expression of cytokeratin 18 compared to basal-like tumors [10].

Thus, KRT 18 can be considered as a suitable candidate marker for assessing response to chemotherapy.

Conclusion

In this work, we analyzed the gene expression levels of ERα, ERβ, and PGR in two groups of patients – one treated with neoadjuvant chemotherapy, and the other, untreated. We have shown that expression levels of steroid receptors depend on the treatment method. Decreased expression of ERα in patients receiving neoadjuvant therapy appears to be associated with increased loss of epithelium and increasing fibrosis. In addition, the damaging effect of cytostatic drugs is characterized by the death of some cells and the development of metabolic disorders in others, one manifestation of which, perhaps, was the reduction in the expression of receptors for steroid hormones. Additionally, the study showed increasing levels of KRT 18 expression with increasing cancer stage and a lower level of expression of this marker in the group with NAC, which speaks in favor of the use of this marker for the assessment of therapeutic pathomorphism. We also saw increased expression of KRT 18 in ER+ tumors, which is associated with the stimulating effect estrogen, has on epithelial growth. In this work, we compared two methods used for determining steroid receptor status in breast cancer cells: IHC method, which is currently the standard in clinical practice, and qPCR, which determines the expression of ER and PR with real-time detection. It was the coincidence of the results of these methods in more than 85% of cases that statistically proved the accuracy of qPCR. Such a high correlation between the level of ERα mRNA and protein content speaks in favor for a transcriptional mechanism regulating the activity of the receptor. This conclusion makes it possible to use qPCR to assess the receptor status of breast cancers.

Thus, the data obtained here can be used in the future for introducing new molecular markers of breast cancer into clinical practice, which will significantly improve approaches to the treatment of each individual patient.

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