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CHARACTERIZATION OF NEW METHYLATION MARKERS AND THE EXPRESSION OF GENES INVOLVED IN CARCINOGENESIS IN BREAST CANCER

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SUMMARY

Modern oncology considers the RARβ2 gene as an important link in the pathogenesis of breast cancer and is being investigated as a new potential biomarker for breast cancer, in particular, a prognosis marker, tumor response to therapy, and metastasis. Point mutations of this gene are rare, but hypermethylation is observed in approximately 15% of breast cancer cases.

The aim of this work was to evaluate RARβ2 hypermethylation in breast cancer and explain it from a biological point of view.

KEYWORDS: breast cancer, RARβ2, breast cancer biomarker, hypermethylation.

Topicality

Breast cancer (BC) is by far the most common type of cancer after lung cancer, which occurs in every 4th woman in her life. Each year, according to the WHO, 2,340,000 new cases of this disease are detected and, unfortunately, 650,000 of them die from this disease.[1,3,4] The development of this complex neoplastic process consists of the following stages: initiation and growth, invasion and metastasis, angiogenesis and possible tumor recurrence. Modern oncology considers the RARβ2 gene as an important link in the pathogenesis of breast cancer and is being investigated as a new potential biomarker for breast cancer, in particular, a prognosis marker, tumor response to therapy, and metastasis.[6,7,10] Point mutations of this gene are rare, but hypermethylation is observed in approximately 15% of breast cancer cases [5]. Most often, tumors with hypermethylation of this gene belong to two molecular genetic subtypes of breast cancer: luminal B and a thrice-negative subtype. It is well known that tumors with hypermethylation of this gene are characterized by an aggressive course, early metastasis, low disease-free and overall survival.[8] Breast tumors are known to exhibit significant variability in cell composition at the genetic level. Hypermethylation of RARβ2 is no exception and is not a sustainable value. Hypermethylation of the number of copies of RARβ2 is observed both between the cells of the same tumor and with the progression of the disease.[2,9,11]

The aim of the study: to evaluate RARβ2 hypermethylation in breast cancer and explain it from a biological point of view.

MATERIAL AND METHODS

In this study, the level of RARβ2 expression and hypermethylation was determined in 80 sick women with primary breast cancer, stages T1-4N0-3M0, who were treated at the Department of Oncammammology of the Republican Specialized Scientific and Practical Medical Center of Oncology and Radiology of the Ministry of Health of the Republic of Uzbekistan, aged 23-45 years.

The material for the study was DNA isolated from paraffin blocks. The isolation procedure consisted of several stages: removal of paraffin, treatment with an enzyme, and extraction of genomic DNA. For dewaxing, 480 µl of PBS and 20 µl of a 10% TWEEN 20 solution were added to a test tube with 3-5 paraffin sections 20 µm thick, the tubes were incubated at 90 ° C for 10 min, then centrifuged for 15 min at 10,000 g and placed on ice for 2 min. The paraffin layer and supernatant formed at the top of the tube were removed. The dewaxed tissue was washed with 1 ml of 96% ethanol. After ethanol was removed, 400 µl of 1M sodium solution of isocyanate was added and left in a thermal shaker for 12 hours at 37 ° C. Then the sample was centrifuged for 20 min at 10000 g and the supernatant was removed, the tissue was washed with 400 µl of PBS. After adding 360 µl of ATL buffering buffer and 40 µl of proteinase, they were incubated for 12-16 hours at 55 ° C. Then, 40 µl of Proteinase L was added twice more at intervals of 6–8 hours, and DNA was extracted after complete tissue lysis. We further studied the status of RARβ2 hypermethylation by evaluating it in the promoter region + 36 + 211 including 10 CG pairs. Statistical processing was performed using SPSS Version 13 software.

RESULTS AND DISCUSSION

The methylation status of the promoter regions of the RARβ2 gene was determined in a group of patients with breast cancer and in morphologically normal tissue samples of this organ. When comparing the results, it was found that methylation of the RARβ2 gene significantly differed between the nodal and diffuse forms of breast cancer. The level of methylation of this gene was higher in patients with diffuse forms of breast cancer. In a detailed comparison of these two subgroups, it turned out that tumors from metachron pairs (M2) make the maximum contribution to the difference. We suggested that the differences found may be related to the age composition of the subgroups - the average age in the group of diffuse tumors is 61.6 years, 55.0 years, and in the nodal subgroup - 49.9 years. Indeed, an analysis of the relationship between the level of methylation of RARB2 and age revealed a weak but significant correlation between these two forms of breast cancer. Hypermethylation of RARβ2 was significantly lower in nodular forms of breast cancer than in diffuse forms. The median value is 5.7%, the range is 3.3-34.1%. For diffuse forms of breast cancer, this indicator was 9.0% - a range of 3.6-15.6%, 9.4% - a range of 4.4-30.1% and 12.2% - a range of 3.2-81%, respectively. The frequencies of RARβ2 hypermethylation obtained in this study were lower than, in most published values. Such discrepancies can be explained by various factors: different methods of analysis and the studied areas, different approaches to the determination of hypermethylation and the formation of the studied groups. Of great importance is the choice of a threshold that distinguishes between high and low methylation levels. In our work, a rather strict approach was used for this: samples were considered hypermethylated in which the level of the studied modification exceeded normal hypermethylation by an amount greater than 2 standard deviations. Finally, the detected frequency of epigenetic disorders can be affected by the characteristics of the analyzed groups, i.e. selection of cases with maximum expression of the studied gene, determining the stage of the disease and the inclusion of cell lines.

When determining the abnormal methylation of the promoters of this gene in DNA, the cytosine in the nucleotide sequence changes to 5-methylcytosines, the HpaII restrictase, does not hydrolyze the DNA at recognition sites and the matrix remains intact, which can be seen by agarose gel electrophoresis in the form of fragments corresponding to the promoter regions of the RARβ2 gene.[5]

According to the figure, methylation of the RARβ2 gene is absent in several patients, and in other patients (4-6-8-11 columns) non-hydrolyzed regions containing methylcytosine, fragments of the promoter region of this gene are detected, which allows us to judge the absence or weak effect of the therapy.

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In order to study the effect of the studied modification of the promoter region of the RARβ2 gene on the expression of hormonal receptors in the tumor, we compared the level of methylation of this gene in neoplasms expressing RE and RP. In the analysis of RE (-) and RE (+) groups, differences in the level of hypermethylation could not be identified (the median values and the range of methylation levels are 10.8% and 10.5%, respectively). A detailed analysis showed that methylation of only one position (+209) is weakly associated with RE expression at the protein level. Further study of the associations between methylation of RARβ2 and expression at the protein level, an additional mRNA measurement was performed. Using real-time PCR, mRNA expression was determined in 33 cases of breast cancer. MRNA methylation did not significantly differ in the case of high (n = 17) and low (n = 16) mRNA expression, median and methylation level range: 11.5% (5.1-26.4%) and 11.2% (4.4-16.6%) respectively. Thus, we were not able to identify the relationship between methylation and RE expression at the level of mRNA or protein. One possible reason for the lack of a link between hypermethylation and expression of hormonal receptors is the detection of a low frequency of hypermethylation in 5 out of 120 analyzed tumors. An analysis of the associations between methylation and molecular and clinical parameters revealed a high occurrence of RARβ2 hypermethylation in luminal B and three-negative subtypes of breast cancer. Hypermethylation was detected in 11/20 (55%) luminal B and in none of the basal carcinomas. The fixed connection was characterized by a poor prognosis.

CONCLUSION

Thus, our study indirectly confirmed the results of the RARβ2 methylation study, in which a high methylation level of this gene is associated with the worst prognosis. Among nodal forms, the proportion of cases with low RARB2 methylation is significantly higher than among diffuse ones, which was 16/68 (24%) and 31/52 (60%).

Our data allowed us to identify the features of cfDNA expression and the level of RARβ2 methylation in various histogenetic subtypes of breast cancer. RARβ2 hypermethylation is associated with an unfavorable clinical course of breast cancer, which allows it to be included in the biomarker system for predicting the course and clinical outcome of the disease, as well as to develop a personalization strategy for the treatment of breast cancer. Epigenetic changes in this gene can be a molecular marker of the early malignant transformation of breast cancer even before its appearance and an

indicator of the prognosis of the disease, as well as in monitoring after specialized therapy.

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