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ONCOLOGY

Search of MicroRNAs Regulating the Receptor Status of Breast Cancer *In Silico* and Experimental Confirmation of Their Expression in Tumors

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MicroRNA whose expression depends on the receptor status of breast cancer were selected using bioinformatic analysis. The expression of 9 microRNAs (16, 17, 21, 27, 125, 146, 155, 200a, and 221) was analyzed in 76 samples of breast cancer with various receptor phenotypes. The expression of microRNAs 155, 27, and 200a did not differ in various types of breast cancer. The data on positive correlation between the expression of microRNA-21 and microRNA-221 and negative receptor status of the tumor were confirmed. The expression of the tumor suppressing microRNA-125b decreased in samples of breast cancer expressing HER2 and ER and in triple negative breast cancer, which characterizes it as a universal marker of breast cancer. An increase in the expression of microRNA-16 was shown in samples of breast cancer expressing HER2 and ER. The expression of microRNA-17 decreased in triple negative breast cancer and increased in ER^+, PR^+, and HER^+ types of breast cancer. MicroRNAs 16, 17, 21, 125b, 146b, and 221 can be promising markers for differential diagnostics of various phenotypes of breast cancer.

Key Words: *microRNA; in silico; breast cancer; receptors of estrogen and progesterone; HER2*

The expression of several genes is changed in breast cancer (BC) cells, but the profile of expression depends on the pathological phenotype of the tumor [8,9]. New data on genome impairments, DNA methylation, and profile of microRNA expression are available for BC subtypes [5]. However, the data of transcriptional, metabolic, and proteomic analysis do not allow to reveal specific markers for the prognosis and treatment of the disease [11,13].

MicroRNAs are important regulators of gene expression at the post-transcriptional level and are considered as promising markers for various types of cancer including BC [10]. MicroRNAs accelerating and in some cases decelerating proliferation in cells of triple negative BC (TNBC) that has no specific treatment besides surgery and chemo- and radiotherapy, were identified [4]. At present time, microRNAs are considered as promising targets not only for diagnostics, but also for the therapy of this tumor [14]. In light of this, further search of microRNAs determining the receptor phenotype of BC is of specific interest.

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Here we performed the bioinformatic analysis in order to select microRNAs specific for various subtypes of BC, and confirmed their expression in the tumors.

MATERIALS AND METHODS

Clinical material. The data of immunohistochemical analysis from 76 patients with BC (T1-4NxM0) aging 45-90 years (mean age of 67.7 years) treated at the Thoracic Department of Novosibirsk Regional Oncology Center in 2014-2016 were used for the characterization of the receptor state of the tumors. TNBC was found in 9 patients, other patients had tumors with phenotypes containing various combinations of receptors ER, PR, and HER2. $HER2^+$ tumors were observed in 14 patients. The samples of BC obtained during surgery were used for analysis of microRNA expression; adjacent non-transformed tissue of the mammary gland served as the control.

Bioinformatic methods. Selected for the investigation microRNAs were determined by bioinformatic analysis using PubMed database. The search was performed within the database, and the parts of articles corresponding to the request or its variations were taken. The data were presented as tables describing the relation between request objects and references of the articles showing these relations. A graphic chart with two types of nodes was created to visualize the information. The nodes contained the information about microRNA with changed expression and ER, PR, and HER2 receptors, vector arrows showing the direction of relations between the nodes, and signs "+" and "–" shown up- and down-regulation, respectively. Not only microRNAs significantly correlating to the changes in the expression of ER, PR, and HER2 receptors (*e.g.* microRNA-221, -21, -27, -155, -200, and -146) were considered, but also microRNA, which correlation with the phenotype of BC was shown once or not at all (*e.g.* microRNA-16 and -17).

Biomolecular methods. RNA was isolated using RealBest Ekstraktsia 1000 kit. Specific primers forming a 5'-hairpin loop were used for reverse transcription. Real-time PCR was performed on a CFX96 amplifier (Bio-Rad Laboratories) using TaqMan technology and Vector-Best reagents according to manufacturer recommendations for estimation of microRNA expression. Small nuclear RNA U6 stably expressed in the mammary gland tissue was used as the reference RNA. Oligonucleotide primers to the analyzed genes were selected using Primer-BLAST software. The changes in microRNA content in the sample in comparison with the control were estimated using a formula:

$$2^{-\Delta\Delta Ct},$$

where $\Delta\Delta Ct=(Ct_{microRNA}-Ct_{U6})_{experiment}-(Ct_{microRNA}-Ct_{U6})_{control}$.

Statistical analysis. The data were analyzed using Statistica 8.0 and SPSS Statistics 22 softwares. Mean, error of the mean, and significance of differences between the parameters were calculated using Mann—Whitney test; χ^2 test was used for estimation of the differences between the samples. The correlation between the samples was analyzed using Pearson's coefficient.

RESULTS

Bioinformatic analysis of microRNAs potentially regulating ER, RP, and HER2 receptors. The number of confirmed studies of the correlation between the expression of microRNA-21 and ER was 1611 (Table 1). Thus, microRNA-21 attracts the maximal interest of researches, and microRNA-515-1 was least interesting. According to the obtained results and analysis of published reports, we selected microRNAs with highest positions in the list of microRNAs evaluated by *in silico* method (microRNA-16, 17, 21, 27, 125b, 146b, 155, 200a, and 221) for further investigation of their expression in tumor tissue and correlation of the expression with the expression steroid hormones.

Analysis of microRNA expression in various types of BC. No correlation to receptor status of BC was observed for microRNA-155 and microRNA-200a (Table 2). Despite enhanced expression of microRNA-27, no significant correlation with the expression of ER and HER2 was observed, even though slight correlation with PR expression was observed. A positive correlation between the hyperexpression of microRNA-221 and negative receptor status of the tumor observed in our study corresponded with previous data [15]. However, the expression of this microRNA was reduced in the tumors expressing PR, ER, and HER2. Changes in the expression of microRNA-21 were found in all study samples of BC, but an increase in the expression by 3-5 times was observed only in TNBC. These data are confirmed by the negative correlation of the expression of this microRNA with study receptors. Intensification of expression of this microRNA in the cells of BC including TNBC was shown by several researches, and this increase correlated with the negative prognosis of TNBC [2,6].

Moreover, a significant reduction in the expression of microRNA-125b in study samples of BC, in some cases to the zero level, was observed. A decrease in the expression of this microRNA was associated with the pronounced expression of HER2 and ER. These results confirm the tumor suppressing effects of microRNA-125b, which was more pronounced in the subtypes of BC expressing ER and HER2, and in TNBC. Tumor suppressing properties of microRNA-125b was shown

TABLE 1. MicroRNAs Potentially Regulating the Expression of Receptors in the Tumors of BC

MicroRNA	Association with ER expression	MicroRNA	Association with ER expression	MicroRNA	Association with ER expression
miR-21	1611	miR-21	1611	miR-21	1611
miR-155	1348	miR-155	1348	miR-125B1	913
miR-145	936	miR-145	936	miR-16-1	769
miR-125b1	913	miR-29a	930	miR-133a1	738
miR-27a	866	miR-125b1	913	miR-221	658
miR-17	821	miR-16-1	769	miR-15a	557
miR-221	658	miR-181a1	744	miR-199a1	551
miR-199a1	551	miR-26a1	622	miR-222	524
miR-222	524	miR-23a	569	miR-214	498
miR-22	473	miR-96	519	miR-200c	480
miR-206	446	miR-126	491	miR-137	479
miR-106b	317	miR-206	446	miR-125a	475
miR-140	278	miR-375	345	miR-22	473
miR-18a	259	miR-200a	334	miR-206	446
miR-34b	245	miR-18a	259	miR-7-1	436
miR-193b	131	miR-219-1	230	miR-205	431
miR-515-1	17	miR-548c	143	miR-135a1	371
		miR-193b	131	miR-23b	315
		miR-548d1	97	miR-27b	311
		miR-423	47	miR-93	290
		miR-561	24	miR-15b	274
				miR-378a	243
				miR-134	168
				miR-139	163
				miR-211	161
				miR-342	119
				miR-193a	107
				miR-548d1	97
				miR-199b	90
				miR-331	60
				miR-28	58
				miR-608	29
				miR-498	23

Note. The first column contains microRNA number, the second column contains the number of interactions between this microRNA and receptor expression in accordance to the data of PubMed database.

in the tissues and cells of the BC [12]. Probably, this microRNA serves as a universal marker of BC independently on the receptor status of the tumor.

The expression of microRNA-146b in tumor tissue was reduced by 2-3 times compared to the control. A significant correlation between this decrease and the expression of ER and HER2 was found. The expression of microRNA-146b was shown to be associated with TNBC [3], but we have not observed this correlation. However, further investigations of the role of

TABLE 2. Correlation between the Expression of microRNA-16, 17, 21, 27, 125, 146, 155, 200a, and 221 with Receptor Status of BC

microRNA	Changes in the expression in the cells of BC	Correlation with PR^+ BC	Correlation with ER^+ BC	Correlation with $HER2^+$ BC	Correlation with TNBC
miR-155	—	—	—	—	—
miR-200a	—	—	—	—	—
miR-27	Significant increase by 2-3 times compared to the control	*r*=-0.53	—	—	—
miR-221	Significant increase by 2-3 times compared to the control in TNBC cells	*r*=-0.88	*r*=-0.67	*r*=-0.87	*r*=0.93
miR-21	Significant increase by 3-5 times compared to the control in TNBC cells	*r*=-0.72	*r*=-0.89	*r*=-0.90	*r*=0.76
miR-125b	Significant increase by 2-3 times compared to the control	—	*r*=-0.55	*r*=-0.87	*r*=-0.74
miR-146b	Significant increase by 2-3 times compared to the control	—	*r*=0.88	*r*=0.76	—
miR-16	Significant increase by 2-5 times compared to the control	—	*r*=0.89	*r*=0.91	—
miR-17	Significant increase by 2 times compared to the control	*r*=-0.66	*r*=-0.59	*r*=-0.78	*r*=0.93

Note. "—", not found.

this microRNA in the maintenance of receptor state of BC are needed. We have also studied microRNA-16, which serves as a part of cluster of miR15/16 genes and inhibits an anti-apoptotic protein BCL2 at post-transcriptional level [1]. A significant increase in the expression level of microRNA-16 was shown in the cells of tumor tissue of $HER2^+$ and ER^+ BC. We also revealed a significant reverse correlation between a decrease in the expression of microRNA-17 and an increase in the expression of ER, PR, and HER2 receptors. However, a positive correlation between a reduction in microRNA-17 expression and absence of receptor expression was observed in TNBC. Together with the literature data, our results reflect a possibility of using of data on microRNA expression for the estimation of receptor status of BC [7].

Thus, microRNA, which expression is changed dependently on the receptor status of BC, have been selected using bioinformatic analysis. Their expression is experimentally shown in tumors of the mammary gland. The correlation is not observed for some of these mRNAs (microRNA-155, 27, and 200a). An association between an increase in the expression of microRNA-21 and microRNA-221 is observed in TNBC, which shows these molecules as promising markers of this type of BC. The observed reduction in the expression of tumor suppressing microRNA-125b in the samples of BC expressing HER2 and ER and in TNBC confirm the versatility of this molecule as a marker of BC. An increase in the expression of microRNA-16 in the samples of BC expressing HER2 and ER reflect the possibility of using it for phenotyping of this type of tumors. We have shown a decrease in the expression of microRNA-17 in TNBC and a negative correlation with ER^+, PR^+, and HER^+ types of BC. A significant reduction in the expression of microRNA-146b in ER^+ and HER^+ types of BC might indicate the tumor suppressing effects of this molecule in tumors with these phenotypes. On the contrary, an increase in the expression of microRNA-16 in ER^+ and HER^+ types of BC reflect the oncogenic properties of this molecule in these phenotypes. Both microRNAs might serve as potential markers for ER^+ and HER^+ BC. Therefore, microRNAs-16, 17, 21, 125b, 146b, and 221 might be of specific interest as markers for differential diagnostics of BC.

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