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Novel miRNA Genes Hypermethylated in Breast Cancer

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Abstract—MicroRNAs play an important role in the regulation of expression of many genes involved in cancer pathogenesis. One of the causes of miRNA level deregulation in tumors is the methylation of CpG islands in the promoter regions of the genes that encode them. Hypermethylation may lead to the suppression of miRNA gene expression and, as a consequence, to a decrease in their inhibitory effect on target gene mRNAs. A search for new miRNA genes hypermethylated in breast cancer has been carried out in the present study. The methylation of five miRNA genes associated with breast cancer (miR-132, miR-1258, miR-107, miR-130b, miR-137) has been as studied using a representative set of 41 breast cancer samples by methylation-specific PCR. Three new genes, *MIR-132, MIR-137* and *MIR-1258*, with a high frequency of hypermethylation (41, 37 and 34%, respectively) have been identified in breast cancer. The methylation of these genes in the breast tissues of ten donors without cancer pathology in anamnesis was only found in single cases. These results enable the involvement of three miRNAs (miR-132, miR-137, miR-1258) and the methylation of the genes that encode them in the pathogenesis of breast cancer to be suggested.

Keywords: hypermethylation, miRNA genes, breast cancer
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INTRODUCTION

Breast cancer (BC) is the most common type of cancer in women, which amounts to 10% of all malignant tumors [1]. Every year, 1.6 million cases of BC are registered in the world; in 2013, more than 50000 new cases of this disease were identified in Russia [2]. Breast cancer is considered to be the main cause of death among women from cancer [1]. The selective hypermethylation of CpG islands of promoter regions of protein coding genes that possess the properties of a tumor suppressor and the suppression of the expression of these genes are observed in cancer [3, 4]. The methylation of CpG islands overlapping promoter regions is also involved in the regulation of microRNA gene expression. It was shown that microRNA genes undergo methylation five to ten times more frequently than protein-coding genes [5]. Methylation may reduce the ability to inhibit target genes by suppressing microRNA gene expression and, thus, significantly affect the regulation of signaling pathways and processes involved in tumorigenesis.

The search for new miRNA genes hypermethylated in BC was carried out in the present study. For this purpose, the methylation frequency of CpG islands of five microRNA genes, *MIR-132*, *MIR-1258*, *MIR-137*, *MIR-107*, and *MIR-130b*, was determined in BC. Previously, it was shown that miRNAs encoded by these genes are involved in the pathogenesis of epithelial tumors of different localizations; in particular, the data on their involvement in invasion, epithelial-mesenchymal transition, metastasis of BC were obtained [6–10].

EXPERIMENTAL

BC samples were collected and clinically characterized in the Research Institute of Clinical Oncology, Russian Cancer Research Center, according to [11, 12]. The samples of malignant tumors from patients who did not receive radiotherapy or chemotherapy before the surgery were analyzed. All breast tumors were classified according to the TNM classification of the Union for International Cancer Control (UICC, 6th ed., 2002) [13] and histologically verified based on the criteria of the World Health Organization's classification of tumors [14]. Additional histological analysis of microsections (3–5 μm) stained with hematoxylin and eosin was performed in order to select samples with a high content of tumor cells (at least 70%). Samples of histologically intact tissue were cut at a distance of at least 2 cm from the tumor; it was histologically confirmed that they are composed of normal breast epithelial cells. Tissue samples were stored at –70°C.

Table 1. Primers, conditions, and length of MSP products

Gene	Primer (5'–3')*	T_a, °C**	Product length, bp	Reference
MIR-107	MF: TGTGTAGTAGTTCGTTTATAGC MR: GACTCTACGACTACTAAATCG	50	220	[17]
	UF: TGTGTAGTAGTTTGTTTATAGTG UR: CCAACTCTACAACTACTAAATC	52	220	
MIR-130b	MF: AAAGATGGAGTCGGTAGGC MR: AAACGCGAAAAATTAAACGA	56	109	[18]
	UF: GTTAAAGATGGAGTTGGTAGGT UR: AAACACAAAAAATTAAACAAAAA	54	112	
MIR-132	MF: GCGTCGGCGTCGTTCG MR: CGCCCCCGCCTCCTTCTA	58	168	***
	UF: GTGTGTGTGTTGTTTG UR: ACCCCCACCTCCTTCTAC	58	141	
MIR-137	MF: TTTTGATTTTTTTCGGTGAC MR: TACCGCTAATACTCTCCTCG	54	98	[19]
	UF: TTTTTTGATTTTTTTTGGTGAT UR: CTACCACTAATACTCTCCTCAA	52	98	
MIR-1258	MF: AGGTCGTGGAAGTTATAGGC MR: CGAACCTACACCTAAACGC	57	126	[20]
	UF: ATTAGGTTGTGGAAGTTATAGGT UR: AACAAACCTACACCTAAACACA	56	126	

* Primer: M, methylated specific to methylated allele; U, unmethylated specific to unmethylated allele.
** MSP was performed in 20 μL of reaction mixture containing 67 mM Tris-HCl, pH 8.8, 16.7 mM $(NH_4)_2SO_4$, 0.01% Tween-20; 1.5–2.5 mM $MgCl_2$, 0.25 mM each dNTP; 10–20 ng DNA; 25 pmol each primer; 0.5 U Hot Start Taq DNA Polymerase (SibEnzyme, Russia).
*** Primers were chosen by the authors.

Paired samples of tumors and histologically intact breast tissues from 41 patients with BC were analyzed, including 29 samples of infiltrating ductal carcinoma and 12 samples of infiltrating lobular carcinoma. Ten samples of breast tissue from women without cancer in anamnesis were used as an additional control. High molecular weight DNA was isolated from the tissue by the standard procedure [11, 12].

The work was carried out in compliance with the principles of voluntariness and confidentiality in accordance with the Fundamentals of Health Protection in the Russian Federation; the resolution of the Ethics Committee of Blokhin Russian Cancer Research Center and the informed consent of patients were obtained.

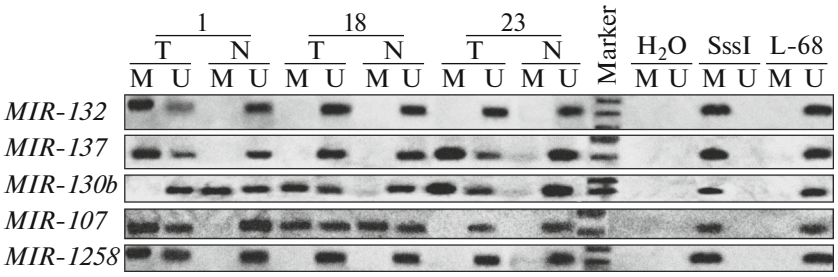
Bisulfite conversion of DNA and methylation-specific PCR (MSP) were performed by the method [15] in the modification of the authors [16] using 0.5–2 μg of DNA. DNA modified by bisulfite was stored at –20°C and used as a matrix during MSP. Primers, annealing temperature (T_a), and the length of MSP products of miRNA genes are shown in Table 1. PCR was performed according to the following program: 95°C for 2 min; 35 cycles of 92°C for 10 s, T_a (Table 1) for 25 s, and 72°C for 25 s; and 72°C for 3 min on a thermocycler T-100 (Bio-Rad, United States). The absence of the MSP product on unconverted DNA was checked using each primer pair. DNA extracted from the cell line L-68 was used as a control for unmethylated alleles. DNA from the cell line L-68, treated with methyltransferase SssI (SibEnzyme, Russia), was used as a positive control of 100% methylation. MSP products were separated by electrophoresis on a 2% agarose gel or 10% polyacrylamide gel. The methylation of samples in which the signal was detected at the level of the marker band with a mass of 7 ng/mL was considered in three independent experiments.

Statistical analysis of the data was performed using the Fisher's exact test; differences were considered significant at $p \le 0.05$.

RESULTS

Methylation Profile of Five microRNA Genes in Breast Cancer

The results of MSP for five microRNA genes (*MIR-132, MIR-1258, MIR-137, MIR-107,* and *MIR-130b*) in representative paired (tumor/normal) DNA samples from breast tissues of BC patients are given in the figure. MSP products with primers



Representative examples of MSP of miRNA genes in paired (tumor/conditionally normal, T/N) DNA samples from BC patients. M (methylated) is the MSP product obtained using primers specific to methylated allele, U (unmethylated) is the MSP product obtained using primers specific to unmethylated allele (Table 1). L-68 are MCP products of DNA from the L-68 cell line; SssI are MSP products of DNA from L-68 cell line treated with methyltransferase SssI (SibEnzyme); H2O is the negative control, MSP products obtained in the absence of DNA sample. MSP products were separated in 2% agarose gel.

towards methylated alleles were identified in a number of tumor samples and in several samples of histologically intact breast tissues from the same patients. In all samples of tumor and normal tissues, MSP products with primers towards unmethylated alleles were detected. At the same time, the MSP products with primers towards methylated alleles in the control DNA of the cell line L-68 and with primers towards unmethylated alleles in DNA of the L-68 cell line artificially methylated by SssI methyltransferase (according to the manufacturer's protocol, SibEnzyme), respectively, were completely absent.

Table 2 summarizes the results of a methylation frequency analysis of these genes using a representative set of paired (tumor/normal) breast tissue samples from 41 patients with BC. A significant (more than two- to threefold) increase in the methylation frequency of three genes in tumor samples compared with histologically normal tissues was found: *MIR-132* (41 vs 12%), *MIR-1258* (34 vs 12%) and *MIR-137* (37 vs 15%). The differences between the data for tumor samples and conditionally normal tissues were statistically significant ($p \le 0.05$ according to Fisher). These results suggest the association of the methylation of these genes with BC pathogenesis.

The differences in the methylation frequency of the genes *MIR-107* and *MIR-130b* were less pronounced (20% vs. 12 and 46 vs. 32%, respectively, see Table 2), which indicates the insignificant contribution of the methylation of these genes to BC pathogenesis.

The methylation of all five genes in donors without cancer in anamnesis was observed in single cases (zero to two out of ten samples, see Table 2).

Association of Hypermethylation with Clinical Characteristics of BC Patients

The data on the methylation of five miRNA genes were compared with the clinical and histological characteristics of 41 patients with BC. On the level of a trend, the higher hypermethylation frequency of these genes was observed in infiltrating ductal BC compared with infiltrating lobular BC: *MIR-132* (48%, 14/29 vs 25%, 3/12), *MIR-1258* (41%, 12/29 to 17%, 2/12) and *MIR-137* (45%, 13/29 vs 17%, 2/12). Moreover, the increased hypermethylation frequency of the *MIR-107* gene was found in poorly differentiated tumors compared with moderately and well differentiated tumors: 63%, 5/8 vs 9%, 3/33, $p = 0.003$. Preliminary data on the higher hypermethylation frequency of the *MIR-1258* gene (2.5 times) in the presence of metastasis in regional lymph nodes or distant organs were also obtained (48%, 10/21 vs 20%, 4/20).

Table 2. Methylation frequency of five microRNA genes in DNA samples of tumor and histologically intact breast tissue of BC patients and healthy donors

DNA sample	*MIR-107*	*MIR-130b*	*MIR-132*	*MIR-137*	*MIR-1258*
BC	8/41, 20%	19/41, 46%	17/41, 41%	15/41, 37%	14/41, 34%
Histologically intact breast tissue from BC patients	5/41, 12%	13/41, 32%	5/41, 12%	6/41, 15%	5/41, 12%
Breast tissue from healthy donors	0/10, 0%	2/10, 20%	0/10, 0%	0/10, 0%	1/10, 10%
p	>0.05	>0.05	***0.005***	***0.042***	***0.035***

DISCUSSION

Thus, the methylation of promoter regions of five miRNA genes (*MIR-132*, *MIR-1258*, *MIR-137*, *MIR-107*, and *MIR-130b*) was studied in a representative set of primary BC samples. Previously, the data on the involvement of each of these genes in pathogenesis of epithelial tumors of different localizations were published. Thus, the epigenetic inactivation of the *MIR-107* and *MIR-130b* genes was observed in pancreatic and ovarian cancer [17, 18]. We did not find a significant increase in the methylation frequency of these genes in breast cancer; however, the hypermethylation of the other three genes (*MIR-132*, *MIR-1258*, and *MIR-137*) was revealed with a frequency of 34–41%, which was not previously known.

miR-132 microRNA is encoded by a gene localized at the region 17p13.3. It is known that the methylation of the *MIR-132* gene is associated with pathogenesis of prostate and pancreatic cancer [21, 22]. At the same time, methylation results in the suppression of *MIR-132* gene expression. In addition, the data on the involvement of miR-132 in the regulation of Akt signaling pathway, as well as on the participation of this microRNA in control of cell adhesion and suppression of metastasis in these cancers were obtained [21, 22]. We found the hypermethylation of the *MIR-132* gene in primary BC, which suggests the involvement of this microRNA in the pathogenesis of this disease. It should be noted that the methylation frequency of the *MIR-132* gene in prostate cancer (42%), as determined in [22], is close to the value that was observed in BC (41%).

miR-137 MicroRNA (1p21.3) is an important factor of differentiation and proliferation, which act as a regulator of specialization pathways of stem cells. Reduced *MIR-137* expression was detected in tumors of different localizations; the role of methylation in the inactivation of this gene was determined [19, 23]. For example, reduced expression of *MIR-137* was revealed in non-small-cell lung cancer, the role of methylation in the suppression of the activity of this gene in cell lines was shown using a demethylating agent [24, 25]. Using the method of bisulfite conversion followed by MSP, we found that the hypermethylation frequency of the *MIR-137* gene promoter region in BC is 37%. We know the only report in which an increase in methylation frequency of the *MIR-137* gene in BC was demonstrated by DNA hybridization on chips [26].

MIR-1258 (2q31.3) methylation during tumorigenesis was detected in lung cancer [20] and in the present work in BC (37% of cases). We know of no other publications that report on the hypermethylation of this microRNA gene. It should be noted that the identification of the association between the hypermethylation of the *MIR-1258* gene and metastasis corresponds to the results obtained in lung cancer [20]. Previously, a decrease in the expression level of *MIR-1258* was found in tumors of several localizations [27]. A comparison of our results and the published data shows that the hypermethylation of the *MIR-1258* gene can be considered to be a mechanism for the suppression of the expression of this gene in tumors.

It is also shown that miR-1258 acts as a negative regulator of heparanase gene expression in tumor cells of breast and lung cancer [27, 28]. High level of heparanase was found in tumors of different localizations, and its increase was observed in metastasis. Our preliminary results on the association of hypermethylation of the *MIR-1258* gene with metastasis are consistent with the data on the decreased level of miR-1258 in metastasis and increased expression of the heparanase gene involved in the regulation of growth and angiogenesis [27, 28]. In the light of these data one can suggest that heparanase gene activation and metastasis, particularly in BC, may be mediated by *MIR-1258* hypermethylation.

Epigenetic markers for diagnosis and prognosis of the course of cancer attract more and more attention. We identified three new microRNA genes (*MIR-132*, *MIR-1258*, and *MIR-137*), subjected to hypermethylation in BC. It was shown that hypermethylation frequency of these genes is associated with clinical characteristics of BC, such as histological type, the degree of differentiation of tumor cells, and metastasis. The obtained results can be used for the development of a molecular diagnosis system of BC based on DNA methylation.

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