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GENETICS

Changes in the Metastatic Properties of MDA-MB-231 Cells after *IGFBP6* Gene Knockdown Is Associated with Increased Expression of miRNA Genes Controlling *INSR*, *IGF1R*, and *CCND1* Genes

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Metastatic cascade is associated with the process of epithelial-mesenchymal transition accompanied by changes in cell proliferation, migration, adhesion, and invasiveness mediated by the insulin-like growth factor (IGF) signal pathway. IGFBP6 protein binds IGF and prevents its interaction with receptors. *IGFBP6* gene knockdown through RNA-interference inhibits cell migration and increased the rate of proliferation of breast cancer MDA-MB-231 cells. *IGFBP6* knockdown cells are characterized by increased expression of *MIR100* and *MIRLET7A2* genes encoding hsa-miR-100-3p, hsa-miR-100-5p, hsa-let-7a-5p, and hsa-let-7a-2-3p miRNA. The target genes of these microRNAs are *IGF2*, *IGF1R*, *INSR,* and *CCND1* associated with IGF signaling pathway and proliferative and migratory activity during the metastatic cascade. A significant decrease in the expression of *INSR* and *CCND1* genes was demonstrated by PCR and microarray analysis.

Key Words: *IGFBP6; MDA-MB-231; breast cancer; metastasis cascade; epithelial-mesenchymal transition*

In accordance with the theory of metastatic cascade, tumor cells undergo a series of transformations that can be divided into a few stages, such as proliferation, adhesion, migration, invasion, escape from the immune response, extravasation, and others. Metastatic cascade is related to two opposite processes — epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET). EMT is associated with a decrease in proliferative activity and an increase in mobility and invasiveness of tumor cells, which is important for initial stages of cell migration from the primary tumor node. During the formation of secondary foci, the cells often undergo MET associate with an increase in division rate and decrease in migration activity [1].

An important role in the regulation of the phenotype and properties of tumor cells is played by insulin-like growth factor (IGF) signal cascade. Among proteins regulating this signal pathway, of specific interest are IGF-binding proteins (IGFBP) preventing IGF interaction with their receptors such as insulin receptor (INSR) and IGF-1 receptor (IGF1R). Moreover, IGFBP can affect the phenotype of tumor cells via dif-

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ferent IGF-independent mechanisms [10]. It was found that suppression of *IGFBP6* gene expression enhanced proliferative activity and inhibited migration activity of triple negative breast cancer (BC) MDA-MB-231 cells. The analysis of the expression of various EMT- and MET-related transcription factors showed that neither of these processes predominated [11]. These data indicate that other mechanisms can be involved into the described phenotypic changes.

Metastatic cascade is regulated by a variety of internal and external factors affecting the tumor cells. MicroRNA molecules, short non-coding RNA regulating gene expression at the posttranscriptional level via interaction with RNA, can significantly contribute to changes in tumor cell properties. The increase in microRNA level can inhibit the expression of the target genes regulated by this molecule and *vice versa*. These processes can significantly modulate cell phenotype, in particular, they can trigger of EMT or MET, change proliferation rate and cell mobility mostly due to the regulation of various signal pathways [6].

Our aim was analysis of the expression of microRNA genes and their target genes under conditions of *IGFBP6* gene knockdown in MDA-MB-231 cells by RNA-interference.

MATERIALS AND METHODS

MDA-MB-231 cells were cultured in standard flasks (25 cm^2; TPP) in complete culture medium containing DMEM, 4.5 g/liter glucose (Gibco), 10% fetal calf serum (Gibco), 2 mM sterile L-glutamine for culture media (PanEco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The medium was changed every 2-3 days.

To suppress *IGFBP6* gene by RNA-interference, the cells were transduced with shRNA molecules (sense 5'-GATCCGCCCAATTGTGACCATCGATTCAAGAGATCGATGGTCACAATTGGGCTTTTTTACGCGTG-3'; antisense 5'-AATTCACGCGTAAAAAAGCCCAATTGTGACCATCGATCTCTTGAATCGATGGTCACAATTGGGCG-3'), which were inserted into a lentiviral vector pLVX shRNA1 (Clonthech Laboratories) as described previously [3,14]. Control cells were transduced with the same vector with shRNA to *Photinus pyralis* luciferase gene. The production, transduction, and selection of the lentiviral vector were performed as described previously [8,10,11].

To isolate RNA from the treatment and control line cells, 2.5 ml cell suspension containing 2×10^5 cells/ml was put in wells of a 6-well culture plate (TPP), and incubated for 48 h in a CO_2 incubators at 37°C and 5% CO_2. In 48 h, the cells in each well were washed 3 times with cold PBS (PanEco) and lysed with Qiazol Lysis Reagent (Qiagen) for further RNA isolation using miRNeasy Mini Kit (Qiagen) [13]. The concentration and quality of the isolated RNA were assessed spectrophotometrically using NanoDrop ND-1000spectrophotometer (Thermo Fisher Scientific) and 2100 Bioanalyzer (Agilent Genomics) [4].

To perform genome-wide transcription analysis, 500 ng RNA of *IGFBP6* gene-knockdown MDA-MB-231 cells and control cells was taken (in 3 replicates). Sample preparation and chip hybridization (Human Transcriptome Arrays 2.0 chips, Affymetrix) were conducted in accordance with manufacturer's protocol. The results were processed using Transcriptome Analysis Console 4.0.1.36 by Gene+Exon-SST-RMA summarization method (analysis version No. 2, ebayes statistical method); *p* level was adjusted for multiple comparison using the Benjamini—Hochberg corrections and without it.

Reverse transcription reaction was performed using MMLV RT kit (Eurogen) according to manufacturer's protocol. In each reaction, 500 ng RNA sample and Random 10 primers were used. The reaction was preformed at 42°C for 60 min and at 70°C for 10 min.

Real-time PCR was performed using the qPCRmix-HS SYBR kit (Eurogen) in a final volume of reaction mixture 25 μl containing 1 μl cDNA obtained after reverse transcription and 5-fold diluted and 250 nM forward and reverse primers. The amplification was performed using a DT-96 detecting amplifier (DNA-Technology) by the following program: 94°C for 10 min, 40 amplification cycles (20 sec at 94°C, 10 sec at 64°C, and 15 sec at 72°C), and registration of melting curve of the PCR product (heating from 56 to 96°C over 20 min). Three replicates of each sample were analyzed. The differences in gene expression were estimated using the ΔΔCt method. The expression of *IGF1R*, *INSR*, and *CCND1* genes was analyzed. *ACTB*, *EEF1A1*, and *HUWE1* genes were used as the reference genes. Primer sequences are presented in the Table 1.

RESULTS

IGFBP6 gene suppression in MDA-MB-231 cells by RNA interference was followed by an increase in the expression of microRNA gene *MIR100* by 3.2 times and *MIRLET7A2* gene by 2.5 times (Table 2). It should be noted that these genes are located in one cluster and are coded in one host-gene *MIR100HG*, while expression was not altered by *IGFBP6* suppression. This gene also contains *MIR125B1* gene, which expression did not significantly change, and *BLID* gene, which expression tended to increase by 2 times (p=0.09 with multiple comparison correction and p=0.016 without correction).

During maturation of the transcription product of *MIR100* gene, hsa-miR-100-5p (previous name hsa-

TABLE 1. Primers for PCR

Gene	Forward primer	Reverse primer
IGF1R	GTGATCCACGACGGCGAGTG	TCCTCACAGACCTTCGGGCAA
INSR	TCTTGCTGAGATGGGAGCCGT	GAACCACACGCATCCTGCCC
CCND1	CCTACTACCGCCTCACACGCT	TGACTCCAGCAGGGCTTCGAT
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAACGCA
EEF1A1	CCCTAAAAGCCAAAATGGGAAA	TAGTGGTGGACTTGCCCGAAT
HUWE1	GCCTGACCTGAGTGGGTTAGTG	CACACTGCTCCAACAGCTTCC

miR-100) and hsa-miR-100-3p (previous name hsa-miR-100*) microRNA appear. During maturation of the transcription product of *MIRLET7A2* gene, hsa-let-7a-5p (previous name hsa-let-7a) and hsa-let-7a-2-3p (previous name hsa-let-7a-2*) microRNA appear. The increase in the expression of *MIR100* and *MIRLET7A2* genes can reflect the increase in the level of mature microRNAs coded in these genes.

The analysis of the target genes of these 4 microRNAs using the DIANA TarBase v8 [7] and miRTarBase 7.0 [2] databases showed that *IGF1R* gene serves as the target of hsa-miR-100-5p, hsa-let-7a-5p, and hsa-let-7a-2-3p; hsa-miR-100-5p and hsa-let-7a-5p regulate the expression of *IGF2* gene; and hsa-let-7a-5p microRNA can also regulate the expression of *CCND1* and *INSR* genes. It is known that IGFBP6 protein can bind IGF2 and prevent the interactions of this protein with other receptors, such as IGF1R and INSR [10]. Suppression of *IGFBP6* gene can trigger an enhanced interaction of IGF2 with its receptors. However, the increase in the expression of the cluster of microRNA genes regulating the expression of *IGF2*, *IGF1R*, and *INSR* can serve as a compensatory mechanism developing in cells in response to introduction of shRNA and IGFBP6 mRNA.

Analysis of the expression of these genes using Affymetrix microchips showed that the expression of *INSR*, *CCND1*, and *IGF1R* genes decreased by 2.2 ($p<0.001$), 2.1 ($p<0.001$), and 1.3 times ($p=0.017$ without correction for multiple comparison, and $p=0.096$ with this correction; Table 2). *IGF2* expression cannot be determined due to cross-detection of *INS* gene mRNA and a readthrough transcript of *INS-IGF2* on the Affymetrix chips. Analysis of the expression of *INSR*, *IGF1R*, and *CCND1* genes by quantitative reverse transcription PCR showed that *IGF1R* expression does not significantly differ in the two types of cells. However, *IGFBP6* knockdown is associated with reduced expression of *INSR* and *CCND1* (by 2.2 times; Fig. 1).

Insignificant changes in *IGF1R* expression can be explained by the fact that partial complementarity of microRNA and target mRNA might not be accompanied by accelerated degradation of mRNA, but only reduced translation of tis transcript [9]. However, the decrease in the expression of *INSR* and *IGF1R* genes can indicate active regulation of these genes, among other things, due to increased microRNA level. INSR and IGF1R receptors are often overexpressed in BC cells *in vitro* and in clinical samples taken from patients; this is associated with enhanced metastasis activity and unfavorable prognosis. The inhibition of these receptors is discussed as a possible way for the therapy of tumors including BC [12]. For cyclin D1 encoded by *CCND1* gene, the relation with metastatic cascade due to the regulation of cell adhesion and invasion was also demonstrated [5].

Thus, suppression of *IGFBP6* gene in BC MDA-MB-231 cells accompanied by changes in the meta-

TABLE 2. Genes of microRNA Significantly Modulated by *IGFBP6* Knockdown and Expression of Their Targets

Gene	$\log_2$ signal in the control	$\log_2$ signal in *IGFBP6* knockdown	Fold increase (+) or fold decrease (-) of expression intensity	*p*
MIRLET7A2	7.5	8.8	+2.5	$p<0.001$
MIR100	6.1	7.8	+3.2	$p=0.049$
IGF1R	4.3	3.9	-1.3	$p=0.096$ ($p=0.017$*)
INSR	6.4	5.2	-2.2	$p<0.001$
CCND1	14.5	13.4	-2.1	$p<0.001$

Note. *Without correction for multiple comparisons.

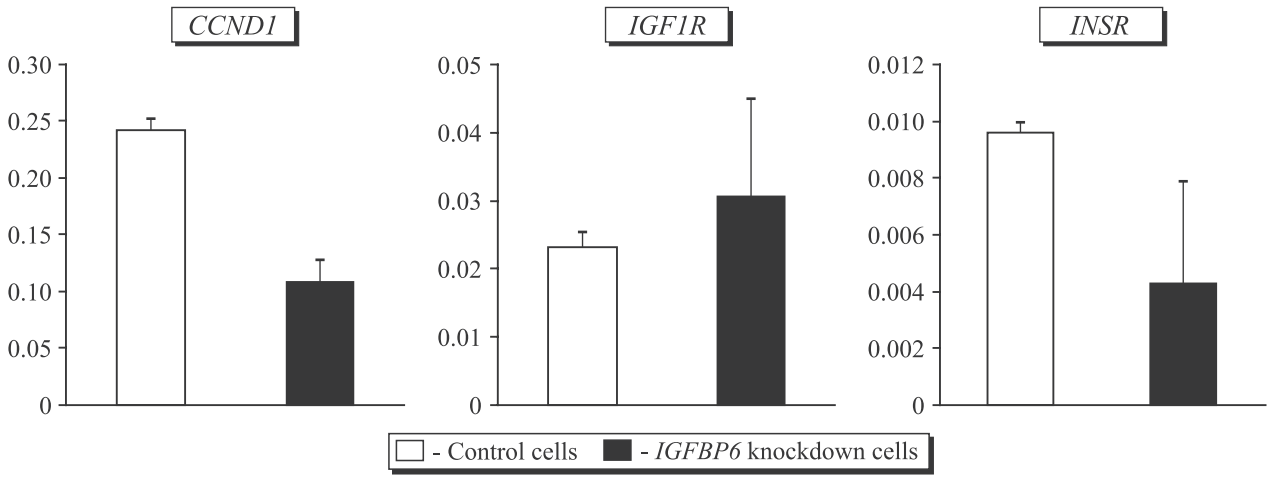

Fig. 1. Expression of *CCND1*, *IGF1R*, and *INSR* genes in MDA-MB-231 cells after introduction of control shRNA and shRNA against *IGFBP6*.

static cascade and balance between EMT and MET processes is associated with enhanced expression of the cluster of microRNA genes *MIR100* and *MIRLET7A2*. These changes can indicate an increase in the content of mature microRNAs encoded by these genes. The genes *IGF2*, *IGF1R*, and *INSR* functionally related to suppressed *IGFBP6* gene and involved in the regulation of metastatic cascade are among the target genes of these mature microRNAs. Suppression of these genes with microRNAs can serve as a intracellular regulatory mechanism triggered as a compensatory response to *IGFBP6* suppression and enhanced stimulation of signal pathways of *INSR* and *IGF1R* receptors.

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