The Influence of microRNAs in Regulation
of Hormone Dependence in Prostate Cancer Cells

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Abstract—A spectrum of differentially expressed microRNAs was determined by the massively parallel sequencing method in normal healthy prostate tissues, in hormone-dependent prostate cancer samples, and in the LNCaP and DU145 cell lines. A set of microRNAs in tumors and prostate cancer (PCa) cell lines was identified on the basis of the changes in expression compared with that in normal prostate tissues. Twenty-seven aberrantly expressed microRNAs were detected in tumor tissues and ten of them showed significant changes in expression in LNCaP and DU145 cells. Seven of them demonstrated the change of the expression in the same direction in all the tumor samples as well as in the PCa cell lines. The expression of miR-148a changed in DU145 cells in the opposite direction compared with that in LNCaP cells and tumors. The expression of let-7c, let-7b, miR-99a, miR-125b-2, miR-100, miR-10a, and miR-31 was reversed in DU145 cells compared with LNCaP cells. However, these microRNAs exhibited no significant changes in expression in tumors. It turns out that the target of miR-148a, let-7b, and microRNAs, included in the miR-99a/let-7c/miR-125b-2 cluster, the expression of which increased in LNCaP cells and decreased in DU145 cells, is the insulin-like growth factor receptor gene 1 (*IGF1R*). The obtained data make it possible to assume that the differences in the effect of microRNAs in cell lines are connected with their repressive influence on *IGF1R* expression in hormone-sensitive LNCaP cells and an absence of such influence in the hormone-independent DU145 cell line.

Keywords: prostate cancer, microRNA, massively parallel sequencing, androgen receptor, insulin-like growth factor 1 (IGF-1) receptor

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INTRODUCTION

MicroRNAs are short, 19—23 nucleotides in size, RNA molecules, which, associating with Argonaute proteins (AGO1—4), form a RISC complex (RNA-induced silencing complex). The function of the mature microRNA is to recognize the target messenger RNAs by the RISC complex. The interaction of this complex with mRNA leads either to its degradation or to the suppression of translation [1, 2]. According to different estimates, from 30 to 60% of human genes are under the direct control of microRNAs [3]. MicroRNAs play a key role in the regulation of major genetic processes, such as cell division [4], apoptosis [5], DNA repair [6], differentiation [7, 8], and the cell's response to stress [9].

Mutations and, as shown in the last 15—20 years, epigenetic disorders of oncogenes and tumor suppressor genes lead to malignant degeneration of cells of various organs, including the prostate. In developed countries, prostate cancer is the second most common cancer among men. It is well known that normal tissue cells, like prostate tumors, need androgens in the early stages of development [10]. However, during development, a malignant tumor of the prostate often acquires the capability of hormone-independent growth and progression. This limits the possibilities of hormone therapy, which is widely used in the treatment of prostate cancer.

Despite intensive research, the mechanism of the origin of hormone-independent prostate tumors remains unclear, and today the role of microRNAs in this process is not clear either. Earlier, microRNAs were detected the expression of which decreases in cells of hormone-independent prostate cancer lines as compared to cells of hormone-dependent lines. These include the miR families miR-99 (miR-99a-99b-100) [11], miR-148a [12], let-7c [13], and others, such as

† Deceased.

Table 1. Clinical characterization of the PCa tissues

No.	Age (years)	TNM classification			Gleason score		Gleason sum	Androgen receptors (+/−)	PSA, ng/mL
1	61	T2	pN0	M0	3	4	7	+	8.7
2	55	T2a	pNx	M0	3	3	6	+	10.0
3	53	T1c	pNx	M0	3	3	6	+	12.0

miR-34a,c, miR-146a, miR-124, miR-31, miR-200b, and miR-185 [14].

It is known that the IGF1R signaling pathway plays a significant role in the development of prostate cancer. The expression level of the *IGF1R* gene in cells of the hormone-independent DU145 line is significantly higher than that in the cells of the hormone-dependent LNCaP line [15, 16]. However, the role of microRNAs in overexpression of *IGF1R* in DU145 cells remains unclear. This paper is devoted to the analysis of the possible role of microRNA-mediated regulation of IGF1R in changing the dependence of prostate cancer cells on hormones.

MATERIALS AND METHODS

Tissue samples. Samples of hormone-dependent tumors of local prostate cancer (PCa) and normal prostate tissues were provided by the Research Institute of Urology and Nephrology of Rostov State Medical University. Normal prostate tissues used as a control were obtained during radical cystectomy. None of the patients received hormone therapy or chemotherapy before surgery. Tissue samples were treated with stabilizing solution of RNAlater (Ambion, United States) immediately after surgery for 24 h and then crushed and frozen for subsequent isolation of RNA. All tumor samples were morphologically characterized according to the TNM system [17] and the Gleason scale [18] (Table 1). The study was approved by the ethics committee of Rostov State Medical University.

Immunohistochemistry. Immunohistochemical staining was performed for morphological characterization of tissue samples and assessment of the status of androgen receptors. Five 4 μm consecutive sections were obtained from formalin-paraffin-fixed tissue samples and mounted on prepared glass. Immunostaining was performed using the Ventana HX Bench-Mark™ system (Ventana Medical Systems, United States) according to the manufacturer's recommendations. Monoclonal mouse antibodies against the androgen receptor were used in a dilution of 1/50 (clone AR441, DAKO, United States). Diaminobenzidine was used as a chromogenic substrate. Hematoxylin and eosin were used for contrasting staining.

Cell lines. The hormone-dependent prostate cancer LNCaP cells were cultured in RPMI-1460 medium, and the hormone-independent DU145 cells were cultured in EMEM medium. Ten volume percent of fetal bovine serum was added to the basic medium. Cells were cultured at 37°C in the presence of 5% CO_2 in 75 cm^2 flasks (with a time interval between consecutive subcultures of 3 days).

RNA isolation. To isolate total RNA from prostate tissue samples, a fragment of 0.5 cm^3 was crushed in liquid nitrogen and placed in QIAzol lysis buffer (Qiagen, United States). To isolate RNA from cell lines, 2×10^6 cells were used. Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, United States) according to the manufacturer's protocol. The miRNeasy Mini Kit (Qiagen, United States) and the RNeasy MinElute Cleanup Kit (Qiagen, United States) were used to isolate the small RNA fractions. Quantitative determination of total RNA was measured on a Qubit 3.0 Fluorometer (Life Science Technologies, United States). For qualitative analysis of total RNA, electrophoresis was carried out in a denaturing 1% agarose gel with formaldehyde.

Massively parallel sequencing. The microRNA expression was determined using massively parallel sequencing on the Illumina platform. For the evaluation of the first repeat in DU145 and LNCaP, the MiSeq system (Illumina Inc., United States) was used; for the second replication of these cell lines and samples of tumor and normal tissues, the HiSeq system (Illumina Inc., United States) was used. In the first series of experiments, the MiSeq system (Illumina Inc., United States) was used for DU145 and LNCaP and HiSeq (Illumina Inc., United States) was used for the other cases. Creating a cDNA library and massively parallel sequencing were performed according to the method described by us earlier [19].

Statistical analysis. In identifying microRNAs, cDNA sequences were compared with canonical microRNA sequences (miRBase, v. 21) using Bowtie, v. 1.2.0 [20]. In this case, one nucleotide was allowed to be replaced in the cDNA sequences of mature microRNA. When normalizing the expression level of the compared variants, the TMM method implemented in the edgeR computer program was used [21]. MicroRNAs are included in the analysis, the mature forms of which are represented in the analyzed sample by no less than 25 copies in a sample of a continuous cell line or samples of prostate tissue and which

Table 2. Changes in expression of microRNAs in prostate cancer cells

microRNA	Cancer 1			Cancer 2			Cancer 3		
	C1	C2	C3	C1	C2	C3	C1	C2	C3
Expression decrease*									
miR-126-3p	−1.9	−1.2	−2.6	−3.0	−2.5	−4.1	−2.3	−1.8	−3.2
miR-144-5p	−3.7	−3.3	−3.0	−3.0	−2.9	−2.6	−3.4	−3.2	−2.8
miR-150-5p	−2.0	−1.4	−1.6	−4.3	−4.0	−4.3	−2.8	−2.5	−2.6
miR-15b-5p	−1.8	−1.7	−2.2	−1.1	−1.2	−1.8	−1.9	−1.9	−2.4
miR-16-5p	−1.1	−0.7	−1.0	−1.6	−1.5	−2.0	−1.2	−1.0	−1.3
miR-21-5p	−0.8	−0.5	−1.0	−1.8	−1.8	−2.4	−1.5	−1.5	−1.9
miR-223-3p	−3.4	−3.7	−3.3	−3.0	−3.5	−3.2	−2.4	−2.9	−2.4
miR-301a-3p	−1.2	−0.6	−1.1	−1.2	−0.8	−1.5	−1.3	−1.0	−1.4
miR-378a-3p	−1.3	−1.2	−1.2	−1.1	−1.2	−1.4	−1.8	−1.9	−1.9
miR-378a-5p	−1.9	−2.2	−1.2	−4.0	−4.5	−3.7	−3.4	−3.9	−2.9
miR-424-5p	−1.3	−0.9	−2.1	−1.9	−1.7	−3.0	−1.8	−1.6	−2.8
miR-451a	−1.8	−1.9	−3.6	−2.4	−2.8	−4.6	−3.1	−3.5	−5.1
miR-454-3p	−1.0	−0.7	−0.9	−1.1	−1.0	−1.3	−1.0	−0.9	−1.0
miR-486-5p	−4.0	−3.9	−2.8	−2.8	−3.0	−2.0	−4.1	−4.2	−3.1
Expression increase*									
miR-1260a	2.1	1.9	2.6	1.5	1.1	1.6	1.7	1.4	2.0
miR-143-3p	2.6	3.2	5.5	3.7	4.0	6.3	3.1	3.5	5.9
miR-148a-3p	4.6	4.4	3.4	3.7	3.4	2.2	2.0	1.6	0.6
miR-148a-5p	1.8	1.8	3.4	2.5	2.2	3.7	1.4	1.1	2.8
miR-148b-3p	2.7	2.9	1.3	3.0	3.0	1.3	2.2	2.2	0.7
miR-152-3p	0.9	1.0	0.9	2.3	2.2	1.9	1.9	1.7	1.7
miR-196b-5p	2.8	3.5	3.8	3.1	3.5	3.7	3.0	3.5	3.8
miR-21-3p	4.3	7.4	1.0	5.5	8.4	1.8	4.5	7.4	1.0
miR-26b-5p	1.2	1.9	2.0	1.1	1.5	1.5	0.7	1.2	1.4
miR-30a-3p	2.2	2.8	2.4	3.6	3.9	3.5	1.8	2.1	1.8
miR-30c-2-3p	3.5	4.0	3.8	5.3	5.6	5.3	3.8	4.1	3.9
miR-582-3p	5.2	5.2	4.4	5.7	5.4	4.5	3.4	3.1	2.4
miR-99a-3p	2.2	2.7	4.1	3.4	3.7	5.0	2.4	2.7	4.2

* Binary logarithm of the ratio of microRNA expression level in prostate cancer cells to that in normal tissue; C1—control 1, C2—control 2, C3—control 3.

showed a change in expression with a false detection rate (FDR) ≤ 0.05.

RESULTS

Using the method of massively parallel sequencing, the spectrum of differential expression of microRNAs was obtained in cells of three samples of hormone-dependent prostate cancer and three samples of normal prostate tissues, as well as in cells of the LNCaP and DU145 lines in duplicate. The expression of microRNAs was compared in cells of each tumor sample and in LNCaP and DU145 cells with expression of microRNAs in cells of each sample of normal prostate tissues. As a result, nine comparisons were performed for tumors and six comparisons were performed for each of the LNCaP and DU145 lines. The analyzed microRNAs showed a statistically significant (FDR ≤ 0.05) change in expression with each comparison in the same direction. Twenty-seven microRNAs (Table 2) were identified in tumor cells, while 116 were identified in LNCaP cells and 87 were identified in DU145 cells (Fig. 1).

Comparison of microRNAs expressed in the same direction in hormone-dependent tumor cells and the LNCaP showed that expression of only 16 microRNAs changed at the same time. And in seven of them, expression changed in the same direction in DU145
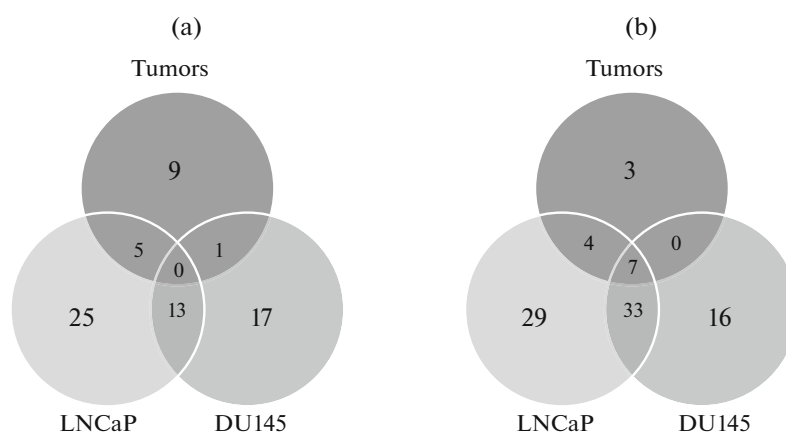
Fig. 1. MicroRNAs expressed in the same direction in the studied lines and prostate cancer. (a) Spectra of microRNAs with expression increased in the same direction in LNCaP and DU145 cells and prostate cancer; (b) spectra of microRNAs with expression reduced in the same direction in LNCaP and DU145 cells and prostate cancer.

cells (Table 3). The expression of these microRNAs reflects changes in metabolism in malignant cells that are not related to their dependence on hormones.

For four of them—miR-150-5p [22, 23], miR-223-3p [24], miR-378a-3p [25], and miR-486-5p [26]—participation in the control of prostate cancer was previously shown. Direct data on the participation in the regulation of prostate carcinogenesis of three other microRNAs included in this group were not found. However, it is known that miR-451a is involved in the control of colorectal cancer and breast cancer [27, 28], miR-126-3p is involved in the control of breast cancer [29] and thyroid cancer [30], and miR-144-5p is involved in the control of bladder cancer [31].

In eight microRNAs of cell lines, the direction of change in expression was reversed when moving from

LNCaP cells to DU145 cells, while the miR-148a expression level was also changed in tumor cells (Table 4). For all five microRNAs that showed an increase in expression in LNCaP cells and, accordingly, its decrease in DU145 cells, the target is the *IGF1R* gene.

DISCUSSION

The key role in the development of prostate cancer is played by the androgen receptor (AR), whose interaction with androgens leads to its activation [32, 33]. Activated AR acquires the ability to move from the cytoplasm to the cell nucleus [34]. Its interaction with AREs (androgen response elements)—canonical DNA sequences in promoter regions of genes—leads to the suppression or activation of their transcriptional

Table 3. Change in expression of microRNAs in the same direction in tumor cells and in both prostate cancer cell lines

microRNA	Cancer**	Cell line											
		LNCaP						DU145					
		experiment I			experiment II			experiment I			experiment II		
		C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
		Change in expression of microRNAs*											
miR-126-3p	↓	−4.6	−4.1	−5.1	−6.2	−5.4	−7.0	−4.2	−3.7	−4.8	−5.9	−5.4	−6.8
miR-144-5p	↓	−8.8	−8.0	−7.0	−10.0	−9.5	−9.1	−8.7	−7.9	−6.9	−10.0	−9.7	−9.2
miR-150-5p	↓	−11.7	−10.7	−10.1	−12.9	−12.2	−12.3	−11.6	−10.6	−10.1	−12.9	−12.4	−12.3
miR-223-3p	↓	−10.8	−10.6	−9.5	−12.1	−12.1	−11.7	−10.8	−10.6	−9.4	−12.1	−12.3	−11.7
miR-378a-3p	↓	−3.4	−3.5	−3.1	−3.3	−3.1	−3.4	−2.4	−2.5	−2.2	−2.2	−2.3	−2.4
miR-451a	↓	−13.7	−13.4	−14.3	−14.9	−14.9	−16.5	−13.6	−13.3	−14.3	−14.9	−15.0	−16.5
miR-486-5p	↓	−2.2	−2.3	−0.8	−2.7	−2.6	−1.7	−4.8	−4.9	−3.5	−5.6	−5.7	−4.6

 * Binary logarithm of the ratio of microRNA expression in cells of the LNCaP and DU145 lines to that in normal tissue.
** The arrow indicates the direction of change in expression of microRNAs in prostate cancer cells. C1—control 1, C2—control 2, C3—control 3.

Table 4. Change in expression of microRNAs in different directions in cancer cells and in both prostate cancer cell lines

microRNA	Cancer**	Cell line											
		LNCaP						DU145					
		experiment I			experiment II			experiment I			experiment II		
		C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
		Change in expression of microRNAs*											
miR-99a-5p	ns	1.2	0.8	6.5	1.6	1.5	6.5	−8.0	−8.4	−2.8	−8.1	−8.5	−3.3
let-7c-5p	ns	2.0	2.0	6.7	2.5	2.7	6.8	−8.9	−8.9	−4.3	−7.4	−7.4	−3.1
miR-125b-2-3p	ns	3.2	3.5	6.6	3.2	3.7	6.3	−8.2	−7.3	ns	−6.6	−6.3	ns
let-7b-5p	ns	2.4	2.3	2.1	2.0	2.1	1.3	−1.1	−1.2	−1.5	−0.7	−0.8	−1.5
miR-148a-3p	↑	3.1	2.8	2.1	1.1	1.1	ns	−0.6	−1.0	−1.7	−3.1	−3.4	−4.5
miR-10a-5p	ns	−2.0	−2.0	−1.2	−2.4	−2.2	−2.0	2.0	2.0	2.7	1.7	1.6	2.0
miR-100-3p	ns	−10.9	−10.0	−8.5	−12.2	−11.5	−10.6	4.3	4.5	5.7	1.0	1.3	2.1
miR-31-5p	ns	−9.2	−8.9	−12.0	−6.2	−6.2	−10.3	5.6	5.3	1.8	4.9	4.6	0.7

 * Binary logarithm of the ratio of microRNA expression in cells of the LNCaP and DU145 lines to that in normal tissue.
** The arrow indicates the direction of change in expression of microRNAs in prostate cancer cells. C1—control 1, C2—control 2, C3—control 3; ns—the differences are not significant.

activity [35]. However, activation of AR and its movement into the cell nucleus can occur in the absence of androgens when the IGF-1 signaling pathway is activated [36]. Over 400 genes are androgen receptor targets [37]. These include the miR-148a gene [38], the direction of expression of which changes to the opposite when moving from LNCaP cells to DU145 (Table 4). The direct targets of this microRNA are DNA methyltransferases DNMT3b [39, 40] and DNMT1 [41], which are responsible for *de novo* methylation and methylation maintenance, respectively (Fig. 2).

In addition, according to miRDB (http://mirdb.org) and TargetScan (http://www.targetscan.org), the *KDM2B* gene encoding lysine-specific demethylase 2B also belongs to the predicted miR-148a targets. KDM2B is part of the polycomb complex 1 (PRC1). Its binding to completely unmethylated DNA of CpG islands leads to lysine monoubiquitinating at position 119 of histone 2A (H2AK119 Ub1) and recruitment to the binding site of polycomb complex 2 (PRC2), of which histone methyltransferase 2 (EZH2) is a component. This leads to trimethylation of lysine in the
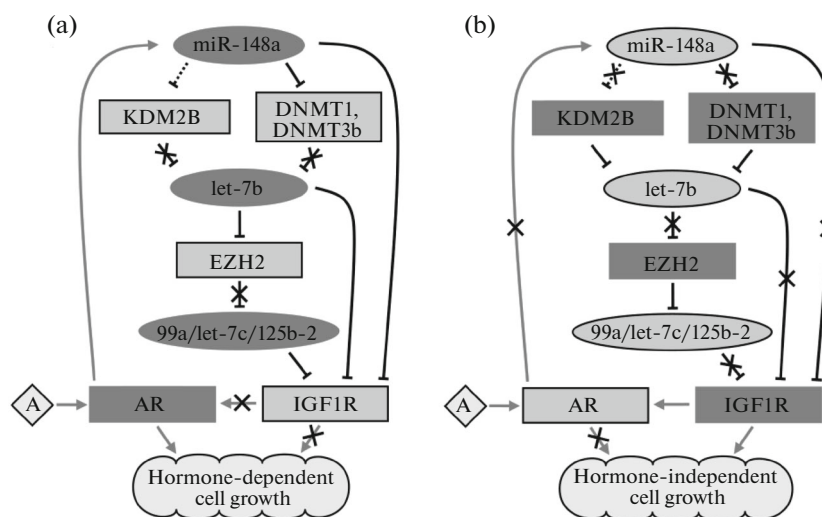

Fig. 2. MicroRNA dependent regulation of IGF1R expression in cells of the LNCaP and DU145 lines. **(a)** Regulation scheme in LNCaP cells; (b) regulation scheme in DU145 cells. (↘) Increased gene expression or protein activation; (⊥) suppression of expression; (✕) absence (weakening) of regulatory action; (☐) increased gene expression; (■) reduced gene expression; (⋮) predicted regulation; (|) verified regulation.

27th position of histone 3 (H3K27me3) and, accordingly, to histone-dependent suppression of the transcriptional activity of genes whose promoters contain CpG islands. In addition, H3K27me3 is a molecular label for *de novo* DNA methylation [42].

It is known that the promoter region of the let-7b microRNA gene, the target of which is histone methyltransferase *EZH2* [43], contains CpG islands and its transcription depends on the level of DNA methylation [44]. Using the example of epigenetic regulation of cell division of mouse embryonic fibroblasts [43] and myeloid cell lines [45], it was shown that activation of KDM2B leads to activation of EZH2 and a decrease in let-7b expression, and, on the contrary, a decrease in expression of KDM2B is accompanied by a decrease in expression of EZH2 and an increase in expression of let-7b. In turn, San et al. [46] showed that EZH2 activity inhibits the activity of the *MIR99AHG* gene. The transcript of this gene contains a miR-99a/let-7c/miR-125b-2 microRNA cluster. The target of microRNAs included in this cluster, as well as miR-148a and let-7b, is the *IGF1R* gene.

For miR-148a, which showed an increase in expression in LNCaP cells, we also observe an increase in expression in hormone-dependent tumor cells. Differences in microRNA expression in LNCaP cells and tumor cells affect let-7c, let-7b, miR-99a, and miR-125b. The nature of these differences remains unclear, but it can be assumed that it is associated with changes in the expression of genes whose proteins are involved in the differential maturation of microRNA, for example, LIN-28 [13, 47]. It has now been shown that IGF1R plays a significant role in the development of hormonal independence of prostate cancer cells [48]. This may, in particular, be associated with androgen-independent activation of androgen receptors [36]. However, Alimir et al. [49] showed that, in the hormone-independent DU145 and PC3 cells, the activity of the *AR* gene is reduced compared with that in hormone-dependent LNCaP cells. In this regard, it is possible that the bypass signaling pathway, dependent on IGF1R, but not on AR, lies in the basis of the division and survival of hormone-independent cells cultured in vitro [50, 51].

Our results suggest that the differences in the effect of microRNAs in the two cell lines studied are mainly related to their repressive effect on the expression of *IGF1R* in cells of the hormone-dependent LNCaP and the lack of this effect in cells of the hormone-independent DU145.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement of compliance with standards of research involving humans as subjects. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants involved in the study.

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