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TUMOR MICROENVIRONMENT-ASSOCIATED miR-7-5p, miR-19a-3p, AND miR-23b-3p EXPRESSION IN PROSTATE CANCER WITH DIFFERENT PROGRESSION RISK

Background. The tumor microenvironment (TME) plays an important role in the occurrence and progression of prostate cancer (PCa). At the same time, the mechanisms and features of the interaction between tumor cells and individual components of the TME in PCa remain not fully elucidated. **The aim** was to study the expression levels of tumor-associated miR-7-5p, miR-19a-3p, and miR-23b-3p in the PCa tissue and to analyze their relationship with the features of TME. **Materials and Methods.** The work is based on the analysis of the results of the examination and treatment of 50 patients with PCa of stages II—IV. The expression of miRNA in the PCa tissue was analyzed by the real-time polymerase chain reaction. The expression of alpha-smooth muscle actin (α-SMA), vimentin (VIM), and CD68 in PCa tissue was determined by the immunohistochemical method. The identification of mast cells in the PCa tissue was assessed by the histochemical method. **Results.** The analysis of the expression levels of tumor-associated miRNAs demonstrated that the tumor tissue of patients with a high risk of PCa progression was characterized by 4.93 ($p < 0.01$) and 8.97 ($p < 0.05$) times higher levels of miR-19a-3p and miR-23b-3p, respectively, compared to similar indicators in the group of patients with a low risk of PCa progression. The levels of miR-7-5p and miR-19a-3p expression in the PCa tissue correlated with the expression level of α-SMA (r = 0.49 and r = 0.45, respectively; $p < 0.05$) and VIM (r = 0.45 and r = 0.46; respectively, $p < 0.05$). A direct relationship (r = 0.44; $p < 0.05$) was established between the level of miR-7-5p expression and the degree of infiltration of the prostate gland tissue by tumor-associated macrophages. **Conclusions.** The features of the expression of tumor-associated miR-7-5p, miR-19a-3p, and miR-23b-3p indicated the prospect of their use as markers of the aggressiveness of the PCa course.

Keywords: prostate cancer, miR-7-5p, miR-19a-3p, miR-23b-3p, risk of progression, tumor microenvironment.

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Research over the past decades has demonstrated the significant role of the tumor microenvironment (TME) in the initiation and progression of neoplasia. The TME is a network of normal untransformed cells, stromal cells, including fibroblasts and immune cells, blood vessels, and extracellular matrix. A complex system of communication between all these components is ensured by the synthesis and release of cytokines, chemokines, growth factors, and other molecular messengers, in particular miRNA [1].

MiRNAs are small non-coding RNAs that regulate gene expression by inducing mRNA degradation and thereby inhibiting protein translation. As pleiotropic modulators of gene expression, miRNAs are involved in various physiological and pathological processes and also play a role in the development of many diseases, including prostate cancer (PCa) [2, 3]. To date, it has been established that more than 50 miRNAs are involved in the initiation, progression, and metastasis of PCa. An important role of miRNAs in the regulation of several key signaling pathways involved in the PCa progression has been shown for the androgen receptor signaling pathway, the PTEN/PI3K/AKT signaling cascade, the p53 signaling pathway, and the Wnt/β-catenin signaling pathway [4]. The importance of miRNA in the processes of apoptosis, tumor growth, metastasis, and sensitivity to drug therapy has been studied and characterized in [5].

Increasing evidence suggests that miRNAs play a key role in TME biology. Providing communication between the TME and the malignantly transformed cells, miRNAs regulate the recruitment of immune cells to the tumor site and their activation, as well as stimulate the secretion of immunosuppressing or immunostimulating factors, as well as participate in the modulation of the antitumor immune response. The expression profile of miRNAs in tumor cells is changed, and these miRNAs are transferred via microvesicles and exosomes to the cells of immune system. As a result, B cells, T cells, natural killers, and M1 macrophages are inhibited while immunosuppressing T-regs, myeloid-derived suppressor cells, and M2 macrophages are stimulated [6—8]. At the same time, the importance of miRNAs in the formation of TME and modulation of the antitumor immune response has only recently become the focus of intensive research and remains to be fully characterized.

Since modern diagnostic and prognostic tools have certain limitations, there is a need to find new markers for therapy stratification taking into account potential genetic and epigenetic differences in neoplasms [9—11]. In this context, high hopes are currently placed on miRNAs, characterized by tissue specificity and high stability both in tumor tissue and in biological fluids. In a previous study, based on bioinformatic analysis, we have identified a panel of miRNAs involved in the formation of reactive TME of PCa and associated with an unfavorable course of the disease [12]. Taking into account the above, the aim of the present work was to study the expression levels of miR-7-5p, miR-19a-3p, and miR-23b-3p in the PCa tissue and to analyze their relationship with the features of TME.

Materials and Methods

The work is based on a retrospective analysis of the results of the examination and treatment of 50 patients with PCa of stages II—IV who were treated in the National Cancer Institute of the Ministry of Health of Ukraine during 2015—2021. The design of the current study was approved by the Institutional Review Board and Research Ethics Committee of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine in accordance with the 1964 Helsinki Declaration and its later amendments. All patients gave informed consent to the use of their clinical data for scientific purposes.

A criterion for inclusion in the study was the absence of special treatment and surgical intervention at the time of diagnosis and low or high PCa progression risk. The diagnosis of PCa was verified by examining histological specimens after transrectal multifocal biopsy of the prostate gland under ultrasound control. The stage of the tumor process was determined according to the international classification (TNM, 8th edition 2017) [13] by the consensus of the International Society of Urological Pathology (ISUP) in 2014 and current standards of diagnosis and treatment of the European Association of Urology, National Comprehensive Cancer Network, and the European Society for Medical Oncology. The risk of PCa progression was determined by the recommendations of the European Association of Urology [15]. The clinicopathological characteristics of PCa patients are shown in Table 1.

Table 1. **Clinicopathological characteristics of patients with PCa**

Index	Number of patients	
	n	%
Total number of patients	50	100
Age (years)		
Average	62.9 ± 5.8	
Range	48—80	
T category by TNM		
T2	28	56.0
T3	20	40.0
T4	2	4.0
N category by TNM		
N0	49	98.0
N1	1	2.0
Gleason's score / Grade group		
6 / GG1	17	34.0
7 / GG2 and GG3	16	32.0
8—10 / GG4 and GG5	17	34.0
Preoperative PSA level (ng/ml)		
≤ 10	15	30.0
> 10	35	70.0
Risk of PCa progression by the EAU classification		
Low	13	26.0
High	37	74.0

The real-time reverse transcription polymerase chain reaction (RT-PCR) was used to study the expression of miRNA in the PCa tissue. Total RNA from paraffin blocks with tissue was isolated using the commercial RNeasy FFPE Kit (QIAGEN, Germany) by the manufacturer's protocol. The amount of isolated RNA was determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). The purity of the isolated RNA was assessed by the ratio of optical absorbance values at wavelengths of 260 and 280 nm. RNA was dissolved in Tris-EDTA buffer and stored at –20 °C.

The RT-PCR was performed using the detection system QuantStudio 5 Dx Real-Time PCR System (Thermo Scientific, USA) with a commercial kit for RT-PCR TaqMan MicroRNA Assay (Thermo Scientific, USA) according to the manufacturer's protocol. Primer sequences for RT-PCR were determined using the resource http://genomics.dote.hu:8080/mirnadesigntool/ and synthesized by Metabion (Germany). MiRNA RNU48 was used as an endogenous control. The fold difference between the expressions of the studied microRNAs was calculated by the formula $2^{-\Delta Ct}$ and expressed as a fold change [17].

Histochemical methods. Mast cells (MCs) were identified by staining tumor tissue sections with toluidine blue (Sigma-Aldrich, USA). Before staining, sections were deparaffinized in xylene and washed in ethanol. The preparations were examined using a Primo Star light microscope (Carl Zeiss, Germany) [18]. The number of MCs was evaluated in 10 fields of view at a magnification of ×400 and the level of their infiltration per mm^2 was determined.

Immunohistochemical method. The immunohistochemical study of the expression of the markers of tumor-associated fibroblasts such as alpha-smooth muscle actin (α-SMA) and vi-

mentin (VIM), or tumor-associated macrophages (CD68) in the PCa tissue was carried out on paraffin sections with a thickness of 5 μm. Unmasking of antigens was carried out in EDTA buffer, pH 8.0 (Diagnostic BioSystems, USA) in a water bath WB-4MS (Biosan, Latvia) for 20 min at 96 °C. Monoclonal antibodies specific for α-SMA (clone 3F11-C2-G6; MyBioSource, Inc., USA), VIM (clone V9; Merck Millipore, Burlington, MA, USA), and CD68 (clone KP-1; Vitro Master Diagnostica, Spain) were used as primary antibodies. To visualize the results of the reaction, a kit of Mouse/Rabbit PolyVue Plus HRP/DAB Detection System reagents (Diagnostic BioSystems, USA) was used by the manufacturer's recommendations. Sections were stained with Mayer's hematoxylin (Thermo Scientific Richard-Allan, USA).

The analysis of α-SMA and VIM expression was performed as described in [18], using the program ImageJ v. 1.42. on 10 arbitrarily selected fields of view at ×400 magnification. The final result of the measurement of the immunopositive PCa tissue area was expressed in relative units (hereafter r.u.).

Statistical analysis of the data was performed using the GraphPad Prism v.8.0 program (GraphPad Software Inc., USA), taking into account the nature of the obtained data distribution. A comparison of the reliability of differences in mean values was carried out using the Mann — Whitney U-test. The difference in the expression levels of the investigated miRNAs depending on the Gleason score was evaluated using the calculation of the Kruskal — Wallis test. Correlation analysis was performed using the Jamovi v.2.4.11.0 program (Computer Software, https://www. jamovi.org) with the calculation of the Spearman correlation coefficient. Multiple linear regression analysis was performed using the STATISTICA 12.0 program (StatSoft, USA). The difference between the indicators of the groups at $p < 0.05$ was considered statistically significant.

Results

The analysis of the obtained results demonstrated a heterogeneous expression of the studied miRNAs in the PCa tissue samples. The highest expression levels in the PCa tissue were found for miR-23b-3p — 22.68 ± 8.10 (Me = 5.41) with individual fluctuations from 1.05 to 132.40 fold change, while the expression levels of miR-7-5p and miR-19a-3p were slightly lower and amounted to 14.53 ± 5.36 (Me = 4.99) with individual fluctuations from 0.03 to 84.44 fold change and 14.05 ± 3.51 (Me = 24.57) with individual fluctuations from 0.23 to 50.87 fold change respectively.

We established a dependence between the relative levels of the investigated tumor-associated miRNAs and the category T by the TNM classification. As shown in Table 2, the levels of miR-7-5p and miR-23b-3p in the PCa tissues of patients with category T3-T4 were 32.2 ($p < 0.01$) and 10.9 ($p < 0.05$) times higher compared to similar indicators of patients with category T2, respectively. No association between miR-19a-3p expression and category T was found. Further analysis established the dependence of the expression of miR-7-5p and miR-19a-3p on Gleason grading. The highest levels of these miRNAs were characteristic of PCa cases with Gleason's score 7 (Table 2). No correlation between miR-23b-3p expression and Gleason grading was found. Also, expression levels of the studied miRNAs in the tumor tissue were not dependent on the preoperative blood PSA level in the PCa patients (Table 2).

Then we performed an analysis of the relationship between the relative expression level of the studied miRNAs in the tumor tissue and the risk of PCa progression (Fig. 1). We established that the tumor tissue of patients with a high risk of PCa progression was characterized by 4.93 ($p < 0.01$) and 8.97 ($p < 0.05$) times higher levels of miR-19a-3p and miR-23b-3p, respectively, compared to similar values in the group of

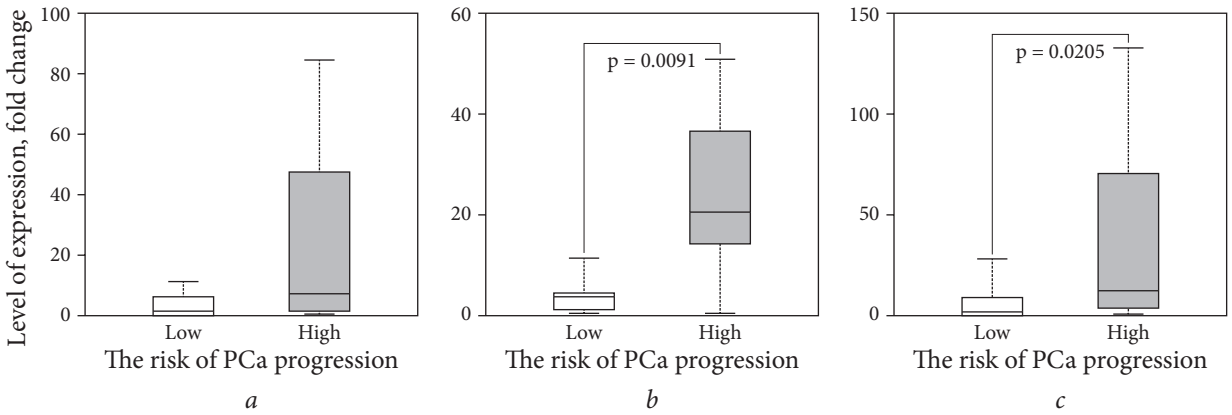

Fig. 1. The relationship between the expression of tumor-associated microRNAs (*a* — miR-7-5p, *b* — miR-19a-3p, *c* —miR-23b-3p) and the risk of PCa progression. The median is indicated by the central line, the first and third quartiles are indicated by the lower and upper limits of the "box", respectively. The whiskers emerging from the rectangles indicate the minimum and maximum values of the indicators

Table 2. **The relationship between the expression of tumor-associated miRNAs and the clinicopathological characteristics of PCa**

Clinicopathological characteristics		*miR-7-5p*		*miR-19a-3p*		*miR-23b-3p*	
		Me (Q1—Q3)	*p*	Me (Q1—Q3)	*p*	Me (Q1—Q3)	*p*
T category by TNM	T2	1.37 (0.38—6.96)	0.0068	2.25 (0.40—17.53)	0.0700	4.22 (3.05—5.71)	0.0473
	T3—T4	44.13 (23.69—78.70)		24.57 (10.50—40.53)		46.04 (1.34—87.11)	
Gleason's score	6	1.16 (0.24—3.32)	0.0197	1.13 (0.42—13.18)	0.0163	4.23 (2.83—5.68)	0.1268
	7	44.13 (19.55—63.23)		38.98 (22.81—47.46)		46.04 (16.29—101.7)	
	8—10	9.67 (0.60—54.15)		15.03 (0.41—27.38)		12.66 (3.05—70.68)	
PSA level (ng/ml)	≤ 10	0.72 (0.19—33.33)	0.2958	0.62 (0.27—38.37)	0.4118	5.37 (1.18—38.63)	0.5531
	> 10	7.05 (0.97—49.55)		16.54 (0.92—27.38)		5.63 (3.34—79.55)	

Table 3. **The data of a predictive model for predicting the risk of PCa progression (multiple linear regression method)**

$R^2 = 0.6643$ $p = 0.0013$	*miR-7-5p*	*miR-19a-3p*	*miR-23b-3p*
β	0.4974	0.5377	-0.0490
p	0.0221	0.0071	0.8098

patients with a low risk of PCa progression. No relationship between the expression of miR-7-5p and the risk of PCa progression was found.

To find out the prognostic value of miR-7-5p, miR-19a-3p, and miR-23b-3p expression indicators, a model for predicting the risk of PCa progression was built (Table 3). It should be noted

that the risk of PCa progression was determined as a dependent variable, and the expression levels of the studied miRNAs were determined as independent variables. The model obtained using the multiple linear regression method had a coefficient of determination $R^2 = 0.6643$ ($p = 0.0013$). The risk of PCa progression was found to be significantly affected by the levels of miR-7-5p ($p = 0.0221$) and miR-19a-3p ($p = 0.0071$) in tumor tissue, while the effect of miR-23b-3p was insignificant ($p = 0.8098$).

Finally, we determined associative relationships between the relative expression levels of miR-7-5p, miR-19a-3p, and miR-23b-3p and certain elements of the stromal microenvironment of PCa. As shown in Fig. 2, the levels of miR-7-5p and miR-19a-3p expression in the PCa tissue directly correlate with the expression levels of α-SMA ($r = 0.49$, $p = 0.0221$ and $r = 0.45$, $p = 0.0450$, respectively) and VIM ($r = 0.45$, $p = 0.0446$ and $r = 0.46$, $p = 0.0382$, respectively), which indicates their participation in the activation of tumor-associated fibroblasts. A direct correlation ($r = 0.44$, $p = 0.0446$) was found between the level of miR-7-5p expression and the degree of infiltration of the PCa tissue by tumor-associated macrophages. The dependence between the miR-23b-3p and miR-7-5p, or miR-19a-3p expression levels was revealed, while there was no association of this miRNA with the components of the stromal microenvironment. No relationship between expression indicators of any of the expression levels of the studied miRNAs and the degree of infiltration of the PCa tissue by MCs was revealed.

Discussion

Recently, the attention of researchers has been focused on the study of the features of the TME of malignant neoplasms of various localizations, including PCa [19—21]. In this context, a large array of data has been accumulated on the significance of the infiltration of the PCa tissue by T-cells [22], macrophages [23], MCs [24], as well as on the influence of fibroblasts on the spatial organization of the neoplasm stroma [25, 26]. However, the mechanisms and features of the interaction between tumor cells and individual components of the TME remain not fully elucidated. Given this, the identification of modulators of the interaction of malignantly transformed cells and cells of the TME with the prospect of their use as prognostic and or predictive markers of PCa is highly relevant.

Our data on the features of the expression of tumor-associated miR-7-5p, miR-19a-3p, and miR-23b-3p indicate the prospects of their use as markers of the PCa course. The highest expression rates of miR-7-5p and miR-23b-3p were recorded when tumors extended beyond the organ capsule. The relationship between the expression levels of miR-7-5p and miR-19a-3p and the Gleason grading, as well as the association of the expression levels of miR-19a-3p and miR-23b-3p with a high risk of PCa progression were established. We have confirmed the prospect of using the studied miRNAs as prognostic markers of PCa using a predictive multiple linear regression model.

Our data are consistent with the results of the study [27] demonstrated that high levels of miR-7-5p in the blood plasma of patients with the Gleason score ≥ 8 were associated with a short period of development of the castration-resistant form of PCa. High levels of miR-19a-3p expression have been shown to stimulate the proliferation, invasion, and migration of PCa cells by inhibiting PMEPA1 and BTG1 [28, 29]. Fu et al. [30] established a direct relationship between the expression of miR-19a-3p and the Gleason score in the PCa tissue and experimentally demonstrated the association of its level with the proliferative and migratory activity of LNCaP and PC3 cells. There are also reports that high levels of miR-19a-3p correlate with low rates of relapse-free survival in patients with PCa, regardless of the Gleason score.

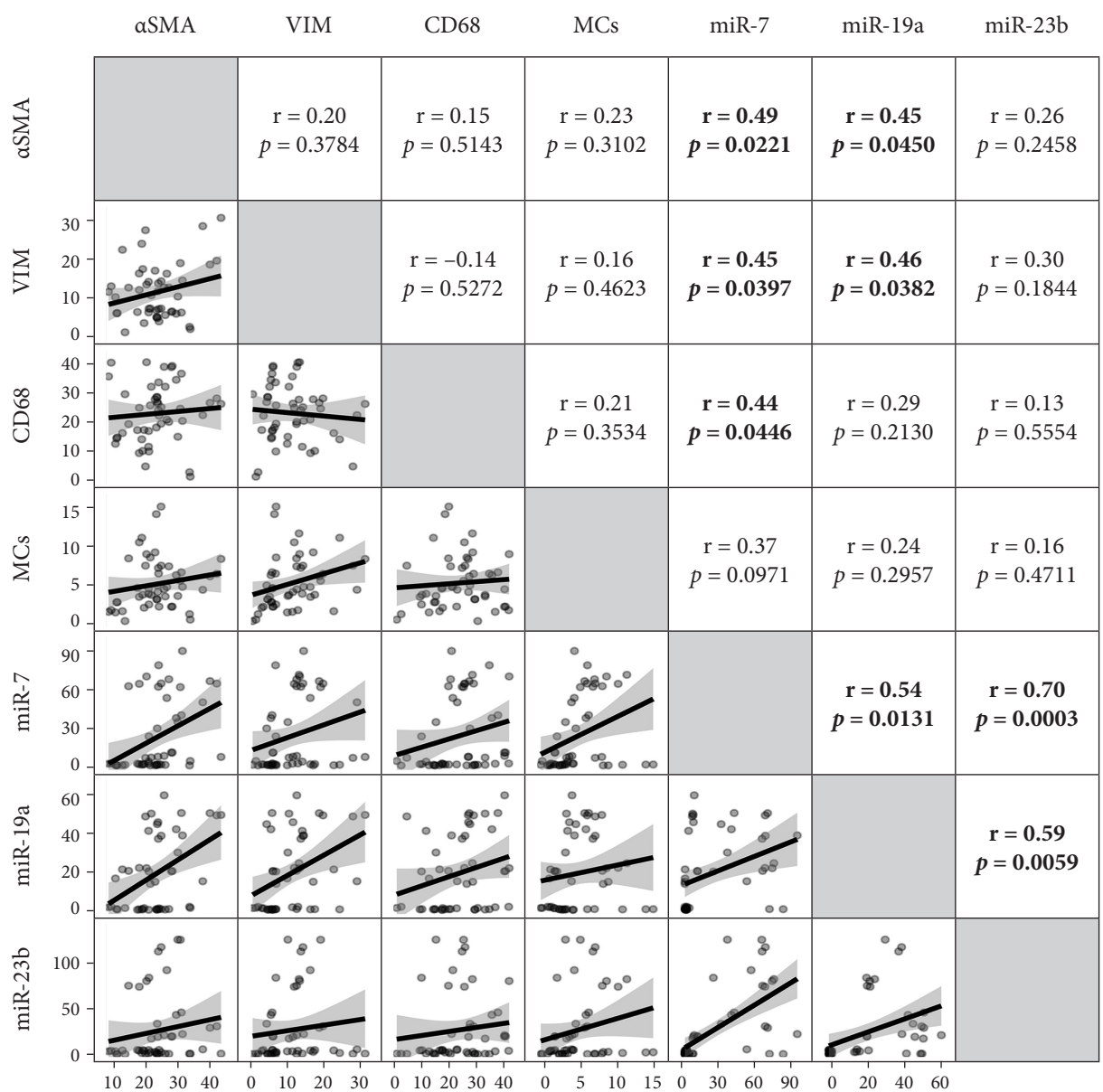

Fig. 2. The relationship between the level of expression of miR-7-5p, miR-19a-3p, and miR-23b-3p in PCa tissue and the features of the TME. The expression levels of the microRNAs under study were determined as fold change, the degree of infiltration of the PCa tissue by macrophages and MCs is expressed in the number of cells per mm^2, and the result of measuring the area of immunopositive PCa tissue by α-SMA and VIM is presented in r.u

According to the report [31], the tumor tissue of patients with locally advanced PCa was characterized by a significantly higher level of miR-23b-3p compared to patients with metastatic PCa. The lowest levels of expression of this miRNA were identified in the PCa tissue of patients with metastases in the regional lymph nodes.

When analyzing the relationship between the expression of the studied miRNAs and the features of the stromal microenvironment of PCa, we established a direct correlation between the

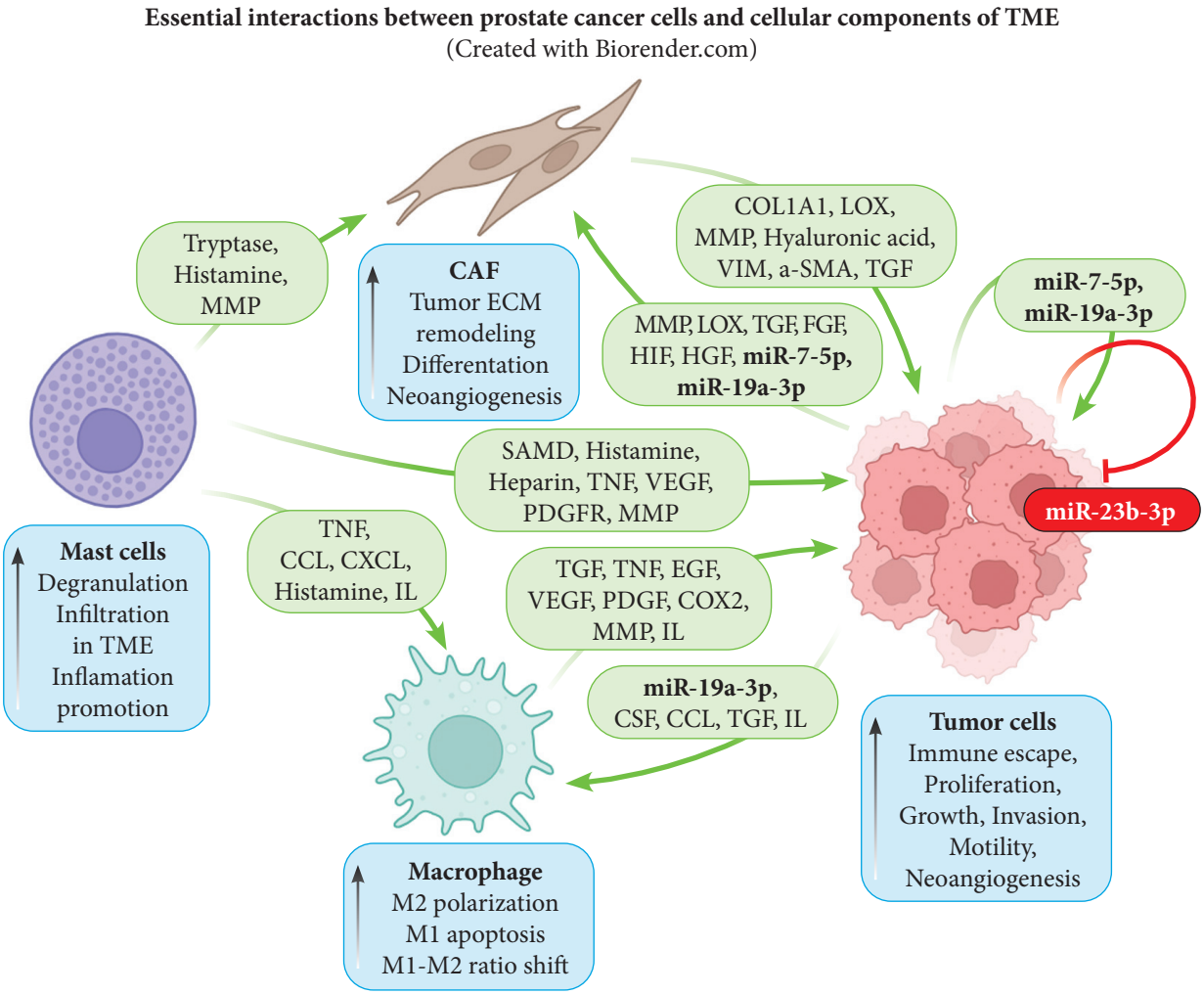

Fig. 3. During PCa progression, tumor cells express several signaling molecules and protein factors that affect TME and cause significant changes in it. In particular, tumor-derived microRNAs simultaneously with growth factors and cytokines promote M2 macrophage polarization. At the same time, M2 macrophages drive tumor invasiveness, activate neoangiogenesis, and play a key role in cancer cells' immune escape. In the same way, microRNA alters the expression of transcription factors in fibroblasts, which leads to their differentiation and increases the number of cancer-associated fibroblasts (CAFs). CAFs are responsible for essential extracellular matrix remodeling through the production of fibrillar proteins and proteinases. MCs in TME are orchestrators of macrophages and CAFs functions, while the main components of their granules regulate PCa cell proliferation and motility: MMP — matrix metalloproteinase, IL — interleukin, VEGF — vascular endothelium growth factor, PDGF — platelet-derived growth factor, TNF — tumor necrosis factor, TGF — transforming growth factor, COX2 — cyclooxygenase 2, EGF — epidermal growth factor, SAMD — sterile alpha motif domain, LOX — lysyl oxidase, HIF — hypoxia-induced factor, HGF — hepatocytes growth factor, FGF — fibroblasts growth factors, COL1A1 — collagen type I alpha 1 chain

expression of miR-7-5p and miR-19a-3p and the expression levels of α-SMA and VIM in the PCa tissue. We found that the level of miR-7-5p expression also correlates with the degree of infiltration of the PCa tissue by tumor-associated macrophages. These facts are consistent with the literature data [32, 33] and allow us to assert the participation of these miRNAs in the formation

of the TME of PCa. Along with this, the study [34] has demonstrated that miR-7-5p participates in the processes of hyaluronan-dependent differentiation of fibroblasts by inhibiting EGFR signaling. In another study [35], it was found that upregulation of FasL with the participation of miR-7-5p led to a shift in the balance toward protumoral M2-macrophages and stimulation of cancer progression. Also, miR-19a-3p was revealed to participate in the suppression of the TGF-β signaling cascade by inhibiting the expression of SMAD2 and SMAD3, as well as in the activation of the proliferation of fibroblasts [36, 37]. Essential relationships between PCa cells, cellular components of TME, and the features of these interactions' regulation are depicted in Fig. 3.

To sum up, the obtained data on the relationship between the expression of miR-7-5p, miR-19a-3p, and miR-23b-3p and the risk of progression and the features of the TME of PCa indicate the prospects of their use for predicting the aggressiveness of the PCa course.

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ЕКСПРЕСІЯ АСОЦІЙОВАНИХ З ПУХЛИННИМ МІКРООТОЧЕННЯМ miR-7-5p, miR-19a-3p та miR-23b-3p В ПУХЛИННІЙ ТКАНИНІ ХВОРИХ НА РАК ПЕРЕДМІХУРОВОЇ ЗАЛОЗИ З РІЗНИМ РИЗИКОМ ПРОГРЕСІЇ

Вступ. Важлива роль у виникненні та прогресії раку передміхурової залози (РПЗ) належить пухлинному мікрооточенню (ПМ). У той же час, механізми та особливості взаємодії між пухлинними клітинами та окремими компонентами ПМ РПЗ залишаються остаточно не з'ясованими. **Мета роботи:** дослідити показники експресії пухлино-асоційованих мікроРНК-7-5p, -19a-3p та -23b-3p у тканині РПЗ, а також аналізувати їх зв'язок з особливостями ПМ. **Матеріали та методи:** Робота базується на аналізі результатів обстеження та лікування 50 хворих на РПЗ II—IV стадій. Дослідження експресії мікроРНК у тканині РПЗ проведено методом полімеразної ланцюгової реакції у реальному часі. Експресію альфа-актину гладких м'язів (α-SMA), віментину (VIM) та CD68 у тканині РПЗ визначали імуногістохімічним методом. Ідентифікацію опасистих клітин у тканині РПЗ здійснювали гістохімічним методом. Статистичний аналіз проводили за допомогою GraphPad Prism v. 8.00, Jamovi v.2.4.11.0 та STATISTICA 12.0. **Результати.** Аналіз рівнів експресії пухлино-асоційованих мікроРНК продемонстрував, що тканина РПЗ хворих із високим ризиком прогресії характеризувалась у 4,93 ($p < 0,01$) та 8,97 ($p < 0,05$) разів вищими рівнями мікроРНК-19a-3p та мікроРНК-23b-3p, відповідно, порівняно з аналогічними показниками в групі пацієнтів низького ризику прогресії. Виявлено, що показники мікроРНК-7-5p та мікроРНК-19a-3p у тканині РПЗ корелювали з рівнем експресії α-SMA (r = 0,49 та r = 0,45, відповідно; $p < 0,05$) та VIM (r = 0,45 та r = 0,46, відповідно; $p < 0,05$). Встановлено існування прямого зв'язку (r = 0.44; $p < 0,05$) між рівнем експресії мікроРНК-7-5p та ступенем інфільтрації тканини РПЗ пухлино-асоційованими макрофагами. **Висновки.** Одержані нами дані щодо особливостей експресії пухлино-асоційованих мікроРНК-7-5p, мікроРНК-19a-3p та мікроРНК-23b-3p вказують на перспективність застосування їх як маркерів агресивності перебігу РПЗ.

Ключові слова: рак передміхурової залози, мікроРНК-7-5p, мікроРНК-19a-3p, мікроРНК-23b-3p, ризик прогресії, пухлинне мікрооточення.