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NANOG AS PROGNOSTIC FACTOR OF PROSTATE CANCER COURSE

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The variability of the clinical course of prostate cancer (PC) indicates the need to find factors that could predict the aggressive potential of neoplasms accounting the biological characteristics of tumor cells. In this context, the role of NANOG, a transcription factor involved in maintaining pluripotency and one of the markers of cancer stem cells (CSCs), is being actively studied today. *Aim*: To investigate the level of NANOG mRNA in tumor tissue of patients with PC and to analyze the possibility of its use as a marker of the disease course. *Materials and Methods*: The study involved 85 patients with PC of stages II–IV. Morphological and immunohistochemical studies were performed on serial paraffin sections of resected PC using monoclonal antibodies to Ki-67 and androgen receptor. *NANOG* and miR-214 mRNA expression in tumor cells was analyzed by real-time reverse transcription polymerase chain reaction. The identification of CSCs was performed by double-labeled immunohistochemical method using primary antibodies to CD24 and CD44. *Results*: We have revealed notable variability of *NANOG* mRNA levels in tumor tissue of patients with PC (mean 4.18 ± 0.65 a.u. with individual deviations from 0.11 ± 0.03 a.u. to 15.24 ± 0.36 a.u.). According to *NANOG* mRNA levels, two groups of the PC patients were delineated: group 1 and group 2, with the average *NANOG* mRNA levels of 2.12 ± 0.16 a.u., and 8.68 ± 1.24 a.u., respectively. The *NANOG* mRNA levels in tumor tissue of PC patients of groups 1 and 2 correlated with preoperative serum prostate-specific antigen level($r = 0.58$; $p < 0.05$ and $r = 0.64$; $p < 0.05$, respectively), tumor volume ($r = 0.42$; $p < 0.05$ and $r = 0.72$; $p < 0.05$, respectively), regional lymph node metastases ($r = 0.70$; $p < 0.05$ and $r = 0.75$; $p < 0.05$, respectively). High *NANOG* mRNA levels in tumor cells were associated with such molecular and biological features of PC as androgen receptor expression ($r = 0.52$; $p < 0.05$), high proliferative activity ($r = 0.60$; $p < 0.05$) and the presence of CSC markers ($r = 0.75$; $p < 0.05$). *Conclusions*: The findings indicate that NANOG is involved in the formation of the PC malignancy and should be further studied as a potential marker for the prediction of the disease course.

***Key Words*: prostate cancer, cancer stem cells, NANOG, microRNA-214, disease course variability.**

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According to the latest statistics, prostate cancer (PC) is among leading causes of cancer-related morbidity and mortality in Ukraine and worldwide [1, 2]. The accumulated clinical experience implies the heterogeneity of this disease, both by morphological and molecular structure and by clinical course, which requires different approaches to diagnosis and therapy. In some cases, the disease is latent and does not affect the quality and life expectancy of patients; in other cases, it manifests itself at late clinical stages, when treatment is already ineffective [3–5].

To determine the treatment strategy in each case, it is necessary to predict the aggressiveness of the PC course. According to the literature, indicators used in clinical practice (the level of prostate-specific antigen (PSA), tumor stage according to TNM system, and Gleason histological classification score) are far from perfect, which can lead to erroneous assessment of the malignancy degree of the disease in about half of the cases, and also complicates the choice of adequate therapy [6, 7]. Assessment of the tumor aggressiveness can be useful for separation of patients requiring active surveillance from those who require the radical treatment, as well as to determine the features of the disease and its potential for progression [8]. In view of this, the search for factors that would allow predicting the PC aggressive potential taking into account the biological characteristics of tumor cells is a priority area of research [9].

In the current literature, the importance of cancer stem cells (CSCs) in the formation of the degree of PC malignancy and intratumoral heterogeneity is actively discussed [10–12]. CSCs are characterized by a wide range of functional properties and are associated with high metastatic activity, resistance to standard radio- and chemotherapy, and the relapse risk, which generally correlates with the unfavorable course of the disease. To date, CSCs have been identified in many malignancies, including PC. A number of markers are used to identify CSCs: CD24, CD44, CD49f, CD133, CD166, α2β1-integrin, and NANOG, a transcription factor identified by Chambers et al. in 2003 [11–13]. NANOG has been shown to be involved in the regulation and function of dozens of genes, providing self-renewal and maintaining pluripotency in undifferentiated cells, in particular in embryonic stem cells [14].

In humans, in addition to the *NANOG* gene, 11 *NANOG* pseudogenes (*NANOGP1–NANOGP11*) were identified, with their sequences being more than 90% identical to the *NANOG*. It is noteworthy that these pseudogenes lack intron-exon repeats and instead have a continuous *NANOG* sequence with 3'-

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Abbreviations used: AR – androgen receptor; CSCs – cancer stem cells; IHC – immunohistochemistry; PC – prostate cancer; PSA – prostate specific antigen.

poly (A) tails. They also differ from the *NANOG* by their promoters. NANOG acts in conjunction with the transcription factors Sox2, Oct3/4 (POU5F1), FoxD3, and is able to activate their expression. NANOG expression is regulated with the involvement of miR-214 and miR-21, which mediate their action through p53 [15–17].

NANOG is thought to play a leading role in the carcinogenesis and progression of some malignancies. It is expressed in embryonic gonocytes, testicular cancer cells *in situ*, seminomas, embryonic cancers and breast cancers. In addition, increased NANOG expression in tumor cells correlates with unfavorable clinical course of cancer [18].

Recent studies on *in vitro* models have demonstrated the presence of NANOG expression in the PC cell lines. Jeter et al. [19] have demonstrated that expression of proteins encoded by the *NANOG* gene and the *NANOGP8* retrogene in human PC cell lines was associated with the presence of CSCs and correlated with such indicators of malignancy as self-renewal ability and colony-forming activity. In particular, overexpression of NANOG in both androgen-sensitive and androgen-refractory PC cell lines promoted the formation and growth of tumors upon their grafting to experimental animals. Recently NANOG overexpression has been shown to be associated with an increase in BCL-2, IGFBP-5, and CXCR4 levels, which makes cells insensitive to androgen deprivation [20]. At the same time, the value of NANOG in predicting the PC course remains poorly understood.

Therefore, the aim of this work was to investigate the level of *NANOG* mRNA in tumor tissue of PC patients and to analyze the possibility of its use as a marker of the disease course.

MATERIALS AND METHODS

The study involved 85 PC patients, stages II–IV, who were treated at the National Cancer Institute of the Ministry of Health of Ukraine during 2015–2017. All patients were thoroughly informed about the study that was approved by the local ethics committee. Clinical diagnosis was established on the basis of determination of PSA in blood serum, finger rectal examination, computer tomography of the pelvic organs and/or transrectal ultrasound examination of the prostate and abdominal cavity, osteoscintigraphy, chest radiography. In all patients, the diagnosis was verified after ultrasound-guided transrectal multifocal biopsy of the prostate. The stage of the tumor process was determined according to the International Classification of Tumors (TNM, 8th edition, 2017).

For morphological examination, the operative material was fixed in 10% neutral formalin solution and further processed by generally accepted histological techniques. From paraffin blocks, sections were prepared that were stained with hematoxylin and eosin; the morphological features of the tumor structure were studied by light microscopy.

Immunohistochemical (IHC) study of the expression of androgen receptors (AR) and Ki-67 in tumor cells was performed on paraffin sections 4–5 microns thick. Monoclonal antibodies specific for AR (clone 441; Thermo Scientific, USA) and Ki-67 (clone MIB- 1; DakoCytomation, Denmark) were used as primary antibodies. A set of Mouse/Rabbit PolyVue Plus HRP/DAB Detection System reagents from Diagnostic BioSystems (USA) was used to visualize the reaction results according to the manufacturer's recommendations. Sections were counterstained with Meyer's hematoxylin. AR expression was considered positive if the number of immunopositive tumor cells > 10%. The number of cells with positive expression of Ki-67 was determined, accounting the degree of manifestation of IHC reaction: "+++" — strong, "++" — moderate, "+" — low, or no expression (0).

Detection of CSC was performed by simultaneous IHC assessment of CD24 and CD44 expression in tumor cells using the MultiVision Polymer Detection System: anti-Mouse-HRP and anti-Rabbit-AP Thermo Scientific (USA) detection system. Monoclonal antibodies specific for CD24 (clone SN3b; Thermo Scientific, USA) and CD44 (clone SP37; Máster Diagnóstica, Spain) were used as primary antibodies in dilutions according to the manufacturer's instructions. The results of IHC reactions were analyzed using an XSP-137-BP optical microscope JNOEC at ×200 or ×400 magnifications.

Total RNA was extracted using "Riboprep" Isolation Kit (Amplisens, Russia). RNA concentration was determined on a "NanoDrop 2000c" spectrophotometer (Thermo Scientific, USA). RNA was dissolved in TE buffer and stored at –20 °C. Single-stranded cDNA of NANOG was synthesized from 100 ng of total RNA using "Reverta-L" kit (Amplisens, Russia). As internal control, the mRNA of β-actin was used. All the primers were synthesized by Metabion, Germany.

For NANOG detection, Q-RT PCR primer sequences were as in [21]: forward 5′-CAGTCTGGACACTGGCTGAA-3′, reverse 5′- CTCGCTGATTAGGCTCCAAC-3′; β-actin: forward 5′-ACAGGGGAGGTGATAGCATT-3′, reverse 5′-GACCAAAAGCCTTCATACATCTC-3′. For miR-214 detection, we used stem-loop primer for reverse transcription 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCATTATT-3′ and forward primer sequence 5′-GTTACAGCAGGCACAGACA-3′. According to the RT-PCR stem-loop miRNA technique, universal reverse primer 5′- GTGCAGGGTCCGAGGT-3′ was used [22]. The primer sequences for miR-214 detection were retrieved using the resource *genomics.dote.hu:8080/mirnadesigntool/*. As an endogenous control, we used microRNA U6 (RNU6-1) [23]. The primer sequences were from the *www.ncbi.nlm.nih.gov* resource: forward, 5′-GCTTCGGCAGCACATATACTAAAAT-3′ reverse, 5′-CGCTTCACGAATTTGCGTGTCAT-3′.

Q-RT PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR System using Maxima SYBRGreen/ROX qPCR Master Mix (Thermo Scientific, USA). Relative expression was identified by comparative CT method. Experiment was performed

in triplicates for each sample. The threshold cycle was averaged in all technical and biological replicas within each sample. Fold change between the studied miRNAs expression relative to control was calculated by the formula $2^{-\Delta\Delta Ct}$ [24].

Statistical processing of the obtained results was performed using the STATISTICA 6.0 program. The following statistical methods were used: standard descriptive, parametric and non-parametric. The critical level of statistical significance was assumed to be 0.05. Pearson's correlation coefficient (r) was used to evaluate the association of expression of the investigated markers with the clinical and pathological characteristics of the PC.

RESULTS AND DISCUSSION

The clinical characteristics of patients with the PC, stages II–IV are shown in Table 1. The number of patients with T2 stage was 42.35%, T3 stage — 57.65%. The age of patients ranged from 46 to 83 years, with an average age of 62.7 ± 4.6 years.

The results of comprehensive examination of patients (radiological, ultrasound, laboratory) showed the presence of metastases in regional lymph nodes in 36.47% cases and the absence of distant metastases in all PC patients.

The morphological study revealed that 65.88% of all tumors studied were moderately differentiated adenocarcinomas (Gleason score < 7) and 34.12% were low-differentiated adenocarcinomas (Gleason score ⩾ 7). In 43.53% of patients, the PSA level was below 10 ng/ml, while in 56.47% of patients this level was higher than 10 ng/ml.

Table 1. Clinical and pathological characteristics of patients with PC

Index	Patients	
	n	%
Total number of the PC patients	85	100
Age (years)		
Median	62.7 ± 4.6	
Range	46–83	
Tumor size (T category)		
T2	49	42.35
T3	36	57.65
Regional lymph node metastases (N category)		
Positive	31	36.47
Negative	54	63.53
Gleason's score		
< 7	56	65.88
⩾ 7	29	34.12
PSA level		
< 10 ng/ml	37	43.53
> 10 ng/ml	48	56.47

The level of *NANOG* mRNA in the tumor tissue of patients with PC was characterized by considerable variability, as evidenced by individual fluctuations from 0.11 ± 0.03 a.u. up to 15.24 ± 0.36 a.u. (4.18 ± 0.65 a.u. on the average).

In order to determine the role of *NANOG* in the formation of the PC malignancy, all patients were divided into 2 groups depending on the *NANOG* mRNA levels — less than 4 a.u. (group 1; n = 54) and higher than 4 a.u. (group 2; n = 31). The average *NANOG* mRNA level in the first group of patients was 4.09 times higher compared with patients in the second group (Table 2).

Next, we analyzed the dependence of *NANOG* mRNA level on the clinical and pathological parameters of patients of both study groups. It is well recognized that the pretreatment PSA level is considered as one of the factors for predicting the aggressiveness of the disease and correlates with the indicators of relapse-free survival of patients [25]. In particular, it was found that at baseline serum PSA level above 50 ng/ml, extracapsular invasion was detected in 80% of patients and metastatic lesions of regional lymph nodes in 66% patients [8, 9]. As can be seen (see Table 2), PSA level > 10 ng/ml was detected in 44.5% of patients of group 1, which is 1.73 times less than in group 2 (77.4%). Patients in both study groups were found to have an increased pretreatment serum PSA levels in association with high *NANOG* mRNA levels in tumor tissue. In particular, in patients of groups 1 and 2 with PSA level < 10 ng/ml, *NANOG* mRNA levels were by 1.95 and 1.81 times lower, respectively, compared with patients with PSA levels > 10 ng/ml.

One of the main criteria that determines the tactics of treatment of patients with PC and correlates with its effectiveness is the size of the tumor. As can be seen from Table 2, the number of T2 tumors in group 1 was twice that in group 2. In patients with neoplasms localized in the prostate, the level of *NANOG* mRNA in tumor tissue was 1.71 and 1.81 times lower than in tumors extending beyond the organ capsule, in groups 1 and 2, respectively.

Another important prognostic PC index is the presence of metastases in regional lymph nodes [26].

Table 2. Relationship between *NANOG* mRNA level in tumor tissue of the PC patients with the main clinical and pathological characteristics

Index	Group 1 (*NANOG* mRNA level < 4 a.u.), n = 54		Group 2 (*NANOG* mRNA level > 4 a.u.), n = 31	
	Number of patients, n/%	*NANOG* mRNA level, a.u.	Number of patients, n/%	*NANOG* mRNA level, a.u.
PSA level				
< 10 ng/ml	30/5.5	1.52 ± 0.18	7 / 22.6	6.11 ± 0.54
> 10 ng/ml	24/44.5	2.97 ± 0.12*	24 / 77.4	11.07 ± 1.72*
Tumor size (T category)				
T2	38/70.4	1.60 ± 0.23	11 / 35.5	7.05 ± 1.21
T3	16/29.6	2.74 ± 0.19*	20 / 64.5	10.44 ± 1.74*
Regional lymph node metastases (N category)				
Positive	41/75.9	1.86 ± 0.26	13 / 41.9	5.36 ± 0.39
Negative	13/24.1	2.57 ± 0.19*	18 / 58.1	11.57 ± 1.38*
Gleason's score				
< 7	42/77.8	2.22 ± 0.34	14 / 45.2	6.22 ± 0.60
⩾ 7	12/22.2	2.07 ± 0.30	17 / 54.8	13.39 ± 1.20*

Note: *$p \leqslant 0.05$ compared to the corresponding characteristics of patients with PC.

According to our results, the number of the PC patients with metastases to regional lymph nodes was significantly higher (2.4 times) in group 2, reaching 58.1%. We determined that the level of *NANOG* mRNA in tumor tissue of patients with regional lymph node metastatic lesions of groups 1 and 2 was 1.38 and 2.5 times higher, respectively, compared with patients without metastases.

When the level of *NANOG* mRNA was analyzed in tumors of patients differed by Gleason score, we found that the highest number of tumors with the Gleason score < 7 was observed in patients with *NANOG* mRNA levels in tumor cells < 4 a.u., whereas most (54.8%) tumors in patients of group 2 had the Gleason score > 7. Analysis of the *NANOG* mRNA level in the tumor tissue of patients of group 1 revealed no association with the Gleason score. We found 2.15-fold increase of *NANOG* mRNA expression associated with an increased Gleason score in patients from group 2 (see Table 2).

The results of Pearson's correlation analysis of NANOG mRNA expression in tumor tissue of patients with PC are presented in Table 3. *NANOG* mRNA levels in both study groups were found to be directly correlated with preoperative PSA levels in patients' serum, disease stage, and presence of regional lymph node metastases. We did not find a correlation between the *NANOG* mRNA level in tumor cells and the Gleason score for a group of patients with *NANOG* mRNA level < 4 a.u. Instead, we revealed a positive stable correlation between these indices in patients of group 2, with *NANOG* mRNA values > 4 a.u.

Therefore, we have demonstrated that increased *NANOG* mRNA expression in tumor tissue is associated with such indices of PC malignancy as high preoperative PSA levels in patients' serum, presence of metastatic lesions of regional lymph nodes, and high Gleason score.

Given the significant variability of the clinical course of PC, due to its biological characteristics, we studied the association between *NANOG* mRNA expression in the PC cells and the characteristics of the molecular profile of tumors.

Because PC is a hormone-dependent neoplasm, it is important to evaluate the receptor status of tumor cells in order to predict the degree of malignancy of this nosological form of cancer. Many studies have shown that ARs can interact with different cell regulatory systems. AR expression correlates with high Ki-67 index and duration of overall and relapse-free survival of patients [27–29]. According to the data presented in Table 4. AR expression was observed in the majority of tumors in patients of groups 1 and 2 (65.6% and 61.3%, respectively) (Fig. 1). It was demonstrated that a high level of *NANOG* mRNA is a characteristic feature of AR-positive tumors in both study groups of patients with PC. Thus, in groups 1 and 2, the *NANOG* mRNA level in AR-negative tumor cells was by 1.22 and 1.57 times lower than in AR-positive tumors, respectively.

An important role in assessing the aggressiveness of the PC course is also played by proliferative activity of tumor cells, the classic marker of which is Ki-67. Increased Ki-67 expression directly correlates with Gleason score, tumor size, and overall PSA, and is associated with the risk of disease recurrence [30–32]. In our study, high Ki-67 expression was detected 2.5-fold more frequently in neoplasms of patients with *NANOG* mRNA levels > 4 a.u. (Fig. 2). It was found that high levels

Table 3. Correlation between *NANOG* mRNA levels and major clinical and pathological parameters of PC

Correlation pairs		Group 1		Group 2	
		r	*p*	r	*p*
NANOG mRNA level	PSA level	0.58	< 0.05	0.64	< 0.05
	Tumor size	0.42	< 0.05	0.72	< 0.05
	Regional lymph node metastases	0.70	< 0.05	0.75	< 0.05
	Gleason score	-0.12	> 0.05	0.54	< 0.05

Table 4. Relationship between *NANOG* mRNA expression in tumor tissue of PC patients and the molecular profile of neoplasms

Index	Group 1 (*NANOG* mRNA level < 4 a.u.), n = 54		Group 2 (*NANOG* mRNA level > 4 a.u.), n = 31	
	Number of patients, n/%	*NANOG* mRNA level, a.u.	Number of patients, n/%	*NANOG* mRNA level, a.u.
AR expression				
Negative	24/44.4	1.93 ± 0.19	12/38.7	6.88 ± 1.69
Positive	30/65.6	2.36 ± 0.16*	19/61.3	10.84 ± 1.38*
Ki-67 expression level				
Low	43/79.6	1.78 ± 0.16	15/48.4	5.21 ± 1.38
High	11/20.4	2.41 ± 0.32*	16/51.6	11.57 ± 0.34*
Expression of CSC markers				
Negative	38/70.4	1.85 ± 0.11	8/25.8	5.98 ± 0.56
Positive	16/29.6	2.39 ± 0.28*	23/74.2	13.39 ± 1.20*

Note: *$p < 0.05$ compared with group 1.

Table 5. Correlation between *NANOG* mRNA level in tumor cells with molecular profile features of PC

Correlation pairs		Group 1		Group 2	
		r	*p*	r	*p*
NANOG mRNA level	AR expression	0.44	< 0.05	0.52	< 0.05
	Ki-67 expression	0.52	< 0.05	0.60	< 0.05
	Micro-RNA-214 level	0.49	< 0.05	0.63	< 0.05
	Number of CSC-positive tumors	0.13	> 0.05	0.75	< 0.05

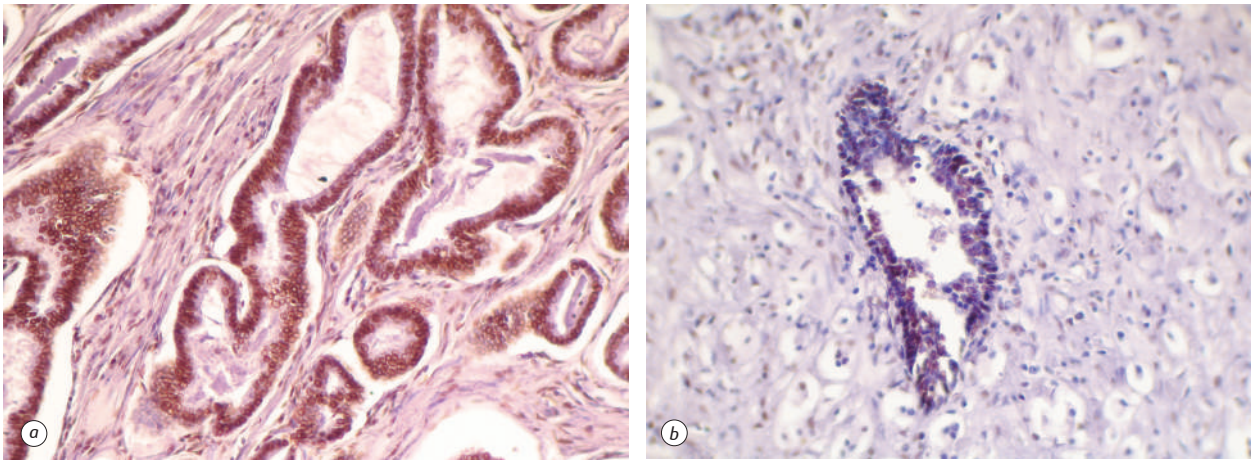

Fig. 1. Presence (*a*) and absence (*b*) of AR expression in tumor tissue of patients with PC. IHC. ×200

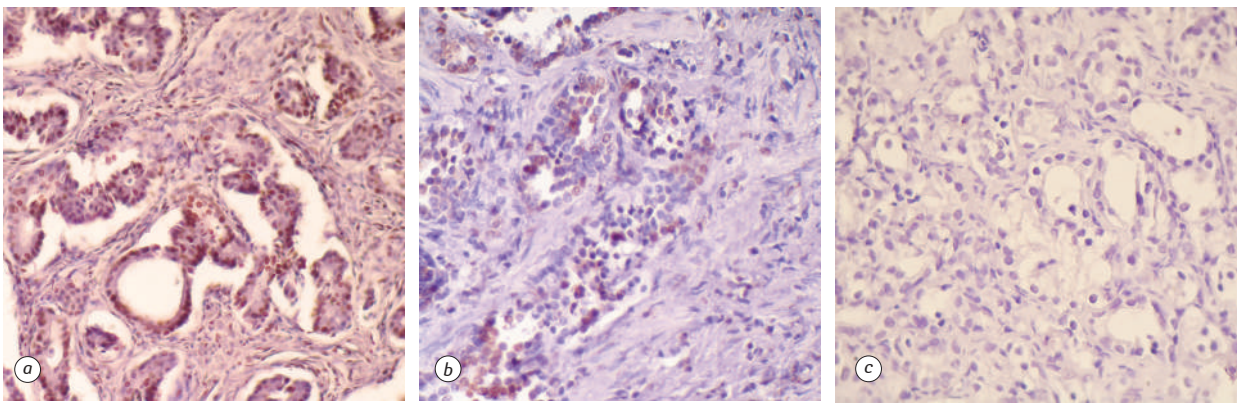

Fig. 2. Expression of Ki-67 in PC cells. *a* — high expression level; *b* — low expression level; *c* — no expression. IHC. × 200

of Ki-67 expression in tumor cells were associated with an increase in *NANOG* mRNA levels by 1.35 and 2.22-fold in patients of groups 1 and 2, respectively.

Given the role of NANOG in the processes of cell renewal and the maintenance of cell pluripotency, we considered it necessary to analyze the dependence of its level on the presence of CSC markers in the PC tissue. In our study, $CD44^{+}CD24^{-}$ expression was not detected in the majority of neoplasms of group 1 patients, while in the majority of investigated cases of group 2, the CSC markers were present (Fig. 3). It was found that high *NANOG* mRNA levels in both study groups were associated with the presence of CSC markers in the PC tissue.

As noted above, an important regulator of *NANOG* expression is microRNA-214, which is also one of the key mediators of the action of 5α-dihydrotestosterone on prostate cells upon malignant transformation. In view of this, we analyzed the dependence of *NANOG* mRNA level on the expression levels of miRNA-214. Patients with *NANOG* mRNA level < 4 a.u. were characterized by significantly lower expression of miRNA-214. In particular, its level in the group 1 was 0.81 ± 0.17 a.u., and in patients of group 2 — 1.47 ± 0.15 a.u.

The results of Pearson's correlation analysis are presented in Table 5. In both groups the level of *NANOG* mRNA correlated with the high expression of AR, Ki-67 and miR-214, and in group 2 — also with the presence of CSC markers in tumor cells.

In conclusion, we have shown that high *NANOG* mRNA levels in tumor cells are associated with such indices of PC malignancy as AR expression, high proliferative activity, and the presence of CSC markers. Therefore, we have demonstrated that NANOG mRNA expression is associated with a high degree of PC malignancy both in clinical terms and in molecular and biological features of tumors.

Presently it is known that NANOG is one of the most important transcription factors involved in embryogenesis. NANOG plays a key role at the preimplantation development stage, and its expression levels progressively decrease during differentiation of embryonic stem cells, thereby regulating embryonic and pre-natal development. Postnatal NANOG is not detected or expressed at very low levels in most human tissues. An increase in NANOG levels occurs in tumor cells during the development and progression of malignant tumors [33]. It is believed that NANOG allows tumor cells to acquire stem cell properties [34], as it was demonstrated in *in vivo* and *in vitro* experiments [35].

Data on the NANOG levels in PC are scarce. According to single observations [33, 36], *NANOG* mRNA expression is identified in 80% of PC samples. At the same time, the highest level of NANOG is observed in malignant transformed cells, whereas samples of patients with benign prostatic hyperplasia are characterized by the absence of this transcription factor.

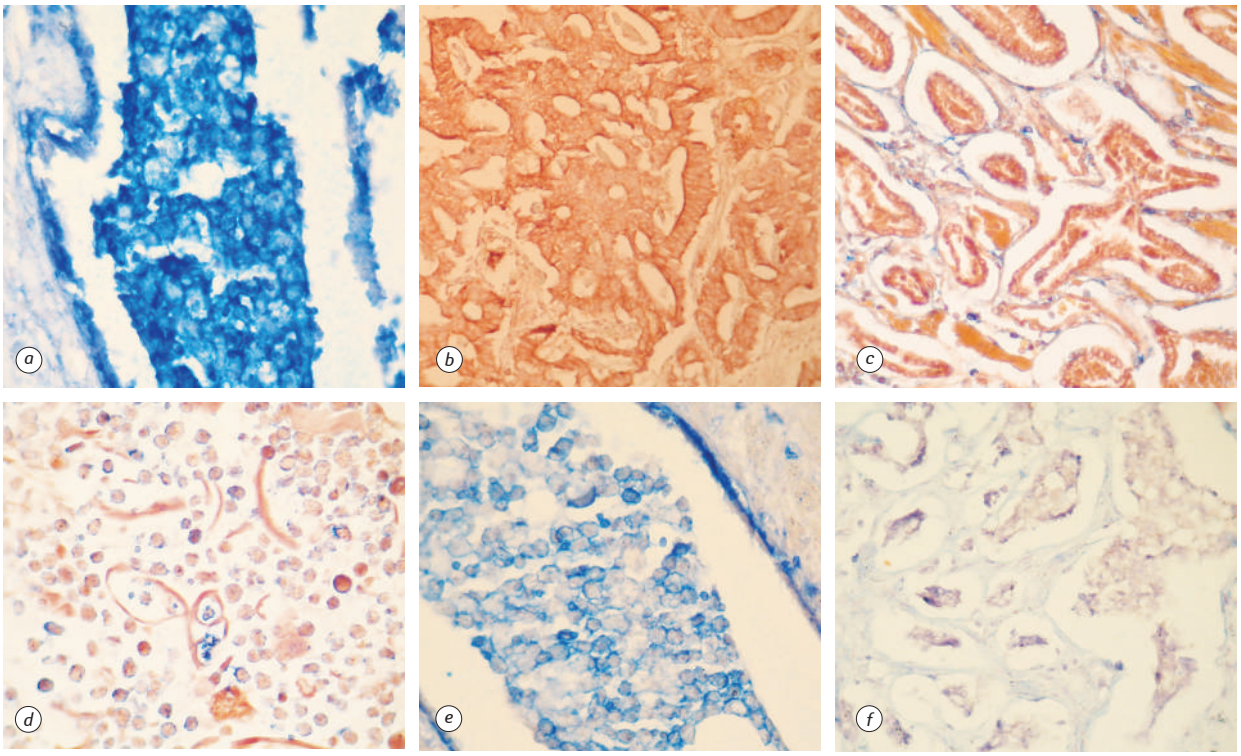

Fig. 3. Expression of CSC markers: CD44 (blue), CD24 (red). Double staining. CD44 expression was homogeneous and located on the cytoplasmic membrane; CD24 was characterized by heterogeneity and detected both on the cell membrane and in the cytoplasm. Tumors of patients with PC were characterized by different numbers of CSC-positive cells: from complete absence up to tumor cell clusters positive by the studied markers: *a* — CD44 positive control, ×400; *b* — CD24 positive control, ×200; *c* — $CD44^{-/low}$ $CD24^{+}$, ×200; *d* — CD44 $CD24^{high}$, ×400; *e* — $CD44^{+}$ $CD24^{-/low}$, ×400; *f* — $CD44^{low}$ $CD24^{low}$, ×400

This indicates that NANOG might be involved in the PC development [37].

Our findings confirm the results of other studies that consider the use of NANOG expression levels as a predictive marker of cancer course. In particular, high levels of NANOG in tumor tissue are associated with aggressive clinical course of lung, breast, stomach, pancreas, ovarian cancer, etc. [38–40].

A positive correlation found between NANOG expression and tumor cell receptor status coincides with the literature data and indicates the possibility of transcription activation of the *NANOG* promoter by 5-alpha-dihydrotestosterone via AR signaling [41].

The obtained data on the increase of the NANOG level in tumor tissue along with high Ki-67 expression levels, as well as a Gleason score of $\geqslant 7$, confirm the involvement of this protein in the proliferation and progression of PC. This observation is further supported by the fact that the NANOG knockdown causes a decrease in colony formation and tumorigenicity in human breast cancer (MCF-7), colon (Colo320), and PC (PC-3, Du145, and LAPC-9) cell lines [35].

Moreover, NANOG overexpression has been found to promote the clonogenicity and proliferation of LNCaP cells under conditions of androgen deprivation and indicate the involvement of this protein in the progression of the hormone-refractory PC [42, 43]. Another evidence of the involvement of this pluripotent transcription factor in the formation of the PC malignancy is the ability of tumor cells to avoid NK cell attack due to NANOG-mediated repression of ICAM1, as revealed by Saga et al. [44].

Thus, the results obtained indicate that NANOG contributes to the PC malignancy, which points to the need for further studies of its potential use as a marker for predicting the course of the disease.

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REFERENCES

1. **Bray F, Ferlay J, Soerjomataram I, *et al.*** Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; **68**: 394–424.

2. Cancer in Ukraine, 2016–2017. Incidence, mortality, activities of oncological service. Bulletin of National Cancer Registry of Ukraine, № 19. Kolesnik OO, ed., Kyiv, 2018. 131 p.

3. **Tolkach Y, Kristiansen G.** The heterogeneity of prostate cancer: a practical approach. Pathobiol 2018, **85**: 108–16.

4. **Shoag J, Barbieri CE.** Clinical variability and molecular heterogeneity in prostate cancer. Asian J Androl 2016; **18**: 543–8.

5. **Mossanen M, Nepple KG, Grubb RL, *et al.*** Heterogeneity in definitions of high-risk prostate cancer and varying impact on mortality rates after radical prostatectomy. Eur Urol Oncol 2018; **1**: 143–8.

6. **Scherbina OV, Sakalo VS, Kovalyov MP, *et al.*** Diagnostics and monitoring of prostate cancer. Oncology 2006; **8**: 322–6 (in Ukrainian).

7. **Shaw GL, Thomas BC, Dawson SN, *et al.*** Identification of pathologically insignificant prostate cancer is not accurate in unscreened men. Br J Cancer 2014; **110**: 2405–11.

8. **Rakul SA, Kamilova TA, Golota AS, *et al.*** Prognostic and predictive biomarkers of prostate cancer. Cancer Urology 2017; **13**: 111–21 (in Russian).

9. **Gorban NA, Karyakin AO.** Prognostic factors in prostate cancer. Cancer Urology 2010; **6**: 47–53 (in Russian).

10. **Li H, Tang DG.** Prostate cancer stem cells and their potential roles in metastasis. J Surg Oncol 2011; **103**: 558–62.

11. **Tu SM, Lin SH.** Prostate cancer stem cells. Clin Genitourin Cancer 2012; **10**: 69–76.

12. **Wang G, Wang Z, Sarkar FH, *et al.*** Targeting prostate cancer stem cells for cancer therapy. Discov Med 2012; **13**: 135–42.

13. **Chambers I, Colby D, Robertson M, *et al.*** Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003; **113**: 643–55.

14. **Loh YH, Wu Q, Chew JL, *et al.*** The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 2006; **38**: 431.

15. **Palla AR, Piazzolla D, Abad M, *et al.*** Reprogramming activity of NANOGP8, a NANOG family member widely expressed in cancer. Oncogene 2014; **33**: 2513-9.

16. **Xu CX, Xu M, Tan L, *et al.*** MicroRNA MiR-214 regulates ovarian cancer cell stemness by targeting p53/Nanog. J Biol Chem 2016; **291**: 34970–8.

17. **Gong S, Li Q, Jeter CR, *et al.*** Regulation of NANOG in cancer cells. Mol Carcinog 2015; **54**: 679–87.

18. **Wong DJ, Liu H, Ridky TW, *et al.*** Module map of stem cell genes guides creation of epithelial cancer stem cells. Cell Stem Cell 2008; **2**: 333–44.

19. **Jeter CR, Yang T, Wang J, *et al.*** Concise review: NANOG in cancer stem cells and tumor development: an update and outstanding questions. Stem Cells 2015; **33**: 2381–90.

20. **Prado MM, Frampton AE, Stebbing J, *et al.*** Gene of the month: NANOG. J Clin Pathol 2015, **68**: 763–5.

21. **Dhamodaran K, Subramani M, Jeyabalan N, *et al.*** Characterization of *ex vivo* cultured limbal, conjunctival, and oral mucosal cells: A comparative study with implications in transplantation medicine. Mol Vis: 2015; **21**: 828–45.

22. **Kramer MF.** Stem-loop RT-qPCR for miRNAs. Curr Protoc Mol Biol 2011; **95**: 1–22.

23. **Torres A, Torres K, Wdowiak P, *et al.*** Selection and validation of endogenous controls for microRNA expression studies in endometrioid endometrial cancer tissues. Gynecol Oncol 2013; **130**: 588–94.

24. **Livak K, Schmittgen T.** Analysis of relative gene expression data using real–time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 2001; **25**: 402–8.

25. **Babaev ER, Matveev VB, Volkova MI.** Predictors of survival in disseminated prostate cancer patients receiving hormonal therapy. Cancer Urology 2011; **7:** 78–83 (in Russian).

26. **Datta K, Muders M, Zhang H, *et al.*** Mechanism of lymph node metastasis in prostate cancer. Future Oncol 2010; **6**: 823–36.

27. **Serpa Neto A, Tobias-Machado M, Wroclawski ML, *et al.*** HER-2/neu expression in prostate adenocarcinoma: A systematic review and meta-analysis. J Urol 2010; **19**: 842–50.

28. **Chen CD, Welsbie DS, Tran C, *et al.*** Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004; **10**: 33–9.

29. **Donovan MJ, Osman I, Khan FM, *et al.*** Androgen receptor expression is associated with prostate cancer-specific survival in castrate patients with metastatic disease. BJU Int 2010; **105**: 462–67.

30. **Tolonen TT, Tammela TLJ, Kujala PM, *et al.*** Histopathological variables and biomarkers enhancer of zeste homologue 2, Ki-67 and minichromosome maintenance protein 7 as prognosticators in primarily endocrine-treated prostate cancer. BJU Int 2011; **108**: 1430–8.

31. **Wolters T, Vissers KJ, Bangma CH, *et al.*** The value of EZH2, p27kip1, BMI-1 and MIB-1 on biopsy specimens with low-risk prostate cancer in selecting men with significant prostate cancer at prostatectomy. BJU International 2010; **106**: 280–6.

32. **Berney DM, Gopalan A, Kudahetti S, *et al.*** Ki-67 and outcome in clinically localised prostate cancer: Analysis of conservatively treated prostate cancer patients from the Trans-Atlantic Prostate Group study. Br J Cancer 2009; **100**: 888–93.

33. **Grubelnik G, Boštjančič E, Pavlič A, *et al.*** NANOG expression in human development and cancerogenesis. Exp Biol Med 2020; **245**: 456–64.

34. **Deng Q, Tang DG.** Androgen receptor and prostate cancer stem cells: biological mechanisms and clinical implications. Endocr Relat Cancer 2015; **22**: 209–20.

35. **Jeter CR, Badeaux M, Choy G**, ***et al.*** Functional evidence that the self-renewal gene NANOG regulates human tumor development. Stem Cells 2009; **27**: 993–1005.

36. **Amini S, Fathi F, Mobalegi J, *et al.*** The expressions of stem cell markers: Oct4, Nanog, Sox2, nucleostemin, Bmi, Zfx, Tcl1, Tbx3, Dppa4, and Esrrb in bladder, colon, and prostate cancer, and certain cancer cell lines. Anat Cell Biol 2014; **47**: 1–11.

37. **Miyazawa K, Tanaka T, Nakai D, *et al.*** Immunohistochemical expression of four different stem cell markers in prostate cancer: High expression of NANOG in conjunction with hypoxia-inducible factor-1α expression is involved in prostate epithelial malignancy. Oncol Lett 2014; **8**: 985–92.

38. **Du Y, Ma C, Wang Z, *et al.*** Nanog, a novel prognostic marker for lung cancer. Surg Oncol 2013; **22**: 224–9.

39. **Zhao L, Liu J, Chen S, *et al.*** Prognostic significance of NANOG expression in solid tumors: a meta-analysis. Onco Targets Ther 2018; **11**: 5515–26.

40. **You L, Guo X, Huang Y.** Correlation of cancer stem-cell markers OCT4, SOX2, and NANOG with clinicopathological features and prognosis in operative patients with rectal cancer. Yonsei Med J 2018; **59**: 35–42.

41. **Ling K, Jiang L, Liang S, *et al.*** Nanog interaction with the androgen receptor signaling axis induce ovarian cancer stem cell regulation: studies based on the CRISPR/Cas9 system. J Ovarian Res 2018; **11**: 36.

42. **Jeter CR, Liu B, Liu X, *et al.*** NANOG promotes cancer stem cell characteristics and prostate cancer resistance to androgen deprivation. Oncogene 2011; **30**: 3833–45.

43. **Jeter CR, Liu B, Lu Y, *et al.*** NANOG reprograms prostate cancer cells to castration resistance via dynamically repressing and engaging the AR/FOXA1 signaling axis. Cell Discov 2016; **2**: 1–19.

44. **Saga K, Park J, Nimura K, *et al.*** NANOG helps cancer cells escape NK cell attack by downregulating ICAM1 during tumorigenesis. J Exp Clin Cancer Res 2019; **38**: 1–13.