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Expression of cancer-associated genes in prostate tumors at mRNA and protein levels

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Aim: To analyze an expression pattern of the cancer-associated genes in prostate tumors at mRNA and protein levels and find putative association between the expression of these genes and the genes, controlling epithelial to mesenchymal cell transition (EMT), the markers of prostate cancer and stromal elements. **Methods:** Relative expression of genes was assessed by a quantitative PCR in 29 prostate cancer tissue samples (T) of different Gleason score (GS) and tumor stage, 29 paired conventionally normal prostate tissue (CNT) samples and in 14 samples of prostate adenomas (A). Immunohistochemistry (IHC) was used to assess protein expression. **Results:** We found significant differences ($p < 0.05$) in RE of three genes (*FOS, PLAU, EPDR1*) between the T, N and A groups. *FOS* was induced in T and CNT, compared with A whereas *PLAU* and *EPDR1* were decreased. Noteworthy, RE of the five genes (*FOS, EFNA5, TAGLN, PLAU* and *EPDR1*) changed significantly, depending on GS ($p < 0.05$) in T, compared to the A and/or CNT groups. The FOS protein signal was higher in adenocarcinomas, compared to hyperplasia. The same trend was demonstrated by q-PCR. *FOS* expression increased upon the tumor development i.e. was higher in the tumors at stage 3-4. *PLAU* expression was decreasing meanwhile, as was shown by q-PCR and IHC. **Conclusions:** IHC data allowed us to understand the high levels of RE dispersion. Mainly, it is due to the expression in other cell types, and not in the prostate gland cells. For the meaningful clustering, prognosis and also for the creation of specific biomarker panels, these two methods should be adequately merged.

Keywords: prostate tumors, gene expression pattern, prostate cancer-associated genes, IHC analysis.

Introduction

Earlier, we have demonstrated that relative expression of seven cancer-associated genes, namely the *TGFB1, IL1B, FOS, EFNA5, TAGLN, PLAU* and *EPDR1*genes, is altered in prostate cancer cell lines [1, 2] and prostate cancer tissues [3]. The proteins, encoded by these genes, play an important role in carcinogenesis and are involved in a number of cellular processes and pathways. For example, *TGFB1* is implicated in the control on EMT and angiogenesis [4]. Importantly, there is an interplay between TGFB1 and androgen receptor signaling pathways, that is crucial for the development and progression of prostate cancer [5]. Usually, *TGFB1* is expressed in reactive tumor stromal cells, i.e. cancer-associated fibroblasts (CAFs) [6]. Another important player is *IL1B*, a pro-inflammatory cytokine, expressing in immune cells and activating the NF-kappa B pathway [7]. High levels of IL1B promote the skeletal colonization and progression of metastatic prostate cancer [8]. FOS is a transcription factor that takes part in many cellular processes, cell proliferation and apoptosis are among those [9, 10]. FOS is involved in the development of castration-resistant prostate cancer and also in metastasizing [15], as well as in the] development of other tumor types [11-14].

EFNA5, TAGLN and *EPDR1* encode proteins of the adhesion machinery, thus controlling the tumor progression [16]. PLAU may regulate migration and invasion upon the development of endometrial [17] and prostate [18] tumors.

Besides alteration of the expression pattern of the described seven genes, we found that the prostate-specific genes [19] and the tumor stromal elements [20] show differential expression in the tissues samples of prostate cancer, compared with the benign tumors. Also, the expression of genes, involved in EMT was altered [21], and in a proportion of prostate tumors the presence of the TMPRSS2:ERG fusion was detected [22].

In the present work we assessed the expression of seven genes (*EFNA5, EPDR1, FOS, IL1B, PLAU, TAGLN* and *TGFB1*) at the mRNA and protein levels and analyzed the putative correlation between the expression of these genes and the prostate-specific genes, tumor stromal elements and genes, controlling EMT.

Materials and Methods

A collection of prostate tissues. Samples of cancer tissue and CNT (at an opposite side of tumor) were frozen in liquid nitrogen immediately after surgical resection at the National Cancer Institute of National Academy of Medical Sciences of Ukraine (NAMU) (Kyiv, Ukraine). Benign prostate tumors (prostate adenoma samples) were collected at the Institute of Urology of NAMU (Kyiv, Ukraine) after radical prostatectomy and were frozen, as described above. All protocols were in accordance with the Declaration of Helsinki and the guidelines, issued by the Ethic Committees of the Institute of Urology of NASU, the National Cancer Institute of MHC and the Institute of molecular biology and genetics of NASU. Experiments were conducted on

29 prostate adenocarcinoma samples of different GS and tumor stages, 29 paired CNT samples and 14 samples of benign prostate tumors (adenomas). Tumor samples were characterized, according to the International System of Classification of Tumors, based on the tumor-node-metastasis (TNM) and the World Health Organization (WHO) criteria. [The] Clinicopathological characteristics (CPC) of adenocarcinomas and the presence and/or absence of the TMPRSS2/ERG fusion that was reported by us earlier [1, 21] are presented in Table 1.

Total RNA isolation and cDNA synthesis. 50-70 mg of frozen prostate tissues were mashed to a powder in liquid nitrogen. Total RNA was extracted by TRI-reagent (SIGMA), according to a manufacturer's protocol. Total RNA concentration was analyzed by a spectrophotometer (NanoDrop Technologies Inc. USA). The quality of the total RNA was determined in a 1 % agarose gel by band intensity of 28S and 18S rRNA (28S/18S ratio). cDNA was synthesized from 1 μg of the total RNA, that was treated with the RNase free DNase I (Thermo Fisher Scientific, USA), using RevertAid H-Minus M-MuLV Reverse Transcriptase (Thermo Fisher Scientific, USA), according to the manufacturer's protocol.

Quantitative PCR (q-PCR). Relative gene expression (RE) levels were detected by q-PCR, using Maxima SYBR Green Master mix (Thermo Fisher Scientific, USA) and Bio-Rad CFX96 Real-Time PCR Detection System (USA) as described earlier [19, 20]. Primers for all genes were selected from a qPrimerDepot (https://primerdepot.nci.nih.gov/) database and confirmed, using an https://www.ncbi.nlm.nih.gov/tools/primer-blast/ algorithm.

Reference gene *TBP* was used for gene expression normalization [3, 23]. Two main models ($2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods), described earlier [19–21] were used for calculation and analysis of RE levels.

Analysis of a protein expression pattern by IHC in prostate tissues. Fresh prostate tissues were fixed in a neutral buffered 4 % formaldehyde solution. After fixation, dehydration, and embedding in paraffin, serial sections were cut at a thickness of 5 μm and stained with hematoxylin/eosin for histological diagnosis.

Expression of the TGFB1, PLAU, FOS, IL1b and TAGLN proteins was assessed, using the specific antibodies by immunohistochemistry. After heating at 56ºC, paraffin was dissolved in xylol and the tissue was rehydrated by stepwise washing with ethanol in phosphate-buffered saline (PBS) (99 %, 90 %, 70 %, and 30 % ethanol). Tissues were then treated with a 2 % solution of hydrogen peroxide in methanol at room temperature for 30 min, to reduce background staining. Epitopes were exposed in a hot citrate buffer in 92°C water bath for 15 min. Antibodies were diluted (1:100 mouse antibodies and 1:100 – rabbit) in the blocking buffer (2 % bovine serum albumin, 0.2 % Tween-20, 10 % glycerol, and 0.05 % NaN3 in PBS). Protein signals were visualized by an EnVision™ Detection Peroxidase/DAB system (Dako, Glostrup, Denmark). Nuclei were stained with Mayer's hematoxylin (Dako).

Statistical analysis. The Kolmogorov-Smirnov test was used to analyze the normality of distribution. The Wilcoxon Matched Pairs test was performed to compare RE in prostate adenocarcinoma and paired CNT, using the $2^{-\Delta Ct}$ model. The Benjamini-Hochberg

procedure with false discovery rate (FDR) 0.10-0.25 was used for multiple comparisons [24]. Differences in RE more, than two-folds were considered as significant, for the $2^{-\Delta\Delta Ct}$ model (i.e. > 2.01 and < 0.49). The Fisher exact test was performed to analyze differences between these sample groups [19, 20]. The Kruskal-Wallis test was used to determine differences between groups of T, CNT and A in $2^{-\Delta Ct}$ model. The Dunn-Bonferoni post hoc test for multiple comparisons was performed to analyze RE differences between pairs of investigated groups. The Spearman's rank correlation test was used to find the putative correlations between RE and CPC of prostate tumors and also between RE levels of the studied genes. The K-Mean clustering was applied for prostate cancer subtyping and also for the specific gene expression profiles, following by the Kruskal-Wallis and Dunn-Bonferoni post hoc tests for detection of RE differences between clusters.

Results

Expression pattern of the EFNA5, EPDR1, FOS, IL1B, PLAU, TAGLN and TGFB1 genes in prostate tissues

According to the Kolmogorov-Smirnov test, RE of investigated genes in the adenoma group did not show the Gaussian distribution (normal); therefore, nonparametric statistical tests and methods were used. We assessed RE levels of seven cancer-associated genes in the paired T/CNT samples, using the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ calculations. The samples were grouped, according to the GS (GS ≤ 7, GS > 7), tumor stage (stage 1-2 and stage 3-4) and by the presence of the TMPRSS2/ERG fusion transcript. Noteworthy, the *EFNA5* and *EPDR1* genes show very low expression in both tumors and normal tissues.

The Wilcoxon Matched paired test in the $2^{-\Delta Ct}$ model showed that only two genes, namely *PLAU* and *IL1B* were differentially expressed in various groups. *PLAU* was de-

Table 1. **Clinico-pathological characteristics and the presence and/or absence of the TMPRSS2/ERG fusion of prostate adenocarcinomas**

Sample N	GS	TNM	Stage	Age	PSA (ng/ml)	Fusion status
1	< 7	T2cNxM0	II	54	27.3	-
2	< 7	T3bNxM0	III	74	23.6	-
3	< 7	T2bNxM0	II	66	6.5	-
4	< 7	T2cNxM0	II	56	25.2	-
5	< 7	T2aNxM0	II	67	18.6	+
6	< 7	T2aN0M0	II	57	9.3	+
7	< 7	T2pN0M0	II	55	5.0	+
8	< 7	T2aN0M0	II	63	13.3	+
9	< 7	T2cN0M0	II	67	29.1	+
10	7	T2aNxM0	II	77	11.7	-
11	7	T2cNxM0	II	69	13.9	-
12	7	T2cNxM0	II	64	19.8	-
13	7	T2aNxM0	II	54	7.1	+
14	7	T1cNxM0	I	68	8.2	+
15	7	T2cNxM0	II	68	19.3	+
16	7	T2aNxM0	II	62	5.6	+
17	> 7	T3aN0M1	IV	76	37.8	-
18	> 7	T2cN0M1	IV	62	22.6	-
19	> 7	T2bNxM0	II	53	6.9	-
20	> 7	T3bNxM0	III	48	51.0	-
21	> 7	T2bNxM0	III	61	0.5	-
22	> 7	T2bN0M0	II	63	20.3	-
23	> 7	T3bN0M0	III	60	12.1	+
24	> 7	T3aN0M0	III	58	25.1	+
25	> 7	T3bN0M0	III	56	84.2	+
26	> 7	T3bNxM0	III	63	20.9	+
27	> 7	T2cN1M0	IV	58	17.0	+
28	> 7	T2bNxM0	II	65	33.0	+
29	> 7	T3bNxM0	III	54	106.0	+

Notes: + — presence of the TMPRSS2/ERG fusion; - — absence of the TMPRSS2/ERG fusion

creased significantly in T, compared with corresponding CNT (N) ($p = 0.0092$). The same was true when the paired adenocarcinomas and CNT with GS ≤ 7 ($p = 0.0258$), a group of paired T/N with stage 3-4 ($p = 0.0206$) and the fusion negative paired T/N ($p = 0.0229$) were analyzed. *IL1B* expressed at significantly lower levels in T with GS > 7 ($p = 0.0192$).

Using the $2^{-\Delta\Delta Ct}$ model and calculations of the Fisher exact test, we found that *EFNA5* ($p = 0.021$) and *PLAU* ($p = 0.038$) were expressed at lower levels in tumors, compared with the paired CNT. Of note, only *IL1B* expressed at lower levels in adenocarcinomas with GS > 7 ($p = 0.030$) and in the adenocarcinoma group where the TMPRSS2/ERG fusion transcript was detected ($p = 0.030$).

Importantly, statistical calculations did not vary, regardless of whether RE fold changes were assessed, according to the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ model.

The changes in RE levels of the investigated genes were calculated for the samples of three groups (T, N, A) (Table 2).

We found that RE values of the majority of the investigated genes fluctuated in each group, especially in adenocarcinomas. Taking into consideration a nature of CNT, the A group was used as the control [19, 21]. Significant RE differences between the groups were detected by the Dunn-Bonferroni post hoc test for multiple comparisons ($p < 0.05$).

We found significant differences ($p < 0.05$) in RE of three genes (*FOS, PLAU, EPDR1*) between the T, N and A groups (Figure 1).

RE in adenoma samples was a normalization point. *FOS* was induced in T and CNT, compared with A whereas *PLAU* and *EPDR1* were decreased. The same character of RE

Table 2. **Descriptive statistics of gene RE in T, N and A groups and significant RE differences by Kruskal-Wallis test***

Gene	Group	Median	25th percentile	75th percentile	p-value *
TGFB1	**T**	0.750	0.254	1.813	
	N	0.707	0.517	1.458	
	A	0.534	0.375	0.846	
IL1B	**T**	0.957	0.369	3.311	
	N	1.352	0.707	3.422	
	A	0.565	0.224	2.383	
FOS	**T**	32.287	14.955	80.560	0.0012
	N	31.950	17.623	57.463	
	A	3.380	0.628	17.489	
EFNA5	**T**	1.741	0.463	6.759	
	N	3.148	1.180	11.277	
	A	1.373	0.788	1.773	
TAGLN	**T**	0.508	0.071	2.154	
	N	0.826	0.162	1.738	
	A	0.450	0.378	0.599	
PLAU	**T**	0.026	0.003	1.000	0.0007
	N	0.112	0.013	1.000	
	A	1.440	0.807	2.041	
EPDR1	**T**	0.088	0.016	0.688	0.0052
	N	0.082	0.028	0.677	
	A	0.945	0.507	1.149	

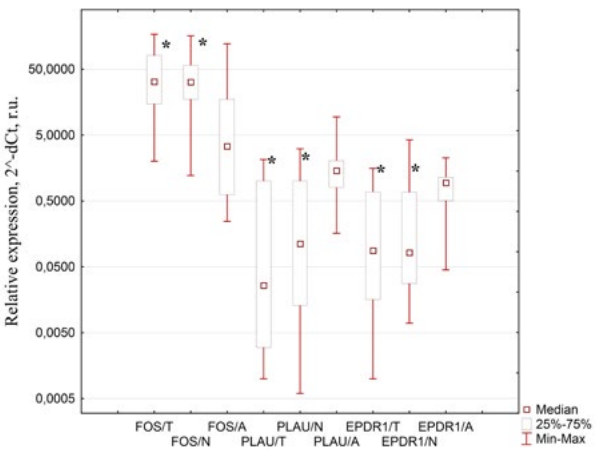

Fig. 1. RE of genes in adenocarcinomas (T), CNT (N) and adenomas (A) with differences. * — significant differences with adenoma group ($p < 0.05$), according to the Dunn-Bonferroni post hoc test.

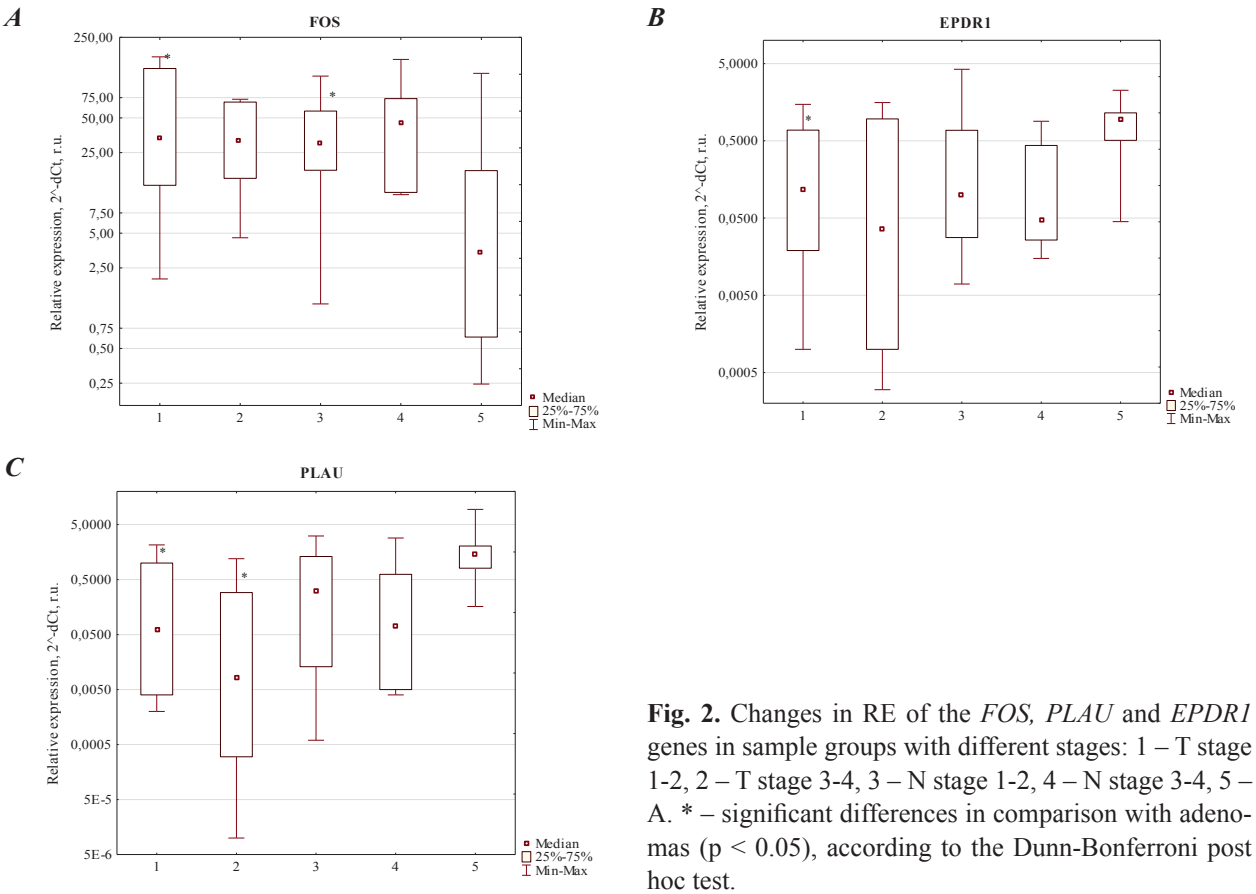

Fig. 2. Changes in RE of the *FOS, PLAU* and *EPDR1* genes in sample groups with different stages: 1 – T stage 1-2, 2 – T stage 3-4, 3 – N stage 1-2, 4 – N stage 3-4, 5 – A. * – significant differences in comparison with adenomas ($p < 0.05$), according to the Dunn-Bonferroni post hoc test.

changes for these three genes we have observed in the groups with different tumor stages (Figure 2). Noteworthy, RE of five genes (*FOS, EFNA5, TAGLN, PLAU* and *EPDR1*) changed depending on GS ($p < 0.05$) in adenocarcinomas, compared to the A group (Figure 3). These genes were expressed similarly in the T and CNT samples.

Next, we calculated the RE pattern for the groups, where the TMPRSS2/ERG fusion was either present or absent (Figure 4). We have found the specific changes in RE of *FOS* and *EPDR1* in the group of samples, where no fusion was detected (Figure 4).

Relations of changes in RE patterns of the investigated genes with CPC and expression of genes, encoding hormone receptors, stromal markers and controlling EMT

The Spearman Rank Order Correlations (r^s) analysis did not show any correlation between RE of the investigated genes and CPC, as we found earlier [3].

We calculated many significant gene-to-gene correlations between RE of the investigated genes in adenocarcinomas (Table 3A). The biggest number of correlations (5 out of 6 calculated) was found for *TGFB1* and

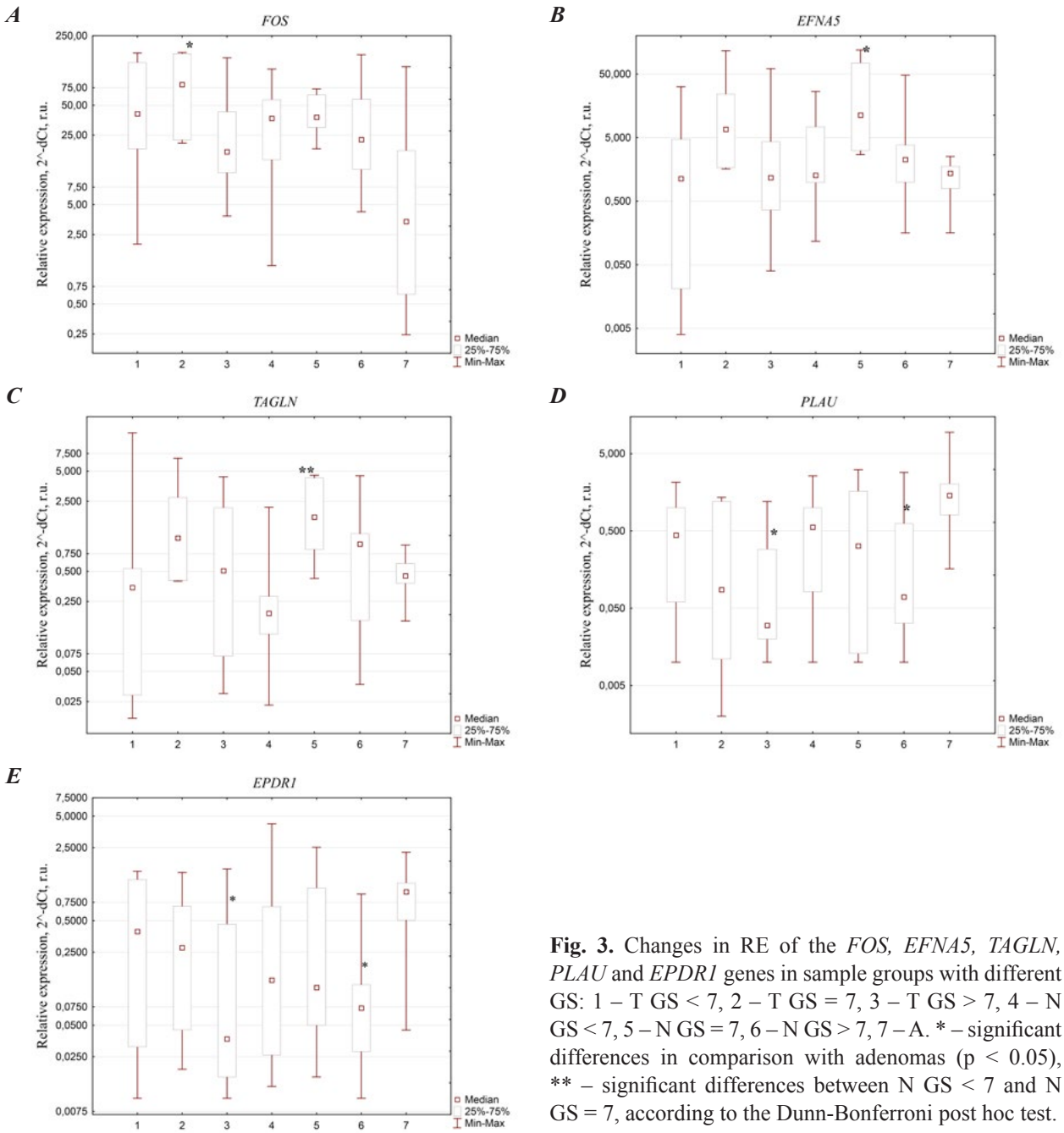

Fig. 3. Changes in RE of the *FOS, EFNA5, TAGLN, PLAU* and *EPDR1* genes in sample groups with different GS: 1 – T GS < 7, 2 – T GS = 7, 3 – T GS > 7, 4 – N GS < 7, 5 – N GS = 7, 6 – N GS > 7, 7 – A. * – significant differences in comparison with adenomas ($p < 0.05$), ** – significant differences between N GS < 7 and N GS = 7, according to the Dunn-Bonferroni post hoc test.

EFNA5. The *FOS* gene showed only 2 correlations.

Earlier, we demonstrated that the expression pattern of the genes, controlling EMT [21],

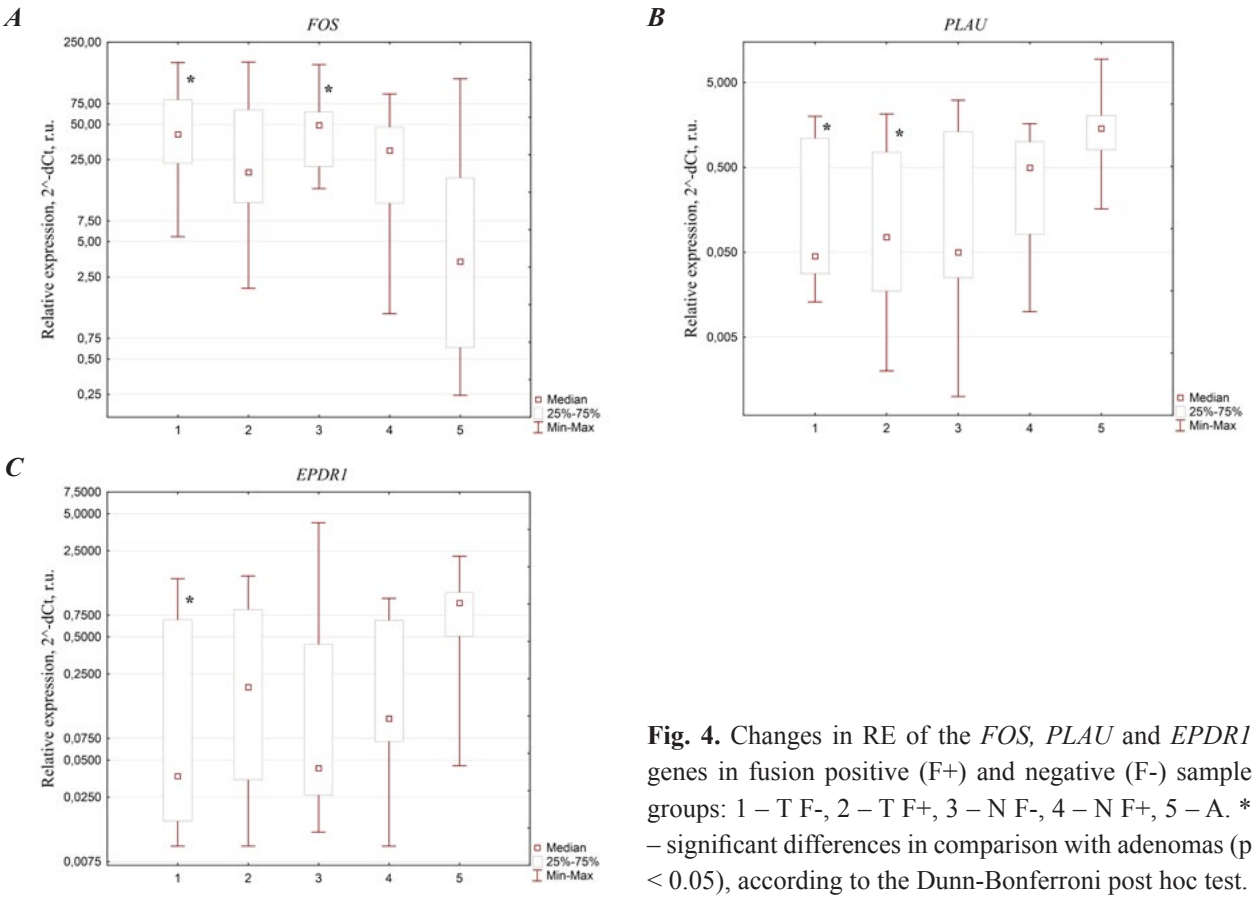

Fig. 4. Changes in RE of the *FOS, PLAU* and *EPDR1* genes in fusion positive (F+) and negative (F-) sample groups: 1 – T F-, 2 – T F+, 3 – N F-, 4 – N F+, 5 – A. * – significant differences in comparison with adenomas ($p < 0.05$), according to the Dunn-Bonferroni post hoc test.

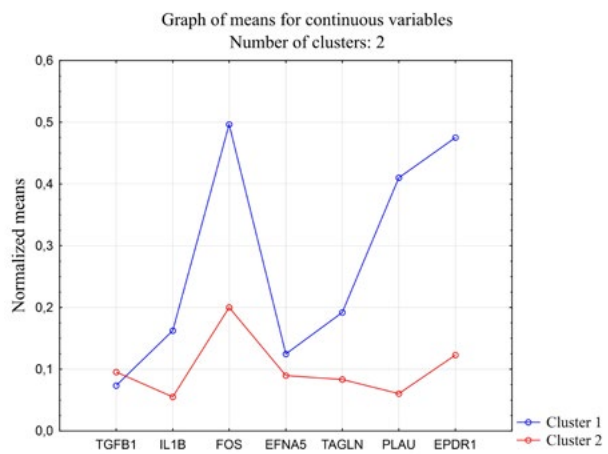

Fig. 5. K-means clustering of prostate adenocarcinomas, depending on RE of the seven genes.

encoding receptors, metabolic enzymes [19] and tumor microenvironment markers [20] dramatically altered in prostate tumors, compared with adenomas. Now we report that expression of the seven presently investigated genes follows many correlations to RE of these genes (Table 3B). We have found that RE of 23 genes (out of 56) correlated significantly with RE of seven presently investigated genes. The most interesting among all the genes is *TAGLN* in this sense.

K-means clustering

Next, we wanted to group the samples of prostate adenocarcinoma, considering RE of

Table 3. **The Spearman Rank Order Correlations (r^s) of RE patterns of the investigated genes (A) in relation to expression of genes, encoding hormone receptors, stromal elements and controlling EMT (B)**

A.

Gene/Gene	*TGFB1*	*IL1B*	*FOS*	*EFNA5*	*TAGLN*	*PLAU*
IL1B	***0.5828***					
FOS	0.3404	***0.6310***				
EFNA5	***0.8873***	***0.6636***	0.3976			
TAGLN	***0.6836***	0.3730	0.3547	***0.6276***		
PLAU	0.3710	0.2797	0.0523	0.3449	0.1875	
EPDR1	**0.5309**	0.3258	0.0471	**0.5065**	0.4328	**0.5598**

B.

Gene/Gene	*TGFB1*	*IL1B*	*FOS*	*EFNA5*	*TAGLN*	*PLAU*	*EPDR1*
CDH2	0.3128	-0.0177	-0.0951	0.3370	**0.4998**	-0.0133	0.2667
FN1	0.4103	0.3064	0.3374	**0.4461**	***0.7703***	0.0636	0.1802
MMP2	0.3956	0.0897	0.0665	0.3614	0.2801	0.3059	0.2001
KRT18	-0.4266	-0.2379	-0.3143	-0.3633	***-0.6806***	-0.1416	-0.0917
CASP3	0.2335	0.2207	0.035	0.3003	**0.5067**	0.1623	0.3983
PTEN	0.2931	0.1246	0.2374	0.4010	0.4028	0.2161	0.0547
PSA	-0.3108	-0.1655	-0.3419	-0.2101	**-0.5185**	-0.1761	-0.0969
HOTAIR #	0.0718	-0.0969	-0.003	0.0887	**0.4854**	-0.1694	0.1826
SCHLAP1 #	-0.3120	-0.3815	**-0.4544**	-0.2763	**-0.4481**	-0.3223	-0.0910
GCR (AG isof)	0.2345	0.0966	0.0374	0.2131	**0.5646**	0.1564	0.2016
SRD5A2	0.3235	**0.4527**	0.1905	**0.4264**	0.0079	0.3383	0.1490
ACTA2	0,3690	0,1453	0,2645	0,3190	***0,6126***	0,2408	0,2097
CXCL12	0,2764	0,2300	0,3212	0,3468	0,3380	0,3710	0,1949
CTGF	0,1803	0,4345	0,4064	0,2037	0,4303	0,3651	0,2378
HIF1A	0,3921	**0,5286**	0,3296	**0,5030**	0,2663	0,2891	0,2704
FAP	0,3197	0,3020	0,1882	**0,4658**	0,2151	0,2748	0,1464
CIAS	0,0315	0,3089	**0,4655**	0,0631	0,1909	0,2728	0,0791
IRF1 (T1)	0,0512	0,3828	0,3724	0,1682	0,1567	0,0059	0,0527
IL1RL1 (T2)	0,0586	-0,0246	0,0749	-0,0320	0,3971	-0,0577	0,1836
IL1R1 (T17)	0,2916	0,2172	0,0502	0,4183	0,2483	-0,0562	0,1336
CCR4	-0,0335	0,1616	0,0227	0,0475	-0,1054	0,3725	0,0012
CCL22	-0,2163	-0,0340	-0,0833	-0,0564	-0,4185	0,1367	-0,0882
NOS2A	0,3901	0,2493	0,1867	0,3084	0,2190	0,1253	0,0781

Note: ***red bold italic – p < 0.001*** **-**, **red bold – p < 0.01**–, red – p < 0.05; # – long non-coding RNA

Table 4. **Prostate adenocarcinomas CPC and RE means of clusters and statistical significant differences between them (Dunn-Bonferroni post hoc test)**

Cluster N	N of cases	%	GS	*TGFB1*	*IL1B*	*FOS*	*EFNA5*	*TAGLN*	*PLAU*	*EPDR1*
1	12	41.38	6	3.346	5.857	85.243	14.463	2.319	0.875	0.744
2	17	58.62	9	4.335	2.008	35.527	10.407	1.007	0.129	0.193
*** p value < 0.05**			*			*			*	*

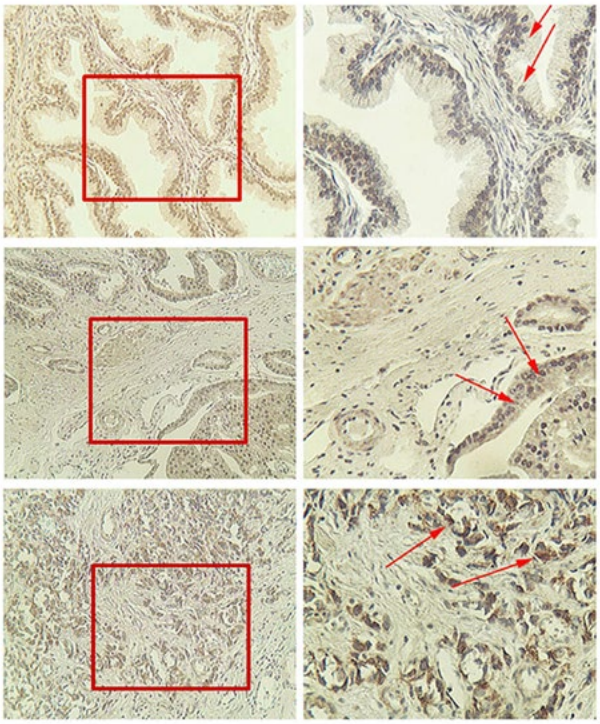

Fig. 6A. IHC on the FOS protein expression. The top row – hyperplasia, the middle row – stage I tumor, the bottom row – stage IV tumor.

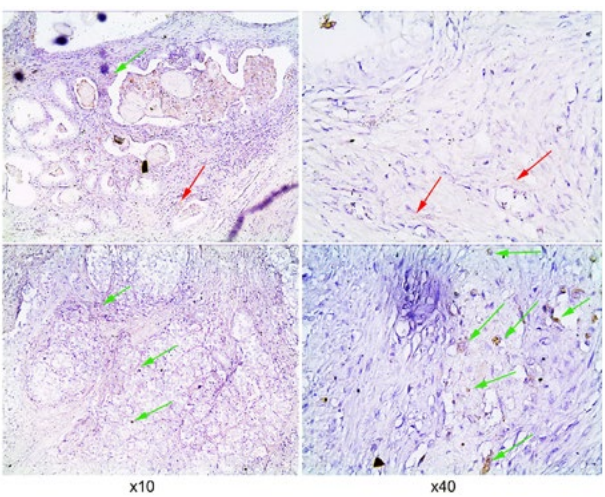

Fig. 6B. IHC on the IL1B protein expression. The top row – hyperplasia, the bottom row – stage IV tumor.

the seven investigated genes and CPC, i.e. GS and tumor stage. The K-means clustering was performed and as a result, two specific clusters were formed, that included all the samples of prostate adenocarcinoma (Figure 5, Table 4). In these clusters, the expression of *FOS*, *PLAU* and *EDPR1* varied significantly. The first cluster contained mainly the tumors with median GS = 6, and the second cluster – with GS = 9. In other words, the second cluster (Cluster 2 in Figure 5) consisted of more aggressive prostate adenocarcinomas.

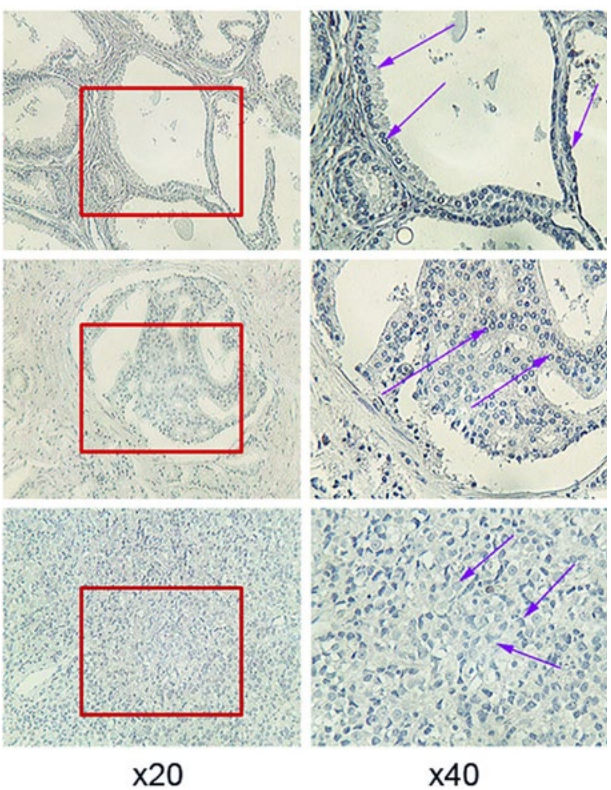

Fig. 6C. IHC on the PLAU protein expression. The top row – hyperplasia, the middle row – stage I tumor, the bottom row – stage IV tumor.

Expression pattern of FOS, IL1B, PLAU, TAGLN and TGFB1 proteins in prostate tissues

Using the IHC, we found that the expression of FOS protein was different in hyperplasia and tumors: the FOS signal was more intensive in low differentiated tumors, compared to prostate hyperplasia (Figure 6A). Notice an increase of the brown signal in the epithelial prostate cells (red arrows). The right panel shows the magnified field, indicated by a red square on the left panel.

The IL1B signal was detected, most probably, in blood cells (Figure 6B). Notice the absence of the brown signal in hyperplasia (red arrows). The brown signal is detected, most probably, in blood cells in tumor (green arrows). More infiltrating lymphocytes were found in low differentiated prostate carcinoma, than in hyperplasia. In epithelial prostate cells IL1B was hardly detectable.

Noteworthy, the PLAU protein showed expression pattern, opposite to FOS – the weak PLAU signal in hyperplasia vanished in highly advanced carcinomas (Figure 6C). Notice a decrease of the brown signal in the epithelial prostate cells (violet arrows). The right panel shows the magnified field, indicated by a red square on the left panel.

The TAGLN protein was not detected in prostate cells in hyperplasia (red arrows, Figure 6D, the top panel). Of note, it was highly expressed in stromal fibroblasts (black

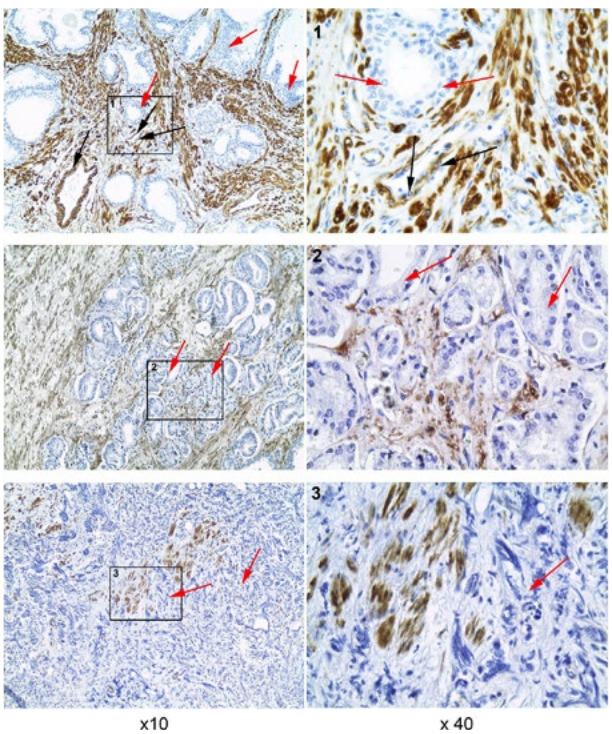

Fig. 6D. IHC on the TAGLN protein expression. The top row – hyperplasia, the middle row – stage I tumor, the bottom row – stage IV tumor.

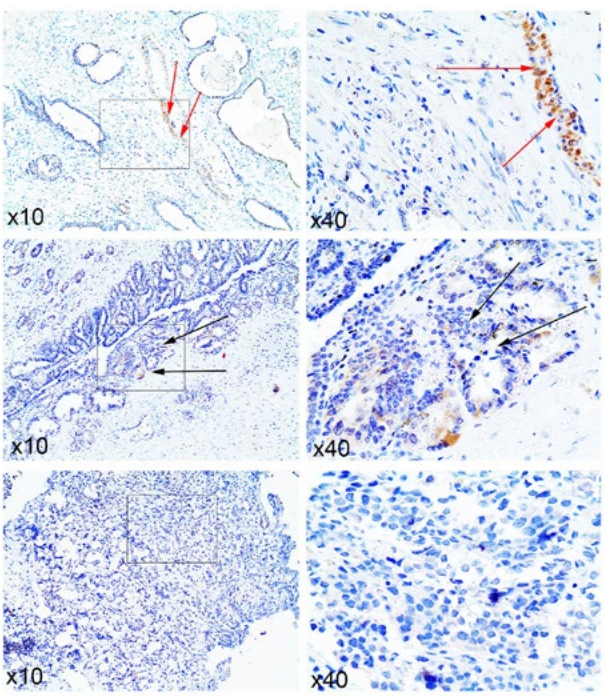

Fig. 6E. IHC on the TGFB1 protein expression. The top row – hyperplasia, the middle row – stage I tumor, the bottom row – stage IV tumor.

arrows, Figure 6D, the top panel). Upon cancer development, the prostate cells remained negative for TAGLN (Figure 6D, the middle and bottom panels). Notice the absence of the brown signal in the epithelial prostate cells (red arrows). The right panel shows the magnified field, indicated by a black square on the left panel. Of note, the stroma cells express TAGLN (black arrows). Due to the fact, that less fibroblasts were present in the Stage IV tumors, the *TAGLN* expression at the mRNA levels was reduced as shown, using the q-PCR.

The TGFB1 signal was quite strong in hyperplasia (red arrows in Figure 6E, the top panel). In moderately differentiated cancers TGFB1 was decreased (black arrows in Figure 6E, the middle panel). In low differentiated tumors the TGFB1 protein was hardly detected (Figure 6E, the bottom panel). Notice the strong brown signal in the epithelial prostate cells in hyperplasia (red arrows). Of note, the TGFB1 signal is decreased in stage I tumor (black arrows) and is absent in stage IV tumor. The right panel shows the magnified field, indicated by a black square on the left panel. In other words, upon the development of prostate cancer the levels of TGFB1 gradually decreased in the prostate tissue cells.

Discussion

In the present paper, we investigated whether the expression of seven genes, namely *EFNA5, EPDR1, FOS, IL1B, PLAU, TAGLN* and *TGFB1,* follows the pattern of the EMT-related genes, the prostate cancer-associated genes and several tumor stromal markers. In addition, we wanted to understand, whether the presence and/or absence of the TMPRSS2/ERG fusion can influence the expression of the abovementioned genes. Moreover, the expression assessment of these seven genes at the mRNA levels was supplemented by the analysis of the encoded proteins (Table 5).

The TGFB1 protein signal decreased upon the tumor progression. The q-PCR analysis demonstrated high levels of dispersion of RE values. The means at the minimum and maximum were scattered for more than 100 fold. Of course, TGFB1 is expressed in various cells. Therefore, we did not demonstrate significant changes in *TGFB1* RE between the groups of prostate tumors.

Table 5. **Expression of the seven genes at the mRNA and protein levels**

Gene	mRNA (q-PCR)			Protein (IHC)		
	A	T, stage 1-2	T, stage 3-4	A	T, stage 1	T, stage 4
TGFB1	+++	+++ &	+++ &	+++ e	++ e	-
IL1B	+	+ &	+ &	+ s	ND	+ s
FOS	+	+++ ↑	++ &	+ e	++ e	+++ e
EFNA5	+	+ ↓	+ ↓	ND	ND	ND
TAGLN	+	+ &	+ &	+++ s	++ s	+ s
PLAU	++	+ ↓	+ ↓	+ e	+ e	- e
EPDR1	+	+ ↓	+ &	ND	ND	ND

Notes: «+++» – high level of expression; «++» – moderate level of expression; «+» – low level of expression; «-» – no expression; «ND» – IHC staining was not done; & – high RE dispersion level; ↑ – significant increase, compared to the A group; ↓ – significant decrease, compared to the A group; e – protein expression in cancer/prostate cells; s – protein expression in stromal/blood cells

IL1B is another gene that was expressed similarly in all tumors. Probably, it is due to the fact, that it is expressed mainly by hematopoietic cells. Using q-PCR, it is impossible to distinguish cell types.

The *FOS, PLAU* and *EPDR1* genes show dependence of RE on the tumor stage, GS and the presence and/or absence of the TMPRSS2:ERG fusion. These three genes demonstrate differential expression in two adenocarcinoma subtypes, as was shown by the clustering analysis. The FOS and PLAU proteins are expressed in the prostate cancer cells. The FOS signal was higher in adenocarcinomas, compared to hyperplasia. The same trend was demonstrated by q-PCR, when the T group was compared to the A group. The *FOS* expression increased upon the tumor development i.e. was higher in tumors at stage 3-4. The *PLAU* expression decreased under the same conditions, as was shown by q-PCR and IHC. *TAGLN* demonstrated RE differences only between the CNT groups with GS < 7 and GS=7. RE of *TAGLN* was quite dispersed in adenocarcinomas. The TAGLN protein was found in the tumor stroma and fibroblasts, but not in the prostate gland cells. *EFNA5* showed the RE differences only between CNT with GS=7 and the adenoma groups.

Matching the expression data at different levels (mRNA and protein), using different statistic methods, allows us to understand and visualize the ambiguous results of the expression of the studied genes in the prostate cancer samples.

Conclusions

The IHC data allowed us to understand high levels of the RE dispersion. Mainly, it is due to the expression in other cell types, not in the prostate gland cells. For the meaningful clustering, prognosis as well as for the creation of specific biomarker panels, these two methods should be adequately merged.

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Експресія пухлино-асоційованих генів у пухлинах передміхурової залози на рівнях мРНК та білків

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Мета: Проаналізувати патерни експресії пухлино-асоційованих генів на рівнях мРНК та протеїнів та вивчити

можливу асоціацію між експресією цих генів та генів, що контролюють епітеліально-мезенхимальний перехід, маркерів раку передміхурової залози та стромальних елементів. **Методи:** Відносні рівні експресії (ВЕ) генів були встановлені за допомогою кількісної ПЛР (кПЛР) у 29 зразках аденокарцином передміхурової залози (П) з різними ступенями Глісона (СГ) та стадіями захворювання, 29 парних умовно-нормальних тканин передміхурової залози (Н) та 14 зразках аденом (А). Імуногістохімія (ІГХ) була використана для встановлення рівнів експресії протеїнів. **Результати:** Виявлено значні відмінності ВЕ ($p < 0.05$) для трьох генів (*FOS, PLAU, EPDR1)* між групами П, Н та А. *FOS* має підвищені рівні ВЕ у групах П та Н у порівнянні з А, тоді як *PLAU* та *EPDR1* навпаки знижені рівні ВЕ у цих групах. Примітно, що п'ять генів (*FOS, EFNA5, TAGLN, PLAU* та *EPDR1*) мають зміни ВЕ в залежності від СГ у П у порівнянні з А та/або Н. Білок FOS має підвищений сигнал у аденокарциномах у порівнянні з аденомами. Ті ж зміни продемонстровані й кПЛР. Експресія *FOS* підвищується при розвитку пухлин, тобто, вона є вищою у пухлинах з 3-4 стадією. Експресія *PLAU* навпаки знижується, як було показано кПЛР та ІГХ методами. **Висновки:** Дані ІГХ дозволили зрозуміти високий рівень дисперсії ВЕ. В основному це пов'язано з експресією генів у інших типах клітин, а не тільки в клітинах передміхурової залози. Для успішної кластеризації, потенційного прогнозу та створення специфічних панелей біомаркерів ці два методи повинні бути адекватно об'єднані.

Ключові слова: рак передміхурової залози, патерни експресії генів, простато-специфічні пухлино-асоційовані гени, ІГХ аналіз.

Экспрессия опухоль-ассоциированных генов в опухолях простаты на уровнях мРНК и белка

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Цель: Проанализировать паттерны экспрессии опухоль-ассоциированных генов на уровнях мРНК и белка и исследовать возможную ассоциацию между экспрессией этих генов и генов, которые контролируют эпителиально-мезенхимальных переход, маркеров рака простаты и стромальных элементов. **Методы:** Уровни относительной экспрессии (ОЭ) генов были установлены с помощью количественной ПЦР (кПЦР) в 29 образцах аденокарцином простаты (О) с различными степенями Глиссона (СГ) и стадиями заболевания, 29 парных условно-нормальных тканей простаты (Н) и 14 образцах аденом (А). Иммуногистохимия (ИГХ) была использована для установления уровней экспрессии белков. **Результаты:** Обнаружены значимые отличия ОЭ ($p < 0.05$) для трех генов (*FOS, PLAU, EPDR1)* между группами О, Н и А. Для гена *FOS* выявлены повышенные урони ОЭ в группах О и Н по сравнению с А, тогда как для *PLAU* и *EPDR1* наоборот обнаружены сниженные урони ОЭ в этих группах. Следует отметить, что пять генов (*FOS, EFNA5, TAGLN, PLAU* та *EPDR1*) имеют отличия ОЭ в зависимости от СГ в О по сравнению с А и/или Н. Белок FOS имеет повышение сигнала в аденокарциномах по сравнению с аденомами. Такие же изменения показал и кПЦР анализ. Экспрессия *FOS* повышается в процессе развития опухолей простаты, то есть она выше в опухолях 3-4 стадии. Экспрессия *PLAU* наоборот снижается, как было показано кПЦР и ИГХ методами. **Выводы:** Данные ИГХ позволили понять причину высокой дисперсии ОЭ. В основном это связано с наличием экспрессии генов в разных типах клеток, а не только в клетках простаты. Для успешной кластеризации, потенциального прогноза и создания специфических панелей биомаркеров эти два метода должны быть адекватно объединены для анализа.

Ключевые слова: рак простаты, паттерны экспрессии генов, простат-специфические опухоль-ассоциированные гены, ИГХ анализ.

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