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 clinical-pathological 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-ΔCt and 2-ΔΔCt 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 92ºC 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-ΔCt 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 $2^{-ΔΔCt}$ 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 $2^{-ΔCt}$ 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 $2^{-ΔCt}$ and $2^{-ΔΔCt}$ 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 $2^{-ΔCt}$ model showed that only two genes, namely PLAU and IL1B were differentially expressed in various groups. PLAU was de-

| Sample N | GS | TNM | Stage | Age | PSA (ng/ml) | Fusion status |
|----------|----|-----|-------|-----|-------------|--------------|
| 1        | <7 | TcNnM0 | 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 | T2aN0M0 | 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 | T2aN0M0 | II    | 62  | 5.6         | +            |
| 17       | >7 | T3aN0M1 | IV    | 76  | 37.8        | -            |
| 18       | >7 | T3cN0M1 | IV    | 62  | 22.6        | -            |
| 19       | >7 | T2bN0M0 | 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.
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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. *ILIB* expressed at significantly lower levels in T with GS > 7 (p = 0.0192).

Using the 2-ΔΔCt 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 *ILIB* 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-ΔCt or 2-ΔΔCt 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

| 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 |          |
| *ILIB* 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.
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...
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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 \textit{FOS}, \textit{PLAU} and \textit{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.

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 $\text{TAGLN}$ in this sense.

\textbf{K-means clustering}

Next, we wanted to group the samples of prostate adenocarcinoma, considering RE of
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Table 3. The Spearman Rank Order Correlations (r*) 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| 0.0350| 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| -0.0030| 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 iso) | 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
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 EDPRI 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. 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.

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 arrows). The right panel shows the magnified field, indicated by a red square on the left panel.

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 |
| *TAGLN* | + | + & | + & | +++ s | ++ s | + s |
| *PLAU* | ++ | + | + | + | + 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 патерні експресії пухлино-асоційованих генів на рівнях мРНК та білків та вивчити
Expression of cancer-associated genes in prostate tumors at mRNA and protein levels

Method: Relative expression levels (REL) of genes were determined using a quantitative PCR (qPCR) in 29 prostate adenocarcinoma samples (P) with different Gleason grades (GG) and stages of the disease, 29 age-matched normal prostate tissue samples (N) and 14 prostate adenoma samples (A). Immunohistochemistry (IHX) was used to determine expression levels of proteins.

Results: Significant differences (p < 0.05) in REL for three genes (FOS, PLAU, EPDR1) were found between groups P, N, and A. FOS showed elevated REL in groups P and N compared to A, whereas PLAU and EPDR1 showed decreased REL in these groups. Notably, five genes (FOS, EFNA5, TAGLN, PLAU, EPDR1) showed changes in REL depending on the GG of P compared to A and/or N. The protein FOS showed a higher signal in prostate adenocarcinomas compared to adenomas. Similar changes were also observed using qPCR analysis. The expression of FOS increased during the development of prostate tumors, i.e., it was higher in tumors of stages 3-4. PLAU expression, on the other hand, decreased, as shown by qPCR and IHX methods.

Conclusions: The IHX data allowed us to understand the high dispersion of REL. This is mainly due to the presence of gene expression in different types of cells, not just in prostate cells. For successful clustering, potential prognosis, and the creation of specific panels of biomarkers, these two methods should be adequately combined for analysis.

Key words: prostate cancer, patterns of gene expression, prostate-specific cancer-associated genes, IHX analysis.