Exp Oncol 2018
40, 4, 315–322

Experimental ONCOLOGY

EXPRESSION PATTERN OF GENES ASSOCIATED WITH TUMOR MICROENVIRONMENT IN PROSTATE CANCER

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Aim: **To assess relative expression (RE) levels of CAF-, TAM-specific, immune defense-associated genes in prostate tumors and to show correlation of RE with clinical, pathological and molecular characteristics, with the aim to define clinically significant specific alterations in a gene expression pattern.** ***Methods:*** **RE of 23 genes was analyzed by a quantitative polymerase chain reaction in 37 freshly frozen samples of prostate cancer tissues of a different Gleason score (GS) and at various tumor stages, compared with RE in 37 paired conventionally normal prostate tissue (CNT) samples and 20 samples of prostate adenomas.** ***Results:*** **Differences in RE were shown for 11 genes out of 23 studied, when tumor samples were compared with corresponding CNTs. 7 genes, namely *ACTA2, CXCL14, CTGF, THY1, FAP, CD163, CCL17* were upregulated in tumors. 4 genes, namely *CCR4, NOS2A, MSMB, IL1R1* were downregulated in tumors. 14 genes demonstrated different RE in TNA at different stages: *CXCL12, CXCL14, CTGF, FAP, HIF1A, THY1, CCL17, CCL22, CCR4, CD68, CD163, NOS2A, CTLA4, IL1R1*. RE changes of 9 genes — *CXCL12, CXCL14, HIF1A, CCR4, CCL17, NOS2A, CTLA4, IL1R1, IL2RA* — were found in tumors with different GS. Moreover, 9 genes showed differences in RE in TNA, dependently on the presence or absence of the TMPRSS2/ERG fusion and 7 genes showed differences in RE of groups with differential *PTEN* expression. Significant correlations were calculated between RE of 9 genes in adenocarcinomas and the stage, and GS; also, between RE of 2 genes and the fusion presence; and between RE of 4 genes and *PTEN* expression.** ***Conclusions:*** **Several gene expression patterns were identified that correlated with the GS, stage and molecular characteristics of tumors, i.e. presence of the TMPRSS2/ERG fusion and alterations in *PTEN* expression. These expression patterns can be used for molecular profiling of prostate tumors, with the aim to develop personalized medicine approaches. However, the proposed profiling requires a more detailed analysis and a larger cohort of patients with prostate tumor.**

Key Words**: prostate tumors, relative gene expression, tumor microenvironment, CAF, TAM, immune-related alterations, clinically significant expression patterns, cancer-host interaction.**

It is well known that tumor initiation and progression, including prostate cancer, depend on genetic and epigenetic changes, leading to transcriptomic and proteomic disorders in cells upon malignant transformation [1–3]. Also, many parameters are altered, that is hierarchically regulated at different levels (organism, organs and systems, tissues and cells) [4–7]. All these processes constitute a common phenomenon named as a tumor-host interaction. One of the disorders in the organism that contributes to the appearance of tumors is chronic inflammation [8, 9]. It could be of different etiologies, such as viral, bacterial, parasitic, chemical or radiation-induced and that of unidentified etiology [8, 10]. However, regardless of its origin, both, the epithelial cells of the organs and the cells of the stroma (fibroblasts, macrophages, endothelial cells, etc.) are involved. Later, all these cells become the tumor microenvironment [11], changing their properties from tumor-suppressive to tumor-stimulating and supporting [12, 13]. For example, fibroblasts are transformed into cancer-associated fibroblasts (CAF) [14, 15] and macrophages change their phenotype to tumor-associated macrophages (TAM) [16–19]. Tumors are filled with tumor-infiltrating leukocytes which suppress immune defense and reactions [20, 21].

We have to mention, that the tumor growth is affected by antimicrobial proteins, secreted by epithelial cells of the prostate. One of such proteins is a product of the *MSMB* gene [22]. Furthermore, elements of a major histocompatibility complex have as immunostimulating as immunosuppressive properties. One of the immunosuppressive glycoprotein is HLA-G [23].

Immunohistochemistry, the new generation sequencing and a real-time polymerase chain reaction (PCR) have been used to identify markers, typical for different types of cells in a tumor microenvironment [15, 16, 20]. We selected a number of genes specific for fibroblasts and CAF, macrophages and TAM and various subpopulations of T-lymphocytes, NK cells. Also, markers of inflammation and nonspecific immune reactions were chosen. The levels of relative gene expression (RE) were assessed and putative correlation with clinical and pathological characteristics (CPC) and molecular features was analyzed. Further, we wanted to monitor the specific alterations in an expression pattern of selected genes that are clinically significant,

Submitted: July 05, 2018.
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Abbreviations used: A – sample of prostate adenomas; CAF – cancer-associated fibroblasts; CNT/N – conventionally normal prostate tissue; CPC – clinical and pathological characteristics; FDR – false discovery rate; GS – Gleason score, IAG – immune-associated genes; PCR – polymerase chain reaction; RE – relative gene expression; st – stage; T – sample of prostate cancer, adenocarcinoma; TAM – tumor-associated macrophages; TNM – International System of Classification of Tumors, based on tumor-node-metastasis.

for the possible application in the molecular profiling of prostate cancers.

MATERIALS AND METHODS

Prostate tissue collection. Tissues of prostate cancer (T) and the paired conventionally normal prostate tissues (CNT, or N from a site opposite to cancer) were frozen in liquid nitrogen directly after surgery. All samples were collected at National Cancer Institute (Kyiv, Ukraine). Benign prostate tumors (prostate adenoma samples — A) were collected with the same procedure at the Institute of Urology (Kyiv, Ukraine) after radical prostatectomy. The samples were collected in accordance with the Declaration of Helsinki and the guidelines, issued by the Ethic Committee of the Institute of Urology of the National Academy of Medical Sciences of Ukraine and the National Cancer Institute and the Ethic Committee of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine. 37 prostate adenocarcinomas of different Gleason score (GS) and stages, 37 paired CNT and 20 samples of benign prostate tumors (A, adenomas) were studied. Tumors 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 classification. CPC of prostate cancer samples were described earlier [24].

Total RNA isolation and cDNA synthesis. 50–70 mg of frozen prostate tissues were mashed to powder in liquid nitrogen. Total RNA was extracted by TRI-reagent (Sigma, USA), according to the manufacturer's protocol. The 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 treated with 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. Levels of a RE of 23 genes were assessed by quantitative PCR using a Maxima SYBR Green Master mix (Thermo Fisher Scientific, USA) on Bio-Rad CFX96 Real-Time PCR Detection System (USA) under the following conditions: 95 °C — 10 min, following 40 cycles of 95 °C — 15 s, 60 °C — 30 s, elongation 72 °C — 30 s. 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.

Four reference genes *TBP*, *HPRT*, *ALAS1* and *TUBA1B* were used for gene expression normalization [25] in comparison with *TBP* [26]. Two main models ($2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods), described earlier [25, 27] were used for calculation and an analysis of RE levels.

Statistical analysis. The Kolmogorov — Smirnov test was applied to assess the normality of distribution. The Wilcoxon Matched Pairs test was performed for comparison of RE in prostate adenocarcinomas and paired normal tissues samples. RE fold differences in $2^{-\Delta\Delta Ct}$ model were considered increased in cases of changes more than 2 fold (RE fold changes > 2.01) and decreased when changes were more than 2 fold (RE fold changes < 0.49), when gene RE were compared in adenocarcinoma versus CNT. The Fisher exact test was calculated to find out differences between these sample groups [24]. The Benjamini — Hochberg procedure with false discovery rate (FDR) 0.10–0.25 was used under multiple comparisons detection [28]. The Kruskal — Wallis test was used to determine differences between experimental groups. The Dunn — Bonferroni post hoc test for multiple comparisons was performed to determine RE differences between pairs of prostate samples. The Spearman's rank correlation test was used to find possible correlations between gene RE and CPC of prostate tumors [25].

RESULTS

We have analyzed RE of 23 genes in prostate T, CNT and A. Eight genes are associated with fibroblasts and CAFs, we called them a CAF group. Six genes are associated with pro-inflammatory and immuno-suppressive macrophages (the TAM group). Nine genes RE is associated with immune defense and reactions (the immune-associated genes (IAG) group).

From the beginning, we have found differences in RE in T, compared with CNT (N) samples. Statistically significant differences in RE in the paired T/N samples are shown in Table 1 (FDR = 0.2).

Table 1. Genes with significant RE differences between prostate adenocarcinoma samples and paired CNT ($2^{-\Delta\Delta Ct}$ model)

Group	Gene	RE fold changes	
		> 2.01	< 0.49
CAF	*ACTA2*	+	
	CXCL14	+	
	CTGF	+	
	THY1	+	
	FAP	+	
TAM	*CD163*	+	
	CCR4		+
	CCL17	+	
	NOS2A		+
IAG	*MSMB*		+
	IL1R1		+

Differences in RE were calculated for 11 genes out of 23 in the paired T/N samples. Increased RE (> 2.01) in T in comparison with the paired N ($p < 0.05$) was observed for 7 genes: *ACTA2, CXCL14, CTGF, THY1, FAP, CD163, CCL17*. Decreased RE (< 0.49) in T in comparison with the paired N ($p < 0.05$) was observed for 4 genes: *CCR4, NOS2A, MSMB, IL1R1.*

Next, RE was analyzed in 3 sample groups, namely T, the paired CNT (N) and A. Furthermore, the GS, stage, presence of the TMPRSS2/ERG fusion [27], levels of *PTEN* expression (low (l) or high (h)) [25, 29] was taken into consideration for grouping of the samples. Summarized data are presented in Table 2.

These data show that the greatest number of changes in RE, for 14 out of 23 genes could be found

Table 2. Significant RE changes according to Dunn – Bonferroni post hoc test for multiple comparisons in investigated genes

Group	Gene	T/N paired	TNA total	TNA/stages	TNA/GS	TNA/fusion status	TNA/*PTEN*, l/h
CAF	*ACTA2*	+					
	CXCL12		+	+	+	+	+
	CXCL14	+	+	+	+	+	+
	CTGF	+	+	+		+	+
	FAP	+	+	+ [#]		+	+
	HIF1A			+	+		
	S100A4						
	THY1	+	+	+			
TAM	*CD68*			+ [#]			
	CD163	+		+		+	
	CCR4	+	+	+	+	+	+
	CCL17	+	+	+	+	+	
	CCL22			+		+	
	NOS2A	+		+	+		
IAG	*CIAS1*						
	CTLA4		+	+	+	+	+
	HLA-G						
	IRF1						
	IL1RL1						
	IL1R1	+	+	+	+		+
	IL2RA				+		
	KLRK						
	MSMB	+					

Note: [#]Presence of RE differences only in *TBP* normalized genes.

in TNA groups, when the stage of tumor was analyzed as well. The expression pattern of these genes and also another 2 genes (*ACTA2* and *MSMB*), for which RE was changed only in the paired T/N samples is shown on Figure. It should be noted, that for two genes (*FAP* and *CD68*) the use of four reference genes and one reference gene for normalization of RE resulted in various fold change differences.

Descriptive statistics of the data on RE in T, N and A samples at the various stages and also significant differences between groups are shown in Table 3. Among genes showing the significant differences in RE (14 genes) eight genes showed the lowest RE in the A group, namely *CTGF, CXCL14, FAP, THY1, CD163, CCL17, CCL22,* and *CTLA4*. Increased RE in T at stages 1–2 ($p < 0.05$) was observed for *CTGF, FAP, THY1, CCL22, CTLA4,* whereas increased RE in T stages 3–4 ($p < 0.05$) was found for *CD163* and *CCL17. CXCL14* was upregulated in all tumors.

CXCL12, HIF1A, CD68, CCR4, NOS2A, and *IL1R* showed the highest RE in the A group *1*. The significantly decreased RE (more than two-fold) in T stages 3–4 ($p < 0.05$) was demonstrated for five genes, namely *HIF1A, CD68, CCR4, NOS2A* and *IL1R1.* Only *CXCL12* was downregulated in all tumors. Significant changes in RE (up- and downregulation) in the CNT group of patients with tumors of the various stages was also found for several of these genes (Table 4). For example, decreased RE of *HIF1A* and *IL1R1* was detected in N compared with A in patients with T stages 3–4. The *CXCL12* was downregulated in all normal tissues. Increased RE in the CNT group was found for four genes. The *CXCL14, CD163* and *CCL17* genes were upregulated in T stages 3–4. One gene (*CTLA4*) was upregulated in all tumors.

Nine genes in groups with different GS showed similar RE changes as genes in groups with different stages (Table 2). *IL2RA* was altered only in groups with different GS. Pairs with RE variability in the TNA group and depending on the presence/absence of the TMPRSS2/ERG fusion and also of *PTEN* RE are shown in Table 4. We have found 10 genes in TNA groups with the different TMPRSS2/ERG fusion status, showing significant differences in RE ($p < 0.05$). The most interesting are genes, which were differently expressed in T with and without the TMPRSS2/ERG fusion. Three genes — *THY1, FAP* and *CCR4* — showed alterations only in T without the fusion, compared with the A group. Four genes (*CTLA4, CCL17, CCL22*) were expressed differently in the CNT group without the fusion, compared with the A group. One gene was (*CD163*) expressed at various levels in the fusion negative compared with the fusion-positive CNT groups.

Eight genes expressed at the differential levels in TNA groups ($p < 0.05$), when *PTEN* mRNA was analyzed. The most interesting among them are *THY1* and *FAP* that were expressed at significantly different RE levels in adenocarcinomas with high *PTEN* (T h) and in adenomas with high *PTEN* RE (A h). *CCR4* and *IL1R1* showed differences in adenocarcinomas with low *PTEN* RE (T l) and adenomas with high *PTEN* RE (A h).

Correlation analysis of RE related to CPC and molecular characteristics of tumors. Using the Spearman's rank correlation (r^s) test, a number of correlations was suggested between the gene expression pattern and CPC, the TMPRSS2/ERG fusion status and *PTEN* levels in prostate adenocarcinomas (Table 5). RE of the nine genes, namely *CXCL14, HIF1A, S100A4, CCL17, CCR4, NOS2A, CTLA4, IL2RA* and *MSMB* correlated with GS. RE of the nine genes (*HIF1A, S100A4, CD68, CD163, CCL17, CCL22, CCR4, NOS2A, IL1R1*) showed significant correlations with the stage. Six genes out of nine belong to the TAM group. *CD163* RE demonstrated the maximal positive correlation with the stage ($r^s = 0.615$). RE of the *THY1* gene correlated significantly with an age. RE of *CXCL14, IL1RL1* and *IRF1* correlated with PSA levels. RE of *ACTA2* and *CXCL12* negatively correlated with the TMPRSS2/ERG fusion status. These

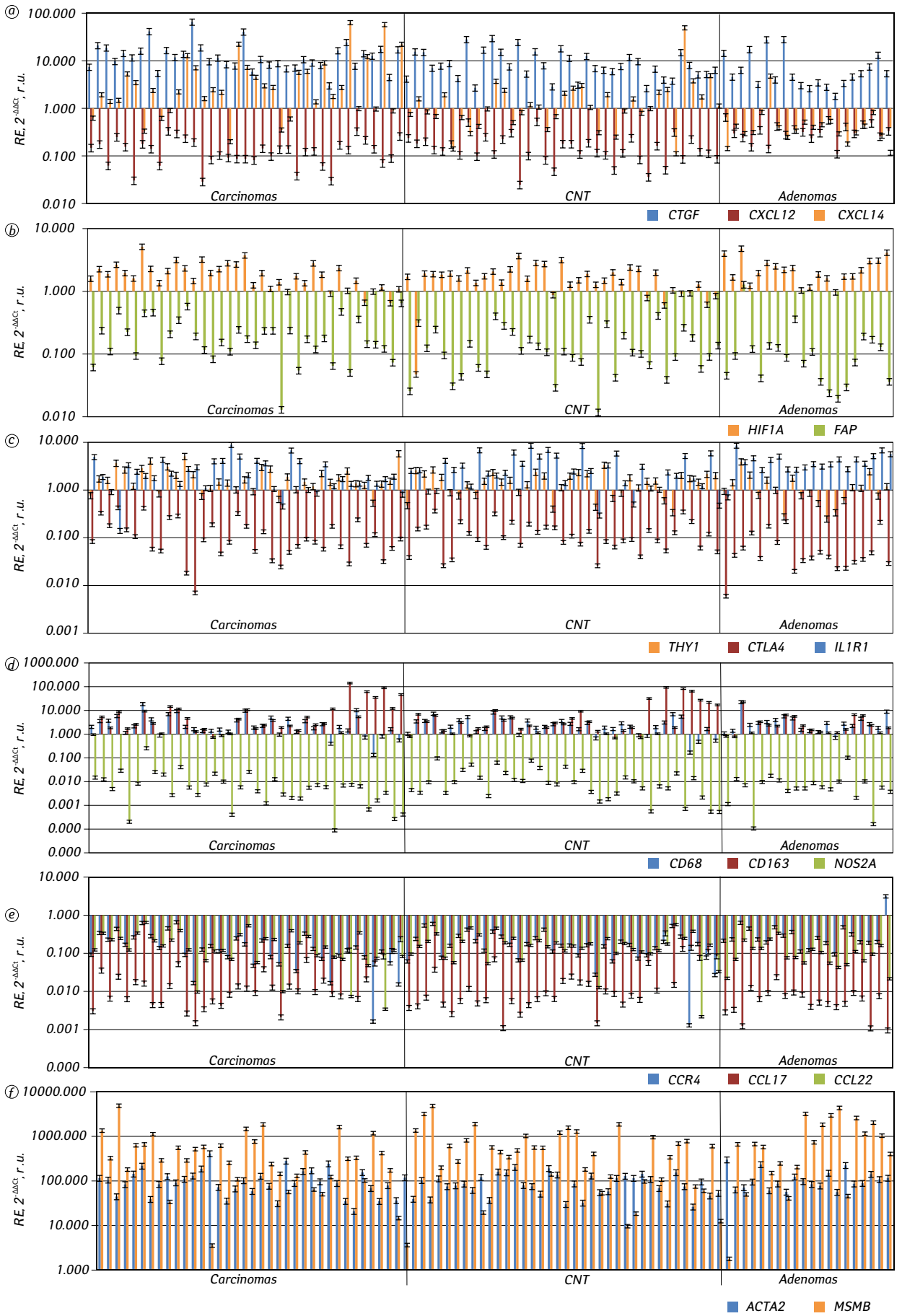

Fig. 1. RE profiles of genes with differences in different stages of disease in prostate adenocarcinomas (carcinomas), CNT and adenomas (*a–e*), *f* — genes with RE differences in paired T/N

Table 3. Descriptive statistics of the RE data in T, N, A sample groups at the various of disease stages and significant differences between pairs of groups

Gene	Group, stage	Median, r. u.	Minimum, r. u.	Maximum, r. u.	25.000th	75.000th	Pairs with differences*
ACTA2	T 1–2 st	89.909	20.917	412.01	62.79	129.333	
	T 3–4 st	96.673	35.072	281.768	76.795	156.101	
	N 1–2 st	86.332	29.923	203.147	65.074	125.702	
	N 3–4 st	55.296	26.263	154.637	37.942	115.354	
	A	92.329	56.143	299.884	74.621	132.95	
CTGF	T 1–2 st	10.691	5.431	65.433	8.107	16.302	T 1–2 st/A
	T 3–4 st	14.209	2.994	24.378	7.745	17.114	T 1–2 st/N 3–4 st
	N 1–2 st	8.358	2.699	29.541	6.176	15.361	
	N 3–4 st	5.196	2.628	14.929	3.964	6.724	
	A	4.583	1.803	28.079	3.287	10.298	
CXCL12	T 1–2 st	0.124	0.029	0.334	0.082	0.166	T 1–2 st/A
	T 3–4 st	0.141	0.03	0.329	0.089	0.216	T 3–4 st/A
	N 1–2 st	0.13	0.025	0.527	0.102	0.213	N 1–2 st/A
	N 3–4 st	0.112	0.036	0.315	0.086	0.163	N 3–4 st/A
	A	0.312	0.11	0.651	0.249	0.417	
CXCL14	T 1–2 st	2.304	0.199	22.365	1.14	5.517	T 1–2 st/A
	T 3–4 st	2.753	0.887	63.428	0.989	22.299	T 3–4 st/A
	N 1–2 st	0.865	0.141	3.715	0.573	1.762	N 3–4 st/A
	N 3–4 st	2.188	0.111	49.347	1.123	3.796	
	A	0.394	0.143	4.779	0.293	0.485	
HIF1A	T 1–2 st	1.965	0.977	5.145	1.531	2.675	T 1–2 st/T 3–4 st
	T 3–4 st	1.023	0.649	2.36	0.921	1.157	T 1–2 st/N 3–4 st
	N 1–2 st	1.866	0.005	3.662	1.446	2.206	T 3–4 st/A
	N 3–4 st	0.92	0.598	1.991	0.786	1.041	N 1–2 st/N 3–4 st
	A	2.068	0.965	4.799	1.645	2.947	N 3–4 st/A
FAP	T 1–2 st	0.176	0.013	0.575	0.11	0.238	
	T 3–4 st	0.142	0.05	0.647	0.073	0.358	T 1–2 st/A
	N 1–2 st	0.109	0.012	0.402	0.054	0.212	
	N 3–4 st	0.091	0.039	0.408	0.067	0.182	
	A	0.091	0.019	1.279	0.039	0.132	
S100A4	T 1–2 st	21.185	2.556	59.247	11.746	29.515	
	T 3–4 st	50.537	8.466	258.211	11.063	94.751	
	N 1–2 st	21.286	3.141	81.063	12.579	32.854	
	N 3–4 st	32.631	5.37	162.32	13.918	81.289	
	A	27.764	8.39	76.886	16.446	36.073	
THY1	T 1–2 st	1.633	0.643	5.119	1.061	2.721	T 1–2 st/A
	T 3–4 st	1.469	0.535	5.83	1.351	1.83	
	N 1–2 st	1.515	0.403	3.351	0.878	2.213	
	N 3–4 st	1.564	0.34	2.158	1.476	2.03	
	A	0.973	0.25	3.938	0.681	1.532	
CD68	T 1–2 st	2.205	0.885	18.661	1.486	4.37	T 1–2 st/T 3–4 st#
	T 3–4 st	0.803	0.134	10.319	0.551	1.625	T 1–2 st/N 3–4 st#
	N 1–2 st	2.358	0.669	8.172	1.641	3.704	
	N 3–4 st	1.651	0.169	6.998	0.539	3.124	
	A	2.731	0.708	22.498	1.471	4.231	
CD163	T 1–2 st	2.478	0.725	14.614	1.515	4.938	T 1–2 st/T 3–4 st
	T 3–4 st	35.354	1.166	142.803	11.618	61.782	T 1–2 st/N 3–4 st
	N 1–2 st	1.985	0.689	9.936	1.248	3.617	T 3–4 st/A, N 3–4 st/A
	N 3–4 st	27.173	1.037	93.815	16.979	64.857	T 3–4 st/N 1–2 st
	A	2.019	0.771	23.007	1.156	4.488	N 1–2 st/N 3–4 st
CCL17	T 1–2 st	0.01	0.001	0.067	0.004	0.017	T 1–2 st/N 3–4 st
	T 3–4 st	0.054	0.006	0.236	0.009	0.116	T 3–4 st/A
	N 1–2 st	0.006	0.001	0.07	0.004	0.011	T 3–4 st/N 1–2 st
	N 3–4 st	0.084	0.011	0.336	0.056	0.132	N 1–2 st/N 3–4 st
	A	0.006	0.001	0.024	0.004	0.009	N 3–4 st/A
CCL22	T 1–2 st	0.2	0.01	0.642	0.117	0.292	
	T 3–4 st	0.074	0.003	0.344	0.048	0.082	T 1–2 st/T 3–4 st
	N 1–2 st	0.158	0.013	0.462	0.104	0.21	T 1–2 st/A
	N 3–4 st	0.163	0.002	0.57	0.097	0.246	T 3–4 st/N 1–2 st
	A	0.086	0.002	0.335	0.056	0.134	
CCR4	T 1–2 st	0.156	0.017	0.654	0.085	0.272	T 1–2 st/T 3–4 st
	T 3–4 st	0.079	0.002	0.142	0.016	0.111	T 3–4 st/A
	N 1–2 st	0.164	0.027	0.592	0.1	0.263	T 3–4 st/N 1–2 st
	N 3–4 st	0.137	0.001	0.523	0.077	0.201	N 3–4 st/A
	A	0.232	0.094	3.138	0.191	0.405	
NOS2A	T 1–2 st	0.007	0	0.257	0.003	0.017	
	T 3–4 st	0.002	0	0.007	0	0.006	T 3–4 st/N 1–2 st
	N 1–2 st	0.01	0.001	0.096	0.004	0.03	
	N 3–4 st	0.002	0.001	0.022	0.001	0.006	
	A	0.006	0	0.103	0.004	0.01	
CIAS	T 1–2 st	0.153	0.051	0.47	0.124	0.202	
	T 3–4 st	0.127	0.034	0.363	0.087	0.238	
	N 1–2 st	0.182	0.046	0.603	0.094	0.271	
	N 3–4 st	0.139	0.074	0.427	0.118	0.196	
	A	0.127	0.052	0.593	0.089	0.221	
CTLA4	T 1–2 st	0.087	0.007	0.425	0.052	0.184	T 1–2 st/A
	T 3–4 st	0.07	0.028	0.239	0.06	0.117	N 1–2 st/A
	N 1–2 st	0.097	0.026	0.365	0.067	0.165	N 3–4 st/A
	N 3–4 st	0.12	0.051	0.349	0.061	0.142	
	A	0.039	0.006	0.219	0.03	0.071	

Table 3 (breakover)

Gene	Group, stage	Median, r. u.	Minimum, r. u.	Maximum, r. u.	25.000th	75.000th	Pairs with differences*
HLA-G	T 1–2 st	137.874	25.081	244.89	88.761	188.734	
	T 3–4 st	162.055	97.866	448.585	125.594	245.191	
	N 1–2 st	119.608	31.088	356.051	70.546	184.076	
	N 3–4 st	141.729	79.736	497.198	119.216	390.41	
	A	104.086	53.32	230.095	78.528	147.66	
IL1R1	T 1–2 st	2.142	0.14	8.884	1.705	4.047	T 3–4 st/A
	T 3–4 st	1.01	0.456	6.783	0.845	1.375	N 3–4 st/A
	N 1–2 st	2.639	0.958	8.55	2.129	5.261	
	N 3–4 st	1.397	0.294	8.454	0.501	2.004	
	A	3.733	0.708	8.572	2.88	4.871	
IL1RL1	T 1–2 st	0.132	0.05	0.494	0.09	0.2	
	T 3–4 st	0.163	0.026	0.37	0.065	0.255	
	N 1–2 st	0.15	0.07	0.842	0.12	0.226	
	N 3–4 st	0.21	0.009	0.335	0.171	0.289	
	A	0.211	0.058	0.701	0.156	0.294	
IL2RA	T 1–2 st	0.107	0.019	0.533	0.08	0.134	
	T 3–4 st	0.08	0.02	0.156	0.055	0.137	
	N 1–2 st	0.092	0.026	0.237	0.072	0.127	
	N 3–4 st	0.076	0.035	0.201	0.059	0.125	
	A	0.072	0.025	0.451	0.036	0.102	
IRF1	T 1–2 st	1.121	0.386	153.203	0.778	1.648	
	T 3–4 st	0.767	0.688	2.926	0.736	0.813	
	N 1–2 st	0.947	0.325	3.361	0.686	1.414	
	N 3–4 st	1.055	0.576	2.59	0.835	1.895	
	A	0.94	0.486	5.527	0.696	1.073	
KLRK	T 1–2 st	0.004	0	0.027	0.002	0.006	
	T 3–4 st	0.002	0	0.053	0	0.008	
	N 1–2 st	0.004	0	0.04	0.001	0.009	
	N 3–4 st	0.006	0	0.022	0.003	0.008	
	A	0.008	0	0.032	0.005	0.016	
MSMB	T 1–2 st	384.275	3.563	1858.97	176.88	715.69	
	T 3–4 st	102.783	3.661	4887.53	56.79	240.35	
	N 1–2 st	564.827	19.582	3196.3	307.6	1121.02	
	N 3–4 st	75.143	9.688	4813.28	18.51	181.68	
	A	670.962	1.793	4367.28	178.17	1933.82	

Note: *Dunn – Bonferroni post hoc method for multiple comparisons with FDR = 0.2 ($p < 0.05$); #presence of RE differences only in *TBP* normalized genes; st – stage; 25.000th – 25th percentile, 75.000th – 75th percentile.

Table 4. RE differences between pairs in TNA groups with TMPRSS2/ERG fusion status (4A) and different *PTEN* RE (4B)

A	TNA/Fusion status		B	TNA/*PTEN* RE (h/l)	
Gene	Pairs	*p*-value*	Gene	Pairs	*p*-value*
THY1	T F–/A	0.0497	*THY1*	T h/A l	0.0431
CXCL12	T F–/A	0.0178	*CXCL12*	T l/A h	0.0000
	T F+/A	0.0000		T h/A h	0.0406
	N F–/A	0.0281		N l/A h	0.0000
	N F+/A	0.0001		T l/N h	0.0358
CXCL14	T F–/A	0.0000	*CXCL14*	T l/A h	0.0000
	T F+/A	0.0001		T h/A h	0.0023
	N F–/A	0.0021		N l/A h	0.0303
	T F–/N F+	0.0256		T l/A l	0.0123
CTGF	T F–/A	0.0211	*CTGF*	T l/A h	0.0294
	T F+/A	0.0153		T h/A h	0.0028
FAP	T F–/A	0.0280		N h/A h	0.0340
	T F–/N F+	0.0419		T h/N l	0.0189
CTLA4	N F–/A	0.0010	*FAP*	T h/A l	0.0374
CD163	N F–/NF+	0.0309	*CTLA4*	N l/A h	0.0437
CCR4	T F–/A	0.0195	*CCR4*	T l/A h	0.0037
	N F+/A	0.0455	*IL1R1*	T l/A h	0.0240
CCL17	N F–/A	0.0225			
CCL22	N F–/A	0.0134			

Note: *Dunn – Bonferroni post hoc method for multiple comparisons with FDR = 0.2, F+ and F– groups with presence (+) and absence (–) of TMPRSS2/ERG fusion, h/l – groups with high (h) and low (l) *PTEN* RE.

both genes belong to the CAF group. RE of other genes from the CAF group, namely *ACTA2, CXCL12, FAP* and *S100A4* positively correlated with *PTEN* levels.

We have to mention, that several genes show correlation in their expression levels. For example, in the T group, expression of *CD163* positively correlated with *S100A4* levels, as did *FAP* and *THY1, CXCL12* and *IL2RA,* and also *CTLA4* and *IL2RA*. The correlation indexes were high — $r^s = |0.633–0.712|$, and correlation was $p < 0.0001$. The largest number of correlations out of 23 investigated genes ($p < 0.01$) demonstrated *CD68, CCR4* and *CCL22*, i.e. each of these genes showed 8 correlations. For *NOS2A*, seven dependences were found, for *HIF1A* — six and for *CTLA4, CIAS, IL2RA* — five.

DISCUSSION

A surgical material of prostate cancer is very heterogeneous, as a rule [30]. Tumor samples usually show a different structure with various sets of cells (i.e. cancerous cells, normal epithelial cells, stromal and immune cells, etc.) [31, 32]. Therefore, a study on specific tumor-host interactions is a very difficult task. To solve this problem, at least, partially, specific markers of different cell types were selected and gene RE was assessed in benign and malignant prostate tumors. We aimed to find the clinically significant alterations in the gene expression pattern. We found significant changes in RE only in a proportion of the investigated genes. Thus, 6 out of 8 genes of the CAF group showed differences in RE in the TNA group, containing tumors at various stages. Of note, the known CAF marker *ACTA2* [33] showed RE changes only in the group of the paired T/N samples.

The *CXCL14, CTGF, FAP* and *THY1* also differentially expressed in tumors and CNT (N), and they showed alterations in RE in the TNA group, including tumors of the various stages. These four genes that are upregulated in tumors encode proteins secreted by CAFs. These proteins play an important role in paracrine interactions in the tumor microenvironment, enhancing tumor progression and me-

Table 5. Spearman rank order correlations (r^s) of CPC and molecular characteristics with genes RE

Gene group	CAF								TAM					
CPC, molecular characteristics	*ACTA2*	*CTGF*	*CXCL12*	*CXCL14*	*HIF1A*	*FAP*	*S100A4*	*THY1*	*CD68*	*CD163*	*CCL17*	*CCL22*	*CCR4*	*NOS2A*
GS	–0.072	–0.133	0.008	0.360	–0.403	–0.021	0.353	–0.056	–0.217	0.224	***0.437***	–0.304	***–0.492***	–0.352
Stage	0.158	0.070	0.151	0.181	**–0.538**	–0.024	0.336	0.012	–0.369	**0.615**	0.435	–0.398	–0.436	***–0.407***
Age	0.180	0.248	0.153	0.148	0.204	0.278	0.027	0.328	0.136	0.193	0.092	0.004	0.193	0.034
PSA, ng/ml	–0.018	0.008	0.069	***0.479***	–0.233	0.098	0.141	0.225	–0.097	0.230	0.324	–0.174	–0.214	–0.169
Fusion status	–0.406	–0.126	–0.415	0.155	0.090	–0.123	–0.176	–0.157	–0.106	–0.143	0.051	0.129	0.090	–0.019
PTEN RE	***0.447***	0.285	**0.529**	0.002	0.146	***0.439***	0.431	0.205	0.234	0.290	–0.007	0.160	0.180	0.183

Gene group	IAG								
CPC, molecular characteristics	*CIAS*	*CTLA4*	*HLA-G*	*IL1R1*	*IL1RL1*	*IL2RA*	*IRF1*	*KLRK*	*MSMB*
GS	–0.247	–0.382	–0.076	–0.272	–0.057	***–0.449***	–0.316	–0.154	***–0.437***
Stage	–0.082	–0.148	0.246	–0.406	0.033	–0.155	–0.303	–0.111	–0.315
Age	0.213	0.172	–0.191	–0.084	0.020	0.023	0.151	0.294	–0.180
PSA, ng/ml	–0.119	–0.117	0.128	–0.321	–0.410	–0.166	–0.417	–0.190	–0.114
Fusion status	–0.114	–0.082	–0.042	0.130	–0.017	0.095	0.066	0.113	0.098
PTEN RE	0.103	0.024	0.192	0.133	0.109	0.026	0.096	0.050	–0.126

Note: $p < 0.05$ (red); ***p*** < 0.01 (red bold italic); **p < 0.001** (red bold).

tastasizing [34]. Moreover, the FAP protein exhibits immune-suppressive properties [35]. RE of *HIF1A* and *S100A4* (also the CAF genes) correlated with the stage of tumors. Only CAF genes demonstrated correlation of RE with molecular characteristics of prostate tumors, namely the TMPRSS2/ERG fusion status and *PTEN* levels. Probably, this indicates the closest and selective type of interaction of the tumor and fibroblasts, as matrix-regulating elements of the prostate stroma [13, 15, 33].

Genes of the TAM group showed altered RE in the TNA group, including tumors at different stages. Some of genes demonstrated RE changes in the TNA group, when GS and the TMPRSS2/ERG fusion status varied. All of the 6 TAM genes correlated with tumor stage. We may speculate on the clinical significance of the selected markers of the TAM group [10, 16, 19]. Genes *CD68* and *NOS2A* are the markers of normal, non-cancerous macrophages [16], whereas *CD163* is the TAM marker [18]. *CCR4, CCL17* and *CCL22* are associated with transition of the macrophage into TAM, i.e. these genes play a role in tumor progression [17].

The smallest number of genes with altered RE is found in the IAG group. Only two genes (*CTLA4* and *IL1R1*) showed changes in RE in the TNA group, including the tumors of different stages. RE of *IL1R1*and *MSMB* was changed in the paired T/N group. It is known that inhibition of *CTLA4* expression is very important in immune checkpoint therapy of prostate cancer, due to the fact that *CTLA4* transmits the immune inhibitory signals to T cells [36]. An IL1R1 signaling suppresses proliferation of mammary tumor cells, early in tumorigenesis [37]. We found the significant decrease of *IL1R1* RE in prostate cancer at the advanced stages of tumor development.

As mentioned earlier, the histological analysis indicated focal manifestations of leukocyte infiltration in the prostate tumors [31]. Therefore, expression of leukocyte genes could vary quite much within even one sample [21, 32]. Based on this, it was important for us to assess the gene expression in the various immune cells. Also, the immune score indexes could have their own independent diagnostic and prognostic values [38]. More suitable markers of prostate carcinogenesis should be studied, to reveal clinically significant alterations of immune response.

The observed high dispersion of the RE values did not make it possible to find the real difference between the groups of samples in all genes. In addition, the lack of the adequate control (a healthy donor's prostate with the relevant age and nationality) also affected experimental results. Moreover, the changes in expression of the studied genes in adenomas and CNT might have a specific character, showing the effect of hypertrophy and hyperplasia in adenomas and the influence of malignant tumors on prostate CNT, especially at the late stages of tumor development [31, 32]. This assumption is confirmed by the results of the present study. We got the different number of genes with RE changes in the group of paired T/N (11 genes) and in adenomas, taking into account the tumor stage (14 genes). Of note, *ACTA2* and *MSMB* showed RE changes only in the paired samples. Unexpectedly, *ACTA2* was highly upregulated in adenomas. Probably, this is due to the specific expression of various *ACTA2* isoforms upon tumor development and the specific expression of ACTA2 in different cell types, like smooth muscle cells [33, 34].

Earlier, we have shown that, based on RE of prostate-specific genes and EMT-associated genes, samples with the same clinical parameters could be grouped into specific RE subtypes [24, 25]. To confirm this, more experiments should be done on the larger patient cohort.

CONCLUSIONS

Several gene expression patterns were identified that correlated with the GS, stage and molecular characteristics of tumors, i.e. presence of the TMPRSS2/ERG fusion and alterations in *PTEN* expression. These expression patterns can be used for molecular profiling of prostate tumors, with the aim to develop personalized medicine approaches. However, the proposed profiling requires a more detailed analysis and a larger cohort of patients with prostate tumor.

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