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EXPRESSION OF STEROID AND PEPTIDE HORMONE RECEPTORS, METABOLIC ENZYMES AND EMT-RELATED GENES IN PROSTATE TUMORS IN RELATION TO THE PRESENCE OF THE TMPRSS2/ERG FUSION

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Aim: **To analyze an expression pattern of the steroid and peptide hormone receptors, metabolic enzymes and EMT-related genes in prostate tumors in relation to the presence of the TMPRSS2/ERG fusion; and to examine a putative correlation between gene expression and clinical characteristics, to define the molecular subtypes of prostate cancer.** ***Materials and Methods:*** **The relative gene expression (RE) of 33 transcripts (27 genes) and the presence/absence of the TMPRSS2/ERG fusion were analyzed by a quantitative PCR. 37 prostate cancer tissues (T) paired with conventionally normal prostate tissue (CNT) and 21 samples of prostate adenomas were investigated. RE changes were calculated, using different protocols of statistics.** ***Results:*** **We demonstrated differences in RE of seven genes between tumors and CNT, as was calculated, using the $2^{-\Delta C_T}$ model and the Wilcoxon matched paired test. Five genes (*ESR1, KRT18, MKI67, MMP9, PCA3*) showed altered expression in adenocarcinomas, in which the TMPRSS2/ERG fusion was detected. Two genes (*INSR*, isoform B and *HOTAIR*) expressed differently in tumors without fusion. Comparison of the gene expression pattern in adenomas, CNT and adenocarcinomas demonstrated that in adenocarcinomas, bearing the TMPRSS2/ERG fusion, genes *KRT18, PCA3,* and *SCHLAP1* expressed differently. At the same time, we detected differences in RE of *AR* (isoform 2), *MMP9, PRLR* and *HOTAIR* in adenocarcinomas without the TMPRSS2/ERG fusion. Two genes (*ESR1* and *SRD5A2*) showed differences in RE in both adenocarcinoma groups. Fourteen genes, namely *AR* (isoforms 1 and 2), *CDH1, OCLN, NKX3-1, XIAP, GCR* (ins AG), *INSR* (isoform A), *IGF1R, IGF1R tr, PRLR, PRL, VDR* and *SRD5A2* showed correlation between RE and tumor stage. RE of four genes (*CDH2, ESR2, VDR* and *SRD5A2*) correlated with differentiation status of tumors (Gleason score). Using the K-means clustering, we could cluster adenocarcinomas in three groups, according to gene expression profiles. A specific subtype of prostate tumors is characterized by the activated ERG signaling, due to the presence of TMPRSS2/ERG fusion, and also by high levels of the androgen receptor, prolactin, *IGF*, *INSR* and *PCA3*.** ***Conclusions:*** **We have found the specific differences in expression of the steroid and peptide hormone receptors, metabolic enzymes and EMT-related genes, depending on the presence/absence of the TMPRSS2/ERG fusion in prostate adenocarcinomas, CNT and adenomas. We showed three different gene expression profiles of prostate adenocarcinomas. One of them is characteristic for adenocarcinomas with the TMPRSS2/ERG fusion. Further experiments are needed to confirm these data in a larger cohort of patients.**

Key Words**: prostate tumors, TMPRSS2/ERG fusion, gene expression patterns, steroid receptors, peptide receptors, EMT regulation.**

Alterations in expression of the androgen receptor (AR) are often associated with development of prostate cancer. It is known already that the AR gene expression is regulated by quite many molecular pathways [1]. Another example of frequent alterations in prostate tumors is formation of gene fusions of androgen dependent gene *TMPRSS2* (transmembrane protease, serine 2) with the ETS (E26 transformation-specific) family in particular with *ERG* (ETS related gene) [2]. Previously, we have shown that the TMPRSS2/ERG fusion is present in prostate adenocarcinoma and even in conventionally normal prostate tissue (CNT) in a group of patients of the Ukrainian population [3]. Therefore, we may speculate that the presence or absence of the gene fusions could be the cause of development of various prostate cancer types with different sensitivity to therapy, recurrence and metastasizing, despite the similar histological characteristics [4].

One of the important characteristics of normal functioning of prostate epithelial cells is sensitivity to steroid and peptide hormones. In the process of cell transformation, tumor cells often lose the sensitivity to hormones and growth factors and also change their metabolism. The AR is a key element of prostate functioning and is involved in malignant transformation. As was shown already, AR signaling plays a primary role in development of androgen resistant and castration-resistant prostate cancer [1]. There are few isoforms of ARs. Some of them are prostate specific. *AR* expres-

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Abbreviations used: A – prostate adenomas; AR – androgen receptor; CNT – conventionally normal prostate tissue; CPC – clinical and pathological characteristics; EMT – epithelial-to-mesenchymal cell transition; FDR – false discovery rate; N – normal/conventionally normal prostate tissue; PSA – prostate-specific antigen; qPCR – quantitative polymerase chain reaction; RE – relative gene expression; RNA – ribonucleic acids; T – prostate cancer, adenocarcinoma; TNM – International System of Classification of Tumors, based on tumor-node-metastasis; WHO – World Health Organization.

sion can change during prostate carcinogenesis. Thus, the overexpression of *AR* isoform A (1 isof) decreases proliferation but accelerates invasion of prostate cancer cells, compared with overexpression of *AR* isoform B (2 isof) [5]. Also, it was proposed that formation of the fusion between *TMPRSS2* and *ERG* might be controlled by androgens [6].

In prostate cells, the most potent AR agonist is dihydrotestosterone. This is a metabolite of testosterone, and the reaction of conversion is catalyzed by SRD5A1 (5α-reductase, type 1) and SRD5A2 (5α-reductase, type 2). The latter are expressed at low levels in normal prostate tissues, but upon prostate cancer progression expression of these enzymes is altered [7]. Noteworthy, prostate cancer is a complex pathology and many other hormone receptors and corresponding pathways are involved in tumor development, especially *GCR* (glucocorticoid receptor, NR3C1 nuclear receptor subfamily 3 group C member 1), *IGF1R* (insulin like growth factor 1 receptor), *ESR1* and *ESR2* (estrogen receptors 1 and 2), *PRLR* (prolactin receptor), *VDR* (vitamin D receptor) and others.

Of note, GCR and AR share several transcriptional targets [8]. All of the three isoforms of GCR (alpha (A), beta (B) and gamma (G)) are very important in development and progression of prostate cancer [9]. In initiation and also in progression of the prostate cancer the IGF network, including INSR (insulin receptor) — (subtypes INSR A and B), IGF1R and IGF2R plays an important role [10–12].

Both estrogen receptors, alpha (*ESRα, ESR1*) and beta (*ESRβ, ESR2*) are associated with development of prostate cancer [13]. It was shown, that the increased expression of *ESRα* is observed upon progression, metastasizing, and in androgen resistant phenotype; *ESRα* could be involved in regulation of expression of the *TMPRSS2-ERG* fusion [14].

PRL (prolactin) can induce growth and survival of prostate cancer cells [15]. The *PRL* expression correlates with the disease severity.

It was shown that vitamin D (calcitriol) influences on prostate cancer cells growth [16]. Furthermore, the *TMPRSS2-ERG* fusion expression is increased upon activation of VDR and AR. Consequently, expression of *TMPRSS2/ERG* leads to inactivation of the VDR signaling [17].

We have shown earlier that several genes, regulating the epithelial-to-mesenchymal cell transition (EMT), such as *CDH1, CDH2, NKX3-1, FN1* and *VIM,* are expressed differently in prostate tumors [18].

In a present work, we aimed to analyze the expression pattern of a group of the cancer-related genes, depending on the presence or absence of the TMPRSS2/ERG fusion in prostate tumors. Also, we wanted to find the putative correlations between gene expression patterns and clinical and pathological characteristics (CPC) to define the molecular subtypes of prostate cancer.

MATERIALS AND METHODS

A collection of prostate tissues samples. 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 (Kyiv, Ukraine). Benign prostate tumors (prostate adenoma samples) were collected at the Institute of Urology (Kyiv, Ukraine) after radical prostatectomy and frozen, as described above. The samples were collected in accordance with the Declaration of Helsinki and the guidelines issued by the Ethic Committee of the Institute of Urology, the National Cancer Institute and an Ethic Committee of the Institute of Molecular Biology and Genetics. Experimental studies were conducted on 37 prostate adenocarcinoma samples of different Gleason score and stages; 37 paired CNT samples; 21 samples of benign prostate tumors (adenomas). Tumor samples were characterized, according to an International System of Classification of Tumors, based on the tumor-node-metastasis (TNM) and the World Health Organization (WHO) criteria. CPC and the presence/absence of the TMPRSS2/ERG fusion, that we have detected earlier [3] are presented on Table 1.

Table 1. CPC and TMPRSS2/ERG status (T/ERG) of prostate adenocarcinoma samples

Sample number	T/ERG	Stage	Gleason score	TNM	PSA, ng/ml
1	–	II	< 7	T2bN0M0	12.8
2	–	II	< 7	T2cNxM0	27.3
3	–	III	< 7	T3bNxM0	23.6
4	–	II	< 7	T2bNxM0	6.5
5	–	II	< 7	T2cNxM0	25.2
6	+	II	< 7	T2aNxM0	18.6
7	+	II	< 7	T2aN0M0	9.3
8	+	II	< 7	T2aN0M0	6.0
9	+	II	< 7	T2pN0M0	5.0
10	+	II	< 7	T2aN0M0	13.3
11	+	II	< 7	T2cN0M0	29.1
12	–	II	7	T2aNxM0	11.7
13	–	II	7	T2cNxM0	13.9
14	–	II	7	T2cNxM0	19.8
15	+	II	7	T2aNxM0	7.1
16	+	I	7	T1cNxM0	8.2
17	+	II	7	T2cNxM0	19.3
18	+	II	7	T2aNxM0	5.6
19	+	II	7	T2cN0M0	14.3
20	+	III	7	T2bN0M0	24.6
21	–	III	> 7	T3bNxM0	86.3
22	–	IV	> 7	T3aN0M1	37.8
23	–	IV	> 7	T2cN0M1	22.6
24	–	III	> 7	T2cN1M0	2.3
25	–	II	> 7	T2bNxM0	6.9
26	–	III	> 7	T3bNxM0	51.0
27	–	III	> 7	T2bNxM0	0.5
28	–	II	> 7	T2bN0M0	20.3
29	+	II	> 7	T2cN0M0	9.7
30	+	III	> 7	T3bN0M0	12.1
31	+	III	> 7	T3aN0M0	25.1
32	+	III	> 7	T3bNxM0	16.0
33	+	III	> 7	T3bN0M0	84.2
34	+	III	> 7	T3bNxM0	20.9
35	+	IV	> 7	T2cN1M0	17.0
36	+	II	> 7	T2bNxM0	33.0
37	+	III	> 7	T3bNxM0	106.0

Note: + presence of TMPRSS2/ERG fusion; – absence of TMPRSS2/ERG fusion.

Total RNA isolation and cDNA synthesis. 50–70 mg of frozen prostate tissues were mashed to a powder in the liquid nitrogen. Total RNA was extracted by TRI-reagent (SIGMA), according to the manufacturer's protocol. Total RNA concentration was analyzed by a spectrophotometer (NanoDrop Tech-

nologies 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 quantitative polymerase chain reaction (qPCR). Relative gene expression (RE) levels of 27 genes (33 transcripts) were detected by qPCR, using Maxima SYBR Green Master mix (Thermo Fisher Scientific, USA) and 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 the different transcripts of *INSR* and *IGF1R* and various isoforms of *GCR* were as published earlier [9, 19]. Primers for others genes were selected, using qPrimerDepot (*https://primerdepot.nci.nih.gov/*).

Four reference genes — *TBP*, *HPRT*, *ALAS1* and *TUBA1B* — were used for normalization of the gene expression [20]. The two main models ($2^{-\Delta C_T}$ and $2^{-\Delta\Delta C_T}$ methods), described earlier [18, 21], were used for the RE level calculation and analysis.

Statistical analysis. The Kolmogorov — Smirnov test was used to analyze the normality of distribution. The Kruskal — Wallis test was used to determine differences by multiple comparison between experimental groups. The Wilcoxon Matched Pairs test was performed to compare RE in prostate adenocarcinoma and paired CNT. RE fold differences in $2^{-\Delta\Delta C_T}$ model were considered significant when expression changes were more, than 2 fold. The Fisher exact test was performed to monitor differences between these sample groups [21]. The Benjamini — Hochberg procedure with false discovery rate (FDR) 0.10–0.25 was used when multiple comparisons were performed [22]. The Dunn — Bonferroni post hoc test was performed to determine RE differences between pairs of prostate samples. The Spearman's rank correlation test was used to find the putative correlations between RE and CPC of prostate tumors and also correlations between RE of investigated 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 — Bonferroni post hoc tests for detection of inter-cluster differences in RE.

RESULTS

Expression of 17 transcripts (11 genes), representing the receptors and metabolic enzymes and also 16 EMT-related transcripts/genes (3 from them are lncRNAs) were studied in prostate adenocarcinomas, CNT and adenomas.

Earlier, we have shown that the TMPRSS2/ERG fusion was expressed in 21 out of 37 adenocarcinomas [3]. In this group, in 16 paired CNT the TMPRSS2/ERG fusion was detected, and 5 CNT did not show this fusion. Thus, we have 3 groups in a set of the paired adenocarcinomas/CNT: 1) T–/N– group — the TMPRSS2/ERG fusion was not detected neither in adenocarcinomas nor in CNT (n = 16); 2) T+/N+ group — the TMPRSS2/ERG fusion was found in both, cancer and CNT (n = 16); 3) T+/N– group — the TMPRSS2/ERG fusion was present in adenocarcinomas, but not in CNT (n = 5).

The Wilcoxon Matched paired test in the $2^{-\Delta C_T}$ model showed the differences in RE of 7 genes, when the paired adenocarcinoma (T) and CNT (N) were compared, regardless presence or absence of the TMPRSS2/ERG fusion (Table 2).

Table 2. RE differences between prostate adenocarcinoma samples and paired CNT with and without fusion status detection (dependent sampling, $2^{-\Delta C_T}$ model)

Gene	Pairs with differences without fusion status detection	*p*-value[&]	Pairs with differences with fusion status	*p*-value[&]
ESR1	T/N	0.010	T+/N+	0.038
			T+/N–	0.043
INSR (B isof)	T/N	0.037	T–/N–	0.039
KRT18	T/N	0.000	T+/N+	0.007
MKI67	T/N	0.017	T+/N+	0.003
MMP2	T/N	0.011	no	–
MMP9	T/N	0.014	T+/N+	0.011
VIM	T/N	0.010	no	–
HOTAIR	T/N	0.007	T–/N–	0.027
PCA3	no	–	T+/N+	0.049

Note: [&]Wilcoxon Matched Pairs test significant with FDR = 0.1.

The following five genes were upregulated in adenocarcinomas, when T/N pairs with the fusion in both, tumor and CNT were analyzed: *ESR1* (p = 0.038),

Table 3. Frequency of RE fold changes ($2^{-\Delta\Delta C_T}$) in prostate adenocarcinoma (T) in comparison with paired CNT (N) in groups with different TMPRSS2/ERG status and statistical significant differences in paired T/CNT in $2^{-\Delta C_T}$ model

Group	N	RE fold changes	*AR (1isof)*	*AR (2isof)*	*ESR1*	*ESR2*	*GCR (AG isof)*	*GCR (in AG)*	*GCR (in B)*	*INSR (A isof)*	*INSR (B isof)*	*IGF1R*	*IGF1R tr*	*PRLR*	*PRL*	*SRD5A1*	*SRD5A2*	*VDR*
1 T–/N–	16	< 0.49	1	4	3	2	0	1	0	0	0	1	3	4	4	2	1	3
		> 2.10	1	2	7[$]	4	0	0	2	2	**4[#]**	2	1	1	2	0	0	1
2 T+/N+	16	< 0.49	3	2	1	3	0	1	1	3	0	3	1	1	0	3	4	2
		> 2.10	1	2	**9[$ #]**	1	1	1	1	0	1	1	0	1	1	2	1	3
3 T+/N–	5	< 0.49	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
		> 2.10	0	1	**3[#]**	2	0	0	0	0	2	1	0	0	0	1	0	0

Group	N	RE fold changes	*CDH1*	*CDH2*	*FN1*	*VIM*	*OCLN*	*MMP2*	*MMP9*	*NKX3-1*	*PSA*	*KRT18*	*MKI67*	*CASP3*	*XIAP*	*PCA3*	*HOTAIR*	*SCHLAP1*
1 T–/N–	16	< 0.49	4	2	3	1	2	0	1	6	5	2	3	1	0	6	2	6
		> 2.10	0	3	3	3	3	1	5	1	3	2	2	0	2	4	**9[$ #]**	6
2 T+/N+	16	< 0.49	5	6	0	0	3	1	0	5	2	0	1	1	0	4	2	3
		> 2.10	0	2	2	3	4	3	**9[$ #]**	2	1	**5[#]**	**7[$ #]**	2	0	**9[$ #]**	7[$]	7[$]
3 T+/N–	5	< 0.49	1	2	0	0	0	0	1	0	1	0	1	0	0	0	1	0
		> 2.10	2	0	0	1	2	0	1	1	1	1	2	0	0	4[$]	1	2

Notes: [$]statistical significant differences between adenocarcinoma and CNT groups by Fisher exact test ($p < 0.05$) ($2^{-\Delta\Delta C_T}$);
[#]statistical significant differences between adenocarcinoma and CNT groups by Wilcoxon Matched Pairs test ($p < 0.05$) ($2^{-\Delta C_T}$).

Table 4. RE differences between pairs of groups with different TMPRSS2/ERG status

Gene/transcript	*p*-value*	Pairs with differences	*p*-value**
AR (2 isof)	0.024	T–/A	0.017
ECR1	< 0.001	T–/A	0.002
		T+/A	< 0.001
		N–/A	0.040
PRLR	0.017	T–/A	0.009
SRD5A2	0.002	T–/A	0.039
		T+/A	0.003
		N+/A	0.020
KRT18	0.007	T+/A	0.008
MMP9	0.001	T–/A	0.003
		N–/A	0.012
OCLN	0.021	no	–
VIM	0.045	no	–
PCA3	0.001	T+/A	0.001
		N+/A	0.001
HOTAIR	0.003	T–/A	0.002
SCHLAP1	0.010	T+/A	0.011

Notes: *Kruskal – Wallis test data significant with FDR = 0.1;
**Dunn – Bonferroni post hoc method for multiple comparisons.

KRT18 ($p = 0.007$), *MKI67* ($p = 0.003$), *MMP9* ($p = 0.011$) and *PCA3* ($p = 0.049$). In adenocarcinomas without fusion *INSR* (B isof) ($p = 0.039$) and *HOTAIR* ($p = 0.027$) were expressed at the higher levels, than in the paired CNT. Only one gene, *ESR1,* showed significant changes in RE in adenocarcinomas with the presence of the fusion, compared with CNT without fusion ($p = 0.043$).

When the $2^{-\Delta\Delta C_T}$ model was used, we found 6 genes with significant differences in RE between adenocarcinomas and CNT (Table 3). Three genes (*MMP9, MKI67,* and *SCHLAP1*) where expressed at the higher levels in tumors, compared with CNT (the T+/N+ group) ($p < 0.05$), two genes (*ESR1* and *HOTAIR*) have shown increased RE in T+/N+ and T–/N– groups ($p < 0.05$). Only one gene, the *PCA3* was significantly increased in T+/N+ and T+/N– groups ($p < 0.05$).

Hence, the data obtained by the two abovementioned models are only partially overlapping. This could be due to different statistical calculations.

Earlier, we have discussed that CNT isolated from patients with prostate tumors do not represent the normal tissue, therefore they can't be considered as an adequate control [18]. In order to avoid working with inadequate controls, adenomas were used as the control instead. Noteworthy, the TMPRSS2/ERG fusion was detected in 4 adenomas as well. No differences in the gene expression patterns were found in these

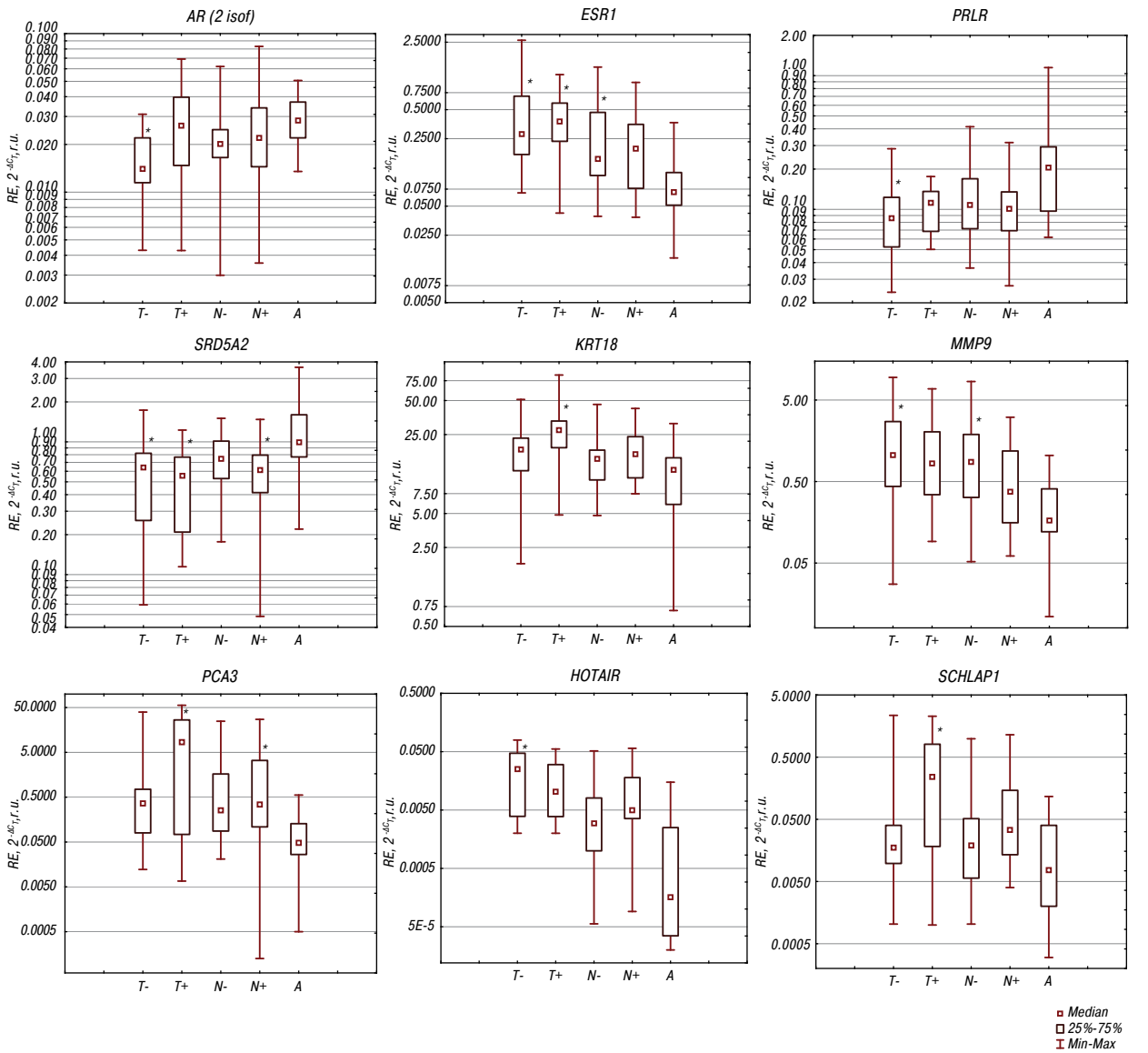

Fig. 1. RE of genes with significant differences between 5 groups with presence (+) or absence (–) of fusion transcript. *$p < 0.05$ in comparision with adenomas group (A) (Dunn — Bonferoni post hoc test for multiple comparisions)

4 adenomas, compared with adenomas without fusion. For further comparison, only the group of adenomas without fusion was analyzed (n = 17). Also, CNT samples without fusion (n = 5) from adenocarcinoma pairs with the fusion were attributed to total CNT fusion negative (N–) group after verification of RE differences in CNT sample groups for all investigated genes.

The Kruskal — Wallis test (with FDR = 0.1) has shown significant differences of RE in 11 out of 33 transcripts/genes between 5 investigated groups (T+, T–, N+, N– and A–), while the Dunn — Bonferroni post hoc method of the multiple comparisons has confirmed changes only for 9 transcripts/genes (Table 4, Fig. 1).

Increased RE levels in the adenocarcinoma and CNT groups, compared to the adenoma group was shown for 6 genes 1) *ECR1* T–/A– group (p = 0.002), T+/A– (p < 0.001), N–/A– (p = 0.040); 2) *KRT18* T+/A– (p = 0.008); 3) *MMP9* T–/A– (p = 0.003), N–/A– (p = 0.012); 4) *PCA3* T+/A– (p = 0.001), N+/A– (p = 0.001); 5) *HOTAIR* T–/A– (p = 0.002), and 6) *SCHLAP1* T+/A– (p = 0.011). Decreased RE levels in the adenocarcinoma and CNT groups, compared to the adenoma group, was detected for 3 genes: 1) *AR* (2 isof) T–/A– (p = 0.0172), 2) *PRLR* T–/A– (p = 0.0088) and 3) *SRD5A2* T–/A– (p = 0.0393), T+/A– (p = 0.0034), N+/A– (p = 0.0203).

Correlations of CPC with RE levels. The Spearman Rank Order Correlations (r^s) analysis of CPC characteristics and RE of a set of genes in prostate adenocarcinomas has revealed a number of positive and negative correlations (Table 5A). For example, there is the reverse correlation between the Gleason score and RE of *ESR2, VDR* and *SRD5A2*: r^s = –0.354, r^s = –0.382 (p < 0.05), r^s = –0.520 (p < 0.01), respectively. Also, RE of *GCR* (in AG) and *PRL* showed the direct correlations with a tumor stage, and 8 genes — *AR* (1 isof), *AR* (2 isof), *INSR* (A isof), *IGF1R, IGF1R tr, PRLR, VDR* and *SRD5A2* showed the negative correlation with the tumor stage. Levels of the PSA in serum correlate negatively with RE of *VDR* and *SRD5A2*.

Correlations of RE levels between investigated genes. Investigation of RE correlations in prostate adenocarcinomas have shown 131 significant correlations (from p < 0.0001 to p < 0.05) (Table 5B). Among them 34 have the highest score r^s = |0,524–0,936| (from p < 0.0001 to p < 0.05). A maximal number of strong RE correlations showed *INSR* (A isof) — 7 correlations, *AR* (1 isof), *GCR* (in AG), *IGF1R*,

Table 5. Spearman Rank Order Correlations (r^s) of CPC with genes RE (5A) and r^s among genes RE in prostate adenocarcinomas (5B)

5A

	AR (1 isof)	AR (2 isof)	*ESR1*	*ESR2*	*GCR* (AG isof)	*GCR* (in AG)	*GCR* (in B)	*INSR* (A isof)	*INSR* (B isof)	*IGF1R*	*IGF1R tr*	*PRLR*	*PRL*	*VDR*	*SRD5A1*	*SRD5A2*
GL	0.023	0.033	-0.238	-0.354	0.157	0.178	0.073	-0.157	-0.304	-0.146	-0.086	-0.009	0.007	-0.382	0.051	**-0.520**
Stage	*-0.381*	*-0.390*	-0.235	0.142	0.261	0.377	-0.087	**-0.478**	-0.187	**-0.441**	**-0.486**	-0.326	**0.437**	**-0.444**	-0.005	-0.395
PSA, ng/ml	-0.147	-0.097	-0.029	-0.271	-0.001	-0.016	-0.304	-0.178	-0.315	-0.319	-0.317	-0.254	-0.168	-0.409	0.067	**-0.461**
Age	-0.154	-0.069	0.113	-0.011	0.211	0.187	0.357	-0.058	-0.066	-0.004	-0.090	-0.056	-0.052	0.261	-0.156	0.032

5B

Gene/ transcript	*T/ERG*	*AR (1 isof)*	*AR (2 isof)*	*ESR1*	*ESR2*	*GCR (AG isof)*	*GCR (in AG)*	*GCR (in B)*	*INSR (A isof)*	*INSR (B isof)*	*IGF1R*	*IGF1R tr*	*PRLR*	*PRL*	*VDR*	*SRD5A1*	*SRD5A2*
AR (1 isof)	0.331	1.000															
AR (2 isof)	**0.486**	**0.665**	1.000														
ESR1	0.156	0.383	0.291	1.000													
ESR2	-0.294	-0.049	-0.096	0.252	1.000												
GCR (AG isof)	-0.118	0.017	0.043	**0.461**	0.297	1.000											
GCR (in AG)	-0.239	-0.071	-0.025	0.267	**0.457**	**0.855**	1.000										
GCR (in B)	-0.007	0.204	0.322	0.307	0.322	***0.539***	***0.584***	1.000									
INSR (A isof)	0.074	**0.518**	0.405	**0.435**	0.301	0.060	0.060	0.217	1.000								
INSR (B isof)	-0.367	0.217	0.202	**0.445**	0.418	0.340	0.388	0.258	***0.589***	1.000							
IGF1R	0.349	***0.532***	**0.488**	0.382	0.119	0.071	0.023	0.303	**0.751**	**0.486**	1.000						
IGF1R tr	0.332	***0.560***	0.461	0.324	0.043	0.008	-0.051	0.230	**0.755**	**0.450**	**0.936**	1.000					
PRLR	0.097	**0.464**	**0.443**	0.322	-0.079	0.087	-0.130	0.162	**0.462**	0.315	**0.446**	**0.458**	1.000				
PRL	-0.314	-0.263	-0.401	-0.293	0.093	-0.053	-0.051	-0.260	-0.391	-0.050	-0.371	-0.325	-0.038	1.000			
VDR	-0.001	0.291	0.280	0.340	0.241	0.063	0.025	0.247	***0.536***	0.278	0.248	0.237	0.232	-0.250	1.000		
SRD5A1	0.194	-0.033	-0.041	-0.091	0.134	-0.029	0.125	-0.163	0.256	0.079	0.249	0.304	-0.227	-0.136	-0.035	1.000	
SRD5A2	-0.222	0.319	0.058	0.328	**0.505**	-0.038	-0.023	0.080	**0.424**	**0.491**	**0.422**	0.398	0.329	-0.029	0.406	0.091	1.000
CDH1	0.125	**0.439**	0.374	0.124	0.116	-0.136	-0.153	0.311	**0.601**	0.296	**0.716**	**0.631**	0.367	-0.335	0.304	0.118	**0.429**
CDH2	-0.358	0.272	0.155	0.118	0.125	**0.518**	**0.530**	0.289	0.120	0.311	-0.007	-0.064	0.253	0.156	0.049	-0.138	0.116
CASP3	-0.045	**0.492**	0.389	0.130	0.104	0.261	0.314	0.340	0.332	0.348	0.248	0.239	0.220	-0.161	0.172	0.174	0.118
FN1	-0.250	0.069	0.099	0.343	0.168	**0.499**	**0.520**	0.362	0.039	0.364	-0.104	-0.140	0.001	-0.057	0.070	-0.229	-0.049
KRT18	**0.449**	-0.064	0.103	-0.313	-0.263	**-0.512**	***-0.527***	-0.328	0.063	-0.381	0.220	0.221	-0.051	-0.153	-0.055	0.258	-0.174
OCLN	**0.467**	**0.631**	**0.516**	0.150	-0.177	-0.190	-0.246	0.044	**0.634**	0.079	**0.619**	**0.619**	0.276	-0.268	0.326	0.267	-0.009
MKI67	0.317	0.356	**0.430**	-0.094	-0.128	-0.248	-0.161	-0.085	0.315	-0.090	0.140	0.186	0.087	-0.129	0.408	0.174	0.032
MMP2	-0.141	-0.053	-0.185	0.149	0.365	0.313	0.374	-0.124	-0.072	0.089	-0.350	-0.305	-0.331	0.056	0.137	0.205	0.205
MMP9	-0.027	-0.278	-0.175	**0.462**	0.355	0.213	0.099	-0.201	0.136	0.109	0.000	0.014	-0.078	0.014	0.208	0.162	0.211
NKX3-1	0.231	**0.540**	**0.538**	0.071	-0.038	-0.104	-0.105	0.366	***0.562***	0.212	**0.632**	***0.592***	**0.495**	-0.293	0.351	0.064	0.307
PSA	0.338	0.346	0.304	-0.330	-0.434	**-0.658**	**-0.717**	-0.307	0.168	-0.305	0.299	0.356	0.202	-0.215	-0.087	0.160	0.037
VIM	-0.235	-0.235	**-0.444**	0.209	0.034	0.268	0.203	-0.316	-0.101	0.226	-0.201	-0.125	-0.164	0.244	-0.116	0.296	0.027
XIAP	0.077	***0.572***	***0.524***	0.235	0.101	0.177	0.191	**0.427**	**0.562**	0.349	0.416	**0.439**	0.389	-0.178	0.355	0.064	0.084
PCA3	0.344	0.151	0.380	-0.092	-0.315	-0.341	-0.388	0.042	0.193	-0.111	0.408	0.402	0.095	-0.304	-0.124	0.054	-0.257
HOTAIR	-0.290	0.025	0.020	0.133	0.006	***0.549***	**0.469**	0.293	-0.053	0.203	-0.136	-0.163	0.300	0.260	0.019	**-0.509**	-0.157
SCHLAP1	0.338	-0.047	0.071	-0.421	***-0.536***	**-0.477**	***-0.545***	**-0.440**	-0.169	***-0.587***	-0.118	-0.098	-0.148	-0.057	-0.258	0.148	**-0.445**

Notes: p < **0.0001 (dark blue bold type),** ***p < 0.001 (dark blue bold+italic type),*** p < **0.01 (red bold type),** p < 0.05 (red).

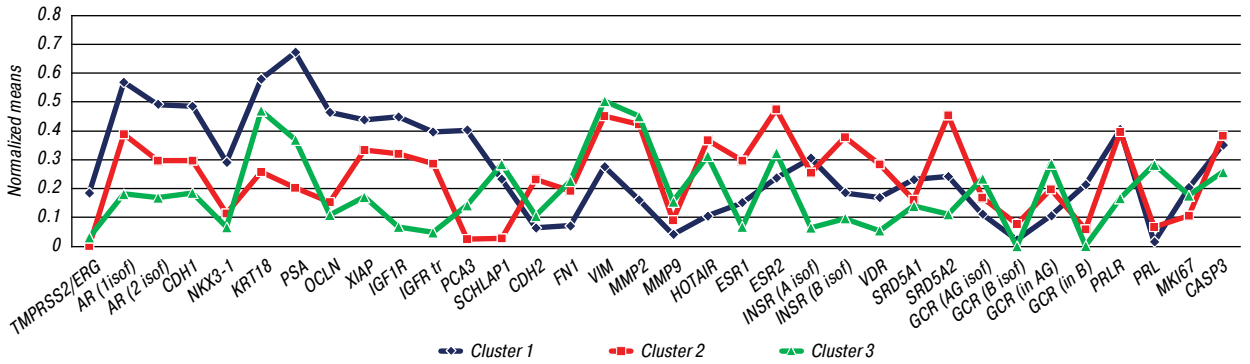

Fig. 2. Prostate adenocarcinomas RE profiling by K-means clustering

IGF1R tr — 6 correlations. This big number of correlations confirms robust relationships between gene expression profiles and the close connections pathways, where these genes belong to.

Expression profiling of adenocarcinomas. To determine the putative molecular subtypes of the prostate adenocarcinomas, showing the certain gene expression profile, the K-means clustering was performed, with analysis of RE of all of the studied genes and CPC (Gleason score and tumor stages) in the adenocarcinoma group. We found three specific clusters (Fig. 2, Table 6), that included 33 out of 37 cancer samples (89%). These clusters showed the significant differences in RE of 21 out of 33 transcripts. The largest distance is between clusters 1 and 3. All three clusters consist of tumors with the various Gleason scores (6, 7, 9).

The cluster 1 contains 12 samples with the TMPRSS2/ERG fusion. Also, in this group the highest expression of *AR*, epithelial markers (*CDH1, NKX3-1, OCLN*) and prostate cancer markers (*PSA, PCA3, KRT18, SCHLAP1*) is detected.

The cluster 3 contains the tumors with the highest Gleason score and a tumor stage index. By other words, the cluster 3 consists of the most aggressive tumors. This assumption is supported by the RE data. For example, in this group we found the lowest expression of *AR*, epithelial markers (*CDH1, OCLN, NKX3-1*), *SRD5A2, INSR* (A and B) and *IGF1R*, and the high levels of *PRL*, lncRNA *SCHLAP1* and *HOTAIR*, and also of mesenchymal markers (*VIM, FN1, MMP2*). We have to mention, however, that the cluster 3 contains the lowest number of samples with the fusion — only 2 out of 8.

The gene expression profile in cluster 2 has a mixed pattern. For example, several epithelial and luminal markers, such as *KRT18, PCA3* and *PSA* show the lowest expression, and other genes, namely mesenchymal markers *CDH2, MMP2, FN1* and *VIM* are highly expressed.

DISCUSSION

The *TMPRSS2-ERG* fusion transcript isoform 2 (EF194202.1) was first detected in prostate tumor samples by Lapointe *et al*. [23]. It is known that formation of this fusion transcript leads to overexpression of the ERG protein, which is involved in the signaling pathways associated with prostate cancer development [24, 25]. We wanted to enlighten the influence of this fusion on expression of some prostate cancer-associated receptors, enzymes and EMT-associated genes. Thus, in paired adenocarcinoma/CNT samples we have found the specific changes in RE in cancers with the fusion for 5 genes, whereas RE alterations for tumors without fusion were found only for 2 genes. The high level of *ESR1* in tumors where the fusion was detected was associated with faster cancer progression [14].

In the present work we found among adenocarcinomas, CNT and adenomas that the *ESR1* and *SRD5A2* genes showed altered expression regard-

Table 6. Prostate adenocarcinomas RE means of clusters and statistical significant differences between them

Cluster number	Number of cases	Percentage (%)	T/ERG status (+)	Stage	Gleason score	*T/ERG*	*AR (1 isof)*	*AR (2 isof)*	*CDH1*	*NKX3-1*	*KRT18*	*PSA*	*OCLN*	*XIAP*	*IGF1R*	*IGFR tr*	*PCA3*	*SCHLAP1*	*CDH2*	*FN1*	*VIM*
1	12	36.4	12	2	7	0.94	2.10	0.03	4.92	0.82	31.78	537.05	1.76	0.48	4.32	4.33	22.61	0.55	0.09	4.5	9.50
2	13	39.4	4	2	6	0.00	1.57	0.02	3.03	0.34	15.11	166.95	0.51	0.38	3.21	3.26	0.22	0.01	0.32	9.42	13.04
3	8	24.2	2	3	9	0.01	0.97	0.01	1.92	0.21	26.15	299.15	0.42	0.23	1.02	0.94	7.98	0.67	0.14	10.8	14.10
p-value < 0.05						*, ***	***	***	***	***	*	*	*, ***		**, ***	**, ***	*	*, **	*		

Cluster number	*MMP2*	*MMP9*	*HOTAIR*	*ESR1*	*ESR2*	*INSR (A isof)*	*INSR (B isof)*	*VDR*	*SRD5A1*	*SRD5A2*	*GCR (AG isof)*	*GCR (B isof)*	*GCR (in AG)*	*GCR (in B)*	*PRLR*	*PRL*	*MKI67*	*CASP3*
1	8.22	1.18	0.01	0.44	0.03	0.48	0.35	0.16	0.10	0.46	2.49	0.00	2.41	8.45	0.12	0.00	0.25	0.35
2	16.43	2.52	0.03	0.81	0.05	0.46	0.60	0.23	0.07	0.82	3.26	4.73	3.65	2.50	0.12	0.00	0.14	0.38
3	16.15	4.28	0.02	0.22	0.03	0.15	0.23	0.09	0.07	0.25	4.15	0.00	4.88	0.85	0.05	0.01	0.23	0.28
p-value < 0.05	*			**		**, ***	*, **	**, ***		**					***	***		

Notes: *differences between 1 and 2 clusters; **differences between 2 and 3 clusters; ***differences between 1 and 3 clusters (Dunn – Bonferroni post hoc method for multiple comparisons).

less presence of the *TMPRSS2-ERG* fusion, while *AR, MMP9* and *HOTAIR* were affected only in cases with no fusion, and expression of *KRT18, PCA3* and *SCHLAP1* were changed in adenocarcinomas with the fusion. Noteworthy, in adenomas we have detected the highest *SRD5A2* RE levels. It is known that increased *SRD5A2* in adenomas provokes hyperplasia extension through NF-kB and AR isoform 7 conferring 5α-reductase inhibitors resistance [26]. From other hand decreased levels of *SRD5A2* in adenocarcinomas is associated with the enhanced cell migration and invasion [7]. Moreover, when *SRD5A2* gene was reintroduced, cell migration and invasion was inhibited, due to F-actin reorganization [27].

The high RE of lncRNA *SCHLAP1* adenocarcinomas with the fusion predict unfavorable prognosis of disease [28]. The other lncRNA, *HOTAIR* when it expressed at the high levels in adenocarcinomas without fusion enhances proliferation and invasion at late stages of prostate cancer [29].

Earlier, we could not find a correlation between frequency of the fusion transcript detection and CPC, such as the Gleason score and stage [3], therefore we didn't analyze TMPRSS2/ERG dependent changes for investigated genes in sample groups with different CPC. Now we found many correlations between CPC and RE of the genes, encoding receptors/enzymes in total group of adenocarcinomas. Eight out of ten significant ($p < 0.01$ to $p < 0.05$) correlations were negative, i.e. expression of these genes was decreasing upon cancer progression. Furthermore, large quantity of the RE correlations of investigated genes allow us to perform the clustering of patients with adenocarcinomas. We clustered prostate adenocarcinomas in three groups, based on the RE of 33 transcripts and also CPC characteristics.

Our experimental data on the RE profiles in prostate adenocarcinomas are in concordance with the literature data [1, 2, 4]. It is widely accepted, that high expression of epithelial and luminal markers is usually accompanied by low expression of mesenchymal markers, and that we have observed in cluster 1. Noteworthy, we showed simultaneous high expression of the fusion transcript, *PCA3* and *NKX3-1* in one cluster. It seems, that the fusion transcript and *PCA3* do not influence negatively on expression of the tumor suppression gene *NKX3-1* and *vice versa*, as they belong to different pathways [1, 4]. At the other hand, the oncogenic PCA3 pathway [28, 30], probably, acts in parallel with the ERG pathway [23, 24].

To summarize subtyping data, it is essential to note specific cluster features. Cluster 1, which contains all fusion positive adenocarcinomas, has the most characteristic expression profile namely fusion positive androgen dependent luminal subtype 1. Probably oncogenic pathways in this group are ERG and PCA3 [25, 30] with high sensitivity to androgens, prolactin, IGF, INS stimulation oncogenic signaling.

We suppose that cluster 3 is another luminal prostate cancer subtype most of all it is fusion negative with androgen independent and castration resistant characteristics [30] (fusion negative androgen independent luminal subtype 2). It has molecular characteristic properties as the lowest expression of *AR*, epithelial markers (*CDH1, OCLN, NKX3-1*), *SRD5A2, INSR* (A and B) and *IGF1R*, high levels of mesenchymal markers (*VIM, FN1, MMP2*) and lncRNAs *SCHLAP1* and *HOTAIR.* Moreover, increasing RE of *HOTAIR* may cause the resistance for enzalutamide [29]. It is unique cluster with the highest *PRL* level, which could promote cancer progression through the PRL/STAT5 signaling pathway [15]. This is could mean prolactin administration of this cluster carcinogenesis.

We assume that cluster 2 is mixed stem-like androgen dependent subtype. The lowest expression of some epithelial and luminal markers *KRT18, PCA3, PSA* and high expression for mesenchymal markers *CDH2, MMP2,* and tendency to RE growth of *FN1,* and *VIM* are characteristics of stem-like (basal) prostate cancer, in spite of high AR, *CDH1, NKX3-1* RE. The highest RE levels of *ESR1, SRD5A2, INSR* B*, PRLR* and lncRNA *HOTAIR* give to this cluster peculiar carcinogenic property.

CONCLUSIONS

We have analyzed RE of 33 transcripts from 27 genes to find alterations in prostate tumors, depending on the presence or absence of the TMPRSS2/ERG fusion. The significant differences of RE ($p < 0.05$) for 7 genes were detected, when compared adenocarcinomas and corresponding CNTs, using the $2^{-\Delta C_T}$ model. Five genes (*ESR1, KRT18, MKI67, MMP9, PCA3*) showed differential expression, when the paired samples with compared that were bearing the fusion; and only two genes (*INSR* (B isof) and *HOTAIR*) — when samples did not expressed the fusion product. When the $2^{-\Delta\Delta C_T}$ model was used, the number of the differentially expressed genes were six (*MMP9, MKI67, PCA3, SCHLAP1*) and two (*ESR1, HOTAIR*) when the tissues expressed the fusion or regardless the presence of the fusion, respectively.

When adenomas, CNT and adenocarcinomas were compared, the *KRT18, PCA3* and *SCHLAP1* genes showed significant differences in RE in adenocarcinomas with the fusion. In adenocarcinomas without the fusion, such properties were shown by the *AR* (2 isof), *MMP9, PRLR* the *HOTAIR* genes. The *ESR1* and *SRD5A2* gene expression was altered in both types of adenocarcinomas.

Using the statistical analysis, we created three clusters of adenocarcinomas, based on gene RE and CPC characteristic. One of clusters was represented by adenocarcinomas with the TMPRSS2/ERG fusion. Further experiments are needed to confirm these data in a larger patient cohort.

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