doi: https://doi.org/10.15407/dopovidi2019.04.086
UDC 577.218+616.65

G.V. Gerashchenko, L.I. Chashchina, A.V. Rynditch, V.I. Kashuba

Institute of Molecular Biology and Genetics of the NAS of Ukraine, Kiev
E-mail: g.v.gerashchenko@imbg.org.ua

The gene expression pattern as a tool for assessment of components of microenvironment and response to anti-cancer therapy of prostate tumors

Presented by Corresponding Member of the NAS of Ukraine A.V. Rynditch

We have analyzed the putative value of the pattern of relative expression (RE) of several genes that might be involved in a response to anti-cancer therapy, namely AR, PTEN, COX2, FASN, HMGCR, LDLR, and CTLA4, in samples of prostate adenoma, adenocarcinoma, and the paired conventional normal tissues. We could propose three subtypes of adenocarcinomas that show the distinct pattern of expression of the above-mentioned genes, characteristics for (1) cancer-associated fibroblasts (CAFs), (2) tumor-associated macrophages (TAMs), and (3) markers of immune response. These groups correlate with the prostate cancer subtypes, that were determined earlier, based on the analysis of RE of the epithelial-to-mesenchymal cell transition (EMT) genes and prostate cancer-associated genes. Noteworthy, the highest correlation was found for genes characteristic of CAFs. This emphasizes the importance of the simultaneous analysis of genes, involved in various intercellular interactions between tumor cells and cells of tumor microenvironment, in prediction of efficacy of anti-cancer therapy. To confirm the presented data, the additional studies on a larger cohort of the prostate cancer patients are required.

Keywords: *prostate tumors, relative gene expression, pharmacological markers, tumor microenvironment, cancer-associated fibroblasts, tumor-associated macrophages.*

An analysis of the gene expression pattern, assessed by a quantitative PCR method, is a powerful approach to solve important questions of modern molecular biology and medicine. Earlier, we used such analysis for the profiling of prostate cancers and creation of a diagnostic panel [1—4]. Next, we wanted to explore the possibility to use the gene expression analysis to predict the efficacy of anti-cancer therapy and to evaluate components of a tumor microenvironment. Both questions are crucial for personalized medical treatment.

It is widely accepted now that one of the most important prediction tools to choose the correct patient treatment is the sensitivity of tumor cells to inhibitors of androgen receptor (AR). These drugs are used at the first line of prostate cancer treatment [5]. Hence, high expression of *AR* is favorable for effective therapy. Of note, low expression of a tumor suppressor *PTEN* or its mutations in prostate tumors results in a better response to inhibitors of the PI3K/AKT/mTOR signaling pathway. Importantly, PTEN itself is the inhibitor of this pathway [6]. That's why the

mutation status and expression levels of *PTEN* serve for the stratification of patients for the choice of therapy [7].

Development of specific drugs to treat prostate cancer is quite slow: only five new drugs were approved by FDA in the last 8 years [8]. However, other approach was proposed — the repositioning of drugs, i.e. when well-known drugs for other diseases are used to treat cancer [9]. Such drugs are, for example, inhibitors of proteins involved in lipid metabolism, namely inhibitors of HMGCR (statins), inhibitors of COX-2 and FASN. Moreover, it is important to assess the levels of *CTLA4* expression, due to the increased use of immunotherapy in cancer treatment [10].

Carcinogenesis is a very complex process involving not only primary cells under transformation, but also cells of microenvironment, such as fibroblasts, macrophages, endothelial cells, many types of immune cells, etc. All these stromal cells of the tumor microenvironment keep the balance between inhibition and stimulation of tumor growth and often can change with time from tumor-inhibiting to tumor-supportive [11]. Obviously, intercellular interactions influence not only the tumor growth, but also the response of tumor cells to treatment and efficacy of anti-cancer therapy.

In the present work, we aimed to cluster components of tumor microenvironment, hoping to predict the efficacy of anti-cancer therapy, using RE of a set of genes, namely *AR, PTEN, COX2, FASN, HMGCR, LDLR*, and *CTLA4*. We also want to relate clusters of components of microenvironment to earlier defined groups (subtypes) of prostate cancer.

Materials and Methods. *A collection of prostate tissues*. Samples of 37 prostate adenocarcinomas (PAC) of different stages and Gleason score and paired conventional normal tissues (CNT), and 20 benign prostate tumors (adenomas, AD) as well were collected as described earlier [2-4].

Total RNA isolation and cDNA synthesis. The total RNA was isolated from 50-70 mg of frozen prostate tissues, using a TRI-reagent (SIGMA), according to manufacturer's protocol. All procedures describing the assessment of the RNA quality, quantity, DNase I treatment, and cDNA synthesis were described earlier [1, 3].

Quantitative PCR (*qPCR*). RE levels of investigated genes were detected by qPCR, using Maxima SYBR Green Master mix (Thermo Fisher Scientific, USA) with a 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 genes were selected from a qPrimerDepot database (https://primerdepot.nci.nih.gov/). Four reference genes (*TBP*, *HPRT*, *ALAS1*, and *TUBA1B*) were used for the gene expression normalization [3, 4]. Two main models for the calculation of RE levels were used, namely the Livak method $2^{-\Delta Ct}$ and the $2^{-\Delta\Delta Ct}$ calculation that show relative quantities and RE fold changes, accordingly [12].

Statistical analysis. The Kolmogorov—Smirnov test was used to analyze the normality of a distribution. The Kruskal—Wallis test and following the Dunn—Bonferoni post hoc test were performed to determine RE differences by multiple comparisons between experimental groups [1, 4]. The Benjamini—Hochberg procedure with false discovery rate (FDR) 0.10—0.25 was used, when multiple comparisons were performed [13]. Clusterization methods (STASISTICA 10 software) were used to cluster components of tumor microenvironment, in particular the K-means clustering. The Spearman rank order correlation test was performed to find putative correlations between clusters [1, 3].

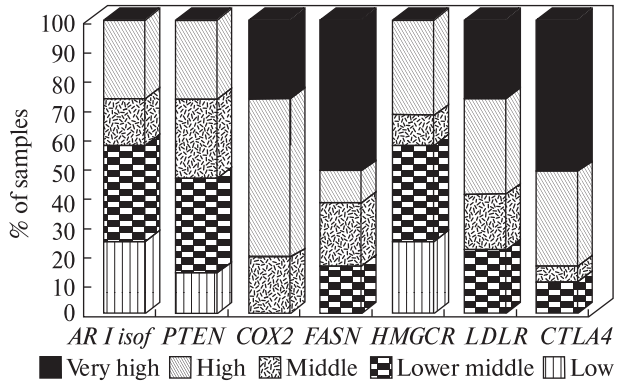

Fig. 1. Ranging of 37 adenocarcinoma samples, based on different levels of pharmacological efficacy

Results and Discussion. RE levels of seven genes often targeted by inhibitory drugs were assessed in PAC, CNT, and AD groups [1, 14]. Surprisingly, no significant differences in RE of these genes were found between groups of PAC and CNT. Instead, differences in RE between PAC and AD as a control were calculated (Table 1).

The analysis of RE levels in PAC and AD groups showed three types of changes: (1) significantly decreased expression in PAC – *AR 1 isof, PTEN*); (2) significant ly increased expression in AD — *COX2, FASN, CTLA4*; (3) no significant changes in RE — *HMGCR, LDLR*. Of note, all genes showed variable expression in individual tumors.

We ranged the putative efficacy (or sensitivity) to anti-cancer therapy as high, middle, lower middle, and low.

RE in AD was used as a control. Therefore, the first threshold level was a median for genes with RE changes of type (1). It could be divided into high and middle ranges. The next threshold level was a 25-th percentile of the AD group. It was divided in middle and lower middle ranges. These values are close to the median of the PAC group. The last threshold level was a 25-th percentile of the PAC group; it was divided in lower middle and low ranges.

Table 1. **The data on RE of pharmacological markers in PAC and AD groups**

Gene	Group	Median	Minimum	Maximum	25-th percentile	75-th percentile	*p*-value*
AR 1 isof	PAC	1.377	0.413	3.353	0.911	2.228	0.021
	AD	2.129	0.879	4.936	1.623	2.672	
PTEN	PAC	8.762	2.278	25.232	6.354	12.322	0.015
	AD	16.989	5.875	90.619	8.964	22.065	
COX2	PAC	1.550	0.291	10.554	0.777	2.319	0.019
	AD	0.542	0.152	4.647	0.256	1.053	
FASN	PAC	3.132	0.351	34.230	1.744	8.048	0.031
	AD	2.252	0.191	4.567	1.241	2.485	
HMGCR	PAC	0.393	0.043	9.724	0.221	0.712	0.058
	AD	0.631	0.331	1.717	0.469	0.901	
LDLR	PAC	0.647	0.052	4.890	0.443	1.227	0.677
	AD	0.605	0.223	1.415	0.360	0.835	
CTLA4	PAC	0.084	0.007	0.425	0.053	0.175	0.016
	AD	0.039	0.006	0.219	0.030	0,071	

N o t e . In bold — a group, that is a basis for ranging. * The Dunn—Bonferroni post hoc test for multiple comparisons.

For the type (2) of RE changes, we proposed an extra range, i.e. a very high level of sensitivity/efficacy. Its threshold level is the median of PAC.

The type (3) of RE changes allow us to range efficacy similarly to type (1), except an additional very high level, if RE of a 75-th percentile of PAC was higher than in AD.

The summarized results of ranging for seven genes are shown on Fig. 1.

It is important to note that the threshold levels of *AR* 1 isoform are very similar to levels of RE means for three subtypes of PAC characterized earlier [1]. Moreover, RE levels of *PTEN* inversely related to pharmacological efficacy of mTOR inhibitors.

The obtained results are our assumptions based on the RE and characteristics of RE changes in groups of prostate tumors. In order to establish the real sensitivity and efficacy for anti-cancer therapy, the clinical studies should be performed. On the other hand, these results suggest the different putative sensitivity to anti-cancer therapy.

As discussed above, the tumor microenvironment plays an important role in the control over the efficacy of anti-cancer therapy. Many components such as CAFs, TAMs, and tumor-infiltrating lymphocytes (TIMs) are known to orchestrate and support the development and expansion of tumors and reduce the effectiveness of antitumor therapy [6, 7]. To define the specific stromal subtypes in prostate cancer, RE of genes characteristic of CAFs (8 genes) (Fig. 2, *a*), TAMs (6 genes) (*b*), and immune-associated genes (IAGs) (9 genes) (*c*) was treated by the K-means clustering. All studied genes were clustered in three PAC groups based on various patterns of RE changes. The first and second clusters contain predominantly tumors at the Stage 1—2, whereas the third cluster consists of the advanced prostate tumors (Stage 3—4). The CAF group shows differences in RE between three clusters for 7 out of 8 genes, i.e.

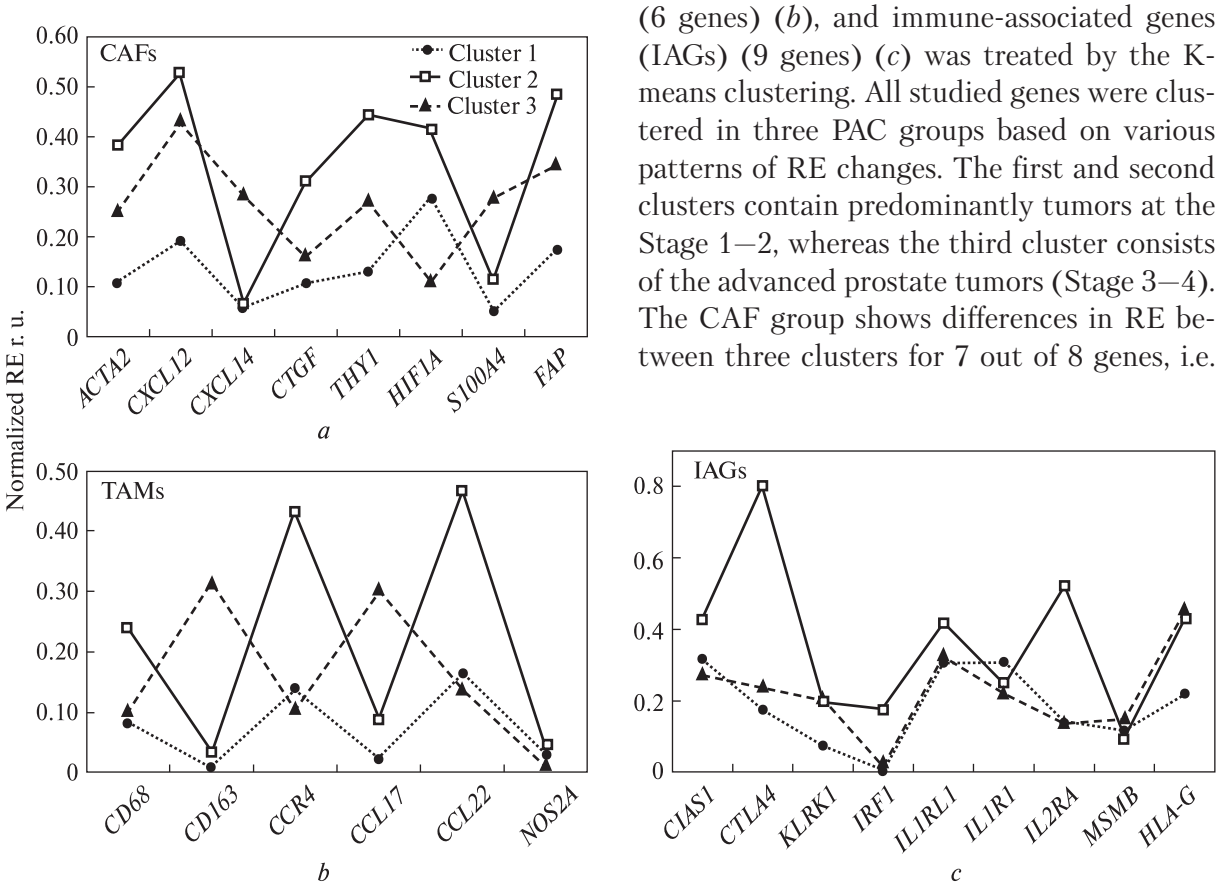

Fig. 2. Profiling of genes, associated with CAFs (*a*), TAMs (*b*), and IAGs (*c*) based on the analysis of gene RE, using the K-means clustering

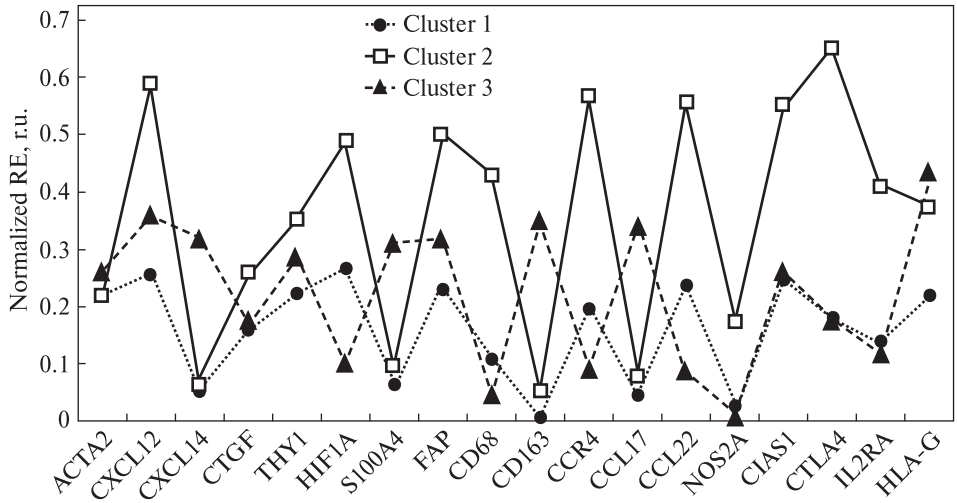

Fig. 3. Profiling of RE in PAC of 18 genes from the CAF, TAM, and IAG groups (total TM group), using the K-means clustering

except *CXCL14*. Nevertheless, the *CXCL14* gene has a tendency to changes in RE. In the TAM group, all 6 genes are expressed differently. The smallest number of genes with RE changes between clusters was calculated for the IAG group: only *CTLA4, IRF1, IL2RA*, and *HLA-G* demonstrated the altered expression pattern.

Based on the obtained data, we performed clustering for the CAF, Tam, and IAG groups for all the genes that showed differences in RE (Fig. 3). We could form also three clusters for PAC. Clusters 1 and 2 consist of samples at Stage 1—2, as was described earlier by us. Cluster 3 contains mainly PAC at Stage 3—4. Of note, differences in RE between clusters vanished for the majority of CAF genes, except for *CXCL12* and *HIF1A*.

Based on these results, we performed a correlation analysis between clusters of different types and between clusters of PAC. To do so, we took into account RE of 33 transcripts from genes associated with EMT and prostate cancer-associated genes (T) determined earlier [3]. The Spearman rank order correlations analysis for all cluster types was performed (Table 2).

One group of clusters (T) contains genes associated with tumor cells, and other three cluster types contain markers of stromal elements described above. All cluster types with stromal elements have high correlations between each other, indicating a significant relationship of CAFs, TAMs, and immune cells upon prostate carcinogenesis.

The most interesting is a high degree of correlation of clusters of microenvironment components with tumor cells. The highest levels of correlation showed the T and CAF groups. Obviously, upon prostate tumor development, there is the strong interaction between the tumor cells and stromal fibroblasts.

Cluster 1 of PACs consists of TMPRSS2-ERG positive androgen-sensitive tumors of the luminal subtype 1. These tumors, in addition to the high sensitivity to androgens, show the high sensitivity to prolactin, insulin growth factor 1, insulin stimulating oncogenic signals and have the activated oncogenic pathways, involving ERG and PCA3. The CAF cluster 1 corresponding to this PAC cluster is characterized by the lowest levels of RE of CAF markers, indicating a small

number of these cellular elements and their low activity, suggesting a favorable prognosis of the disease.

For cluster 2 of tumor markers and characteristics of CAF, tumors are of the same stage, as in cluster 1. However, several epithelial and luminal markers (*KRT18, PCA3, PSA*) showed the lowest expression, while mesenchymal markers (*CDH2, MMP2, FN1, VIM*) were highly expressed. This corresponds to a stem-cell-like (basal) subtype of prostate cancer [3]. Simultaneously highly expressed *ESR1, SRD5A2, INSRB, PRLR*, and lncRNA *HOTAIR* suggest that this cluster includes tumors with enhanced carcinogenic properties. The CAF cluster 2 includes samples with the highest RE of six genes (*ACTA2, CXCL12, CTGH, THY1, HIF1A, FAP*) associated with a poor prognosis of a course of disease.

Table 2. **The Spearman rank order correlations (r^s) between different groups of clusters with prostate cancer-associated genes and tumor microenvironment markers in PACs**

Cluster groups	T	CAF	TAM	IAG
T	1.0000			
CAF	**0.8219**	1.0000		
TAM	*0.5838*	**0.7616**	1.0000	
IAG	*0.6109*	**0.7380**	**0.7827**	1,0000

Note. Italic — $p < 0.001$; bold — $p < 0.0001$.

Cluster 3 contains advanced tumors at Stage 3-4. Mainly tumors are androgen independent and castrate-resistant of luminal subtype. The CAF characteristics are between clusters 1 and 2. However, samples in this cluster show very high level of RE of the TAM marker CD163, which is usually expressed in M2 macrophages. Obviously, tumor supportive macrophages contribute to progression of cancer disease [15].

We could distinct two molecular subtypes of PACs of the luminal type (clusters 1 and 3) with specific tumor gene expression characteristics at early and advanced stages. To these two PAC subtypes, two different CAF subtypes with high and low expression of CAF markers correspond. Similar trend was observed for the TAM, IAG, and total stromal clusters. Cluster 2 characterizes early prostate cancers with increased RE levels of basal and tumor stem cells markers in T cluster and specific RE levels of CAF, TAM, IAG genes. Thus, we have found three additional adenocarcinoma subtypes of stromal elements, which matched with three prostate cancer subtypes described by us earlier. These data are very important for better diagnosis and stratification of prostate tumors for the selection of personal treatments.

To summarize the obtained data, we have analyzed the putative value of the pattern of relative expression (RE) of several genes that might be involved in a response to anti-cancer therapy, namely *AR, PTEN, COX2, FASN, HMGCR, LDLR*, and *CTLA4*. We could propose three subtypes of adenocarcinomas that show the distinct pattern of expression of the above-mentioned genes characteristic of (1) CAFs, (2) TAMs, and (3) markers of immune response. These groups correlate with the prostate cancer subtypes that were determined earlier, based on the analysis of RE of the epithelial-to-mesenchymal cell transition (EMT) genes and prostate cancer-associated genes. Noteworthy, the highest correlation was found for genes characteristic of CAFs. This emphasizes the importance of the simultaneous analysis of genes involved in various intercellular interactions between tumor cells and cells of tumor microenvironment in prediction of efficacy of anti-cancer therapy. To confirm the presented data, the additional studies on a larger cohort of the prostate cancer patients are required.

REFERENCES

1. Gerashchenko, G. V., Mankovska, O. S., Dmitriev, A. A., Mevs, L. V., Rosenberg, E. E., Pikul, M. V., Marynychenko, M. V., Gryzodub, O. P., Stakhovsky, E. O. & Kashuba, V. I. (2017). Expression of epithelial-mesenchymal transition-related genes in prostate tumours. Biopolym. Cell, 33, No. 5, pp. 335-355. doi: https://doi.org/10.7124/bc.00095E
2. Gerashchenko, G. V., Rynditch, A. V. & Kashuba, V. I. (2019). Development of gene expression panels to determine prostate cancer. Dopov. Nac. acad. Nauk Ukr., No. 1, pp. 100-106. doi: https://doi.org/10.15407/dopovidi2019. 01.100
3. Gerashchenko, G. V., Mevs, L. V., Chashchina, L. I., Pikul, M. V., Gryzodub, O. P., Stakhovsky, E. O. & Kashuba, V. I. (2018). 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. Exp Oncol., 40, No. 2, pp. 101-108.
4. Gerashchenko, G. V., Grygoruk, O. V., Kononenko, O. A., Gryzodub, O. P., Stakhovsky, E. O. & Kashuba, V. I. (2018). Expression pattern of genes, associated with tumor microenvironment in prostate tumors. Exp. Oncol., 40, No. 4, pp. 315-322.
5. Aoun, F., Bourgi, A., Ayoub, E., El Rassy, E., van Velthoven, R. & Peltier, A. (2017). Androgen deprivation therapy in the treatment of locally advanced, nonmetastatic prostate cancer: practical experience and a review of the clinical trial evidence. Ther. Adv. Urol., 9, No. 3-4, pp. 73-80. doi: https://doi.org/10.1177/1756287217697661
6. Matsumoto, C. S., Almeida, L. O., Guimarães, D. M., Martins, M. D., Papagerakis, P., Papagerakis, S., Leopoldino, A. M., Castilho, R. M. & Squarize, C. H. (2016). PI3K-PTEN dysregulation leads to mTOR-driven upregulation of the core clock gene BMAL1 in normal and malignant epithelial cells. Oncotarget, 7, No. 27, pp. 42393-42407. doi: https://doi.org/10.18632/oncotarget.9877
7. Jamaspishvili, T., Berman, D. M., Ross, A. E., Scher, H. I., De Marzo, A. M., Squire, J. A. & Lotan, T. L. (2018). Clinical implications of PTEN loss in prostate cancer. Nat. Rev. Urol., 15, No. 4, pp. 222-234. doi: https://doi.org/10.1038/nrurol.2018.9
8. New Drugs at FDA: CDER's New Molecular Entities and New Therapeutic Biological Products. Retrieved from https://www.fda.gov/drugs/developmentapprovalprocess/ druginnovation/default.htm
9. Turanli, B., Grøtli, M., Boren, J., Nielsen, J., Uhlen, M., Arga, K. Y. & Mardinoglu, A. (2018). Drug repositioning for effective prostate cancer treatment. Front. Physiol., 15, No. 9, 500. doi: https://doi.org/10.3389/fphys.2018.00500
10. Montironi, R., Santoni, M., Sotte, V., Cheng, L., Lopez-Beltran, A., Massari, F., Matrana, M. R., Moch, H., Berardi, R. & Scarpelli, M. (2016). Emerging immunotargets and immunotherapies in prostate cancer. Curr. Drug. Targets, 17, No. 7, pp. 777-782.
11. Komohara, Y. & Takeya, M. (2017). CAFs and TAMs: maestros of the tumour microenvironment. J. Pathol., 241, No. 3, pp. 313-315. doi: https://doi.org/10.1002/path.4824
12. Livak, K. & Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25, No. 4, pp. 402-408. doi: https://doi.org/10.1006/meth.2001.1262
13. Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B (Methodological), 57, No. 1, pp. 289-300.
14. Gerashchenko, G. V., Kononenko, O. A., Bondarenko, Yu. M., Stakhovsky, E. O. & Kashuba, V. I. (2018). Expression patterns of genes, regulating lipid metabolism in prostate tumors. Biopolym. Cell, 34, No. 6, pp. 445–460. doi: https://doi.org/10.7124/bc.000990
15. Takahashi, H., Sakakura, K., Kudo, T., Toyoda, M., Kaira, K., Oyama, T. & Chikamatsu, K. (2017). Cancer-associated fibroblasts promote an immunosuppressive microenvironment through the induction and accumulation of protumoral macrophages. Oncotarget, 8, No. 5, pp. 8633-8647. doi: https://doi.org/10.18632/oncotarget.14374

Received 21.02.2019

Г.В. Геращенко, Л.І. Чащина, А.В. Риндич, В.І. Кашуба

Інститут молекулярної біології і генетики НАН України, Київ
E-mail: g.v.gerashchenko@imbg.org.ua

ПАТЕРНИ ЕКСПРЕСІЇ ГЕНІВ ЯК ЗАСІБ ДЛЯ ОЦІНКИ КОМПОНЕНТІВ МІКРООТОЧЕННЯ ТА ВІДПОВІДІ НА АНТИРАКОВУ ТЕРАПІЮ ПУХЛИН ПЕРЕДМІХУРОВОЇ ЗАЛОЗИ

Проаналізовано потенційні значення діапазонів відносної експресії ряду генів, які можуть бути залучені у відповідь на протиракову терапію, а саме *AR, PTEN, COX2, FASN, HMGCR, LDLR* і *CTLA4*, у зразках аденом, аденокарцином і парних умовно-нормальних тканин передміхурової залози. Визначено три підтипи аденокарцином, які показують чітку картину експресії досліджуваних генів, які характеризують: 1) пухлиноасоційовані фібробласти; 2) пухлиноасоційовані макрофаги; 3) маркери імунної відповіді. Ці групи корелюють з підтипами раку передміхурової залози, які були визначені раніше на основі аналізу відносної експресії генів, асоційованих з епітеліально-мезенхімальним переходом, і генів, пов'язаних з раком передміхурової залози. Звертає на себе увагу, що найбільша кореляція була знайдена для цих підтипів раку й групи генів пухлиноасоційованих фібробластів. Це підкреслює важливість одночасного аналізу генів, залучених до різних міжклітинних взаємодій між клітинами пухлин і клітинами пухлинного мікрооточення, в прогнозуванні ефективності протиракової терапії. Для підтвердження одержаних даних необхідні додаткові дослідження на більшій вибірці хворих на рак передміхурової залози.

Ключові слова: *пухлини передміхурової залози, відносна експресія генів, фармакологічні маркери, пухлинне мікрооточення, пухлиноасоційовані фібробласти, пухлиноасоційовані макрофаги.*

А.В. Геращенко, Л.И. Чащина, А.В. Рындич, В.И. Кашуба

[1] Институт молекулярной биологии и генетики НАН Украины, Киев
E-mail: g.v.gerashchenko@imbg.org.ua

ПАТТЕРНЫ ЭКСПРЕССИИ ГЕНОВ КАК СПОСОБ ОЦЕНКИ КОМПОНЕНТОВ МИКРООКРУЖЕНИЯ И ОТВЕТА НА АНТИРАКОВУЮ ТЕРАПИЮ ОПУХОЛЕЙ ПРОСТАТЫ

Проанализированы потенциальные значения диапазонов относительной экспрессии ряда генов, которые могут быть вовлечены в ответ на противораковую терапию, а именно *AR, PTEN, COX2, FASN, HMGCR, LDLR* и *CTLA4*, в образцах аденом, аденокарцином и парных условно-нормальных тканей простаты. Определены три подтипа аденокарцином, показывающие четкую картину экспрессии исследуемых генов, которые характеризуют: 1) опухолеассоциированные фибробласты; 2) опухолеассоциированные макрофаги; 3) маркеры имунного ответа. Эти группы коррелируют с подтипами рака простаты, которые были детектированы ранее на основе анализа относительной экспрессии генов, ассоциированных с эпителиально-мезенхимальным переходом, и генов, связанных с раком простаты. Важно отметить, что наибольшая корреляция была найдена для этих подтипов рака и группы генов опухолеассоциированных фибробластов. Это подчеркивает важность одновременного анализа генов, участвующих в различных межклеточных взаимодействиях между клетками опухолей и клетками опухолевого микроокружения, в прогнозировании эффективности противораковой терапии. Для подтверждения полученных данных необходимы дополнительные исследования на большей выборке больных раком простаты.

Ключевые слова: *опухоли простаты, относительная экспрессия генов, фармакологические маркеры, опухолевое микроокружение, опухолеассоциированные фибробласты, опухолеассоциированные макрофаги.*