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Development of gene expression panels to determine prostate cancer

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The aim of this investigation is to prove a modified algorithm for statistical approaches to develop gene expression panels for the detection of prostate tumors. According to Classification and Regression tree models and RE differences between adenocarcinoma (T) and adenoma (A) groups, we have chosen 31 transcripts for MDR analysis. Among them, there were 15 transcripts of (epithelial-mesenchymal transition (EMT) and prostate-cancer associated (PrCa-associated) genes and 16 transcripts of cancer-associated fibroblasts (CAF), tumor-associated macrophages (TAM), immune-associated genes (IAG)), which have shown some datasets with high statistical parameters. The highest diagnostic levels are manifested by expression panels developed from all 5 gene groups: PCA3, HOTAIR, ESR1, IL1R1 ($Se = 0.97$, $Sp = 0.85$, $Ac = 0.93$, $OR = 204$); CDH2, KRT18, PCA3, HOTAIR, ESR1, IL1R1 ($Se = 1.0$, $Sp = 0.8$, $Ac = 0.93$, $OR > 500$). We propose an improved algorithm for the gene expression data analysis to develop diagnostic panels with good and excellent diagnostic levels for the prostate tumor stratification in a group of patients from the Ukrainian population. Our data require a more detailed analysis and a larger cohort of patients with prostate tumor.

Keywords: prostate tumors, gene expression panels, MDR analysis, gene expression panels, cancer-associated genes.

5 main functions of a biomarker/panels of biomarkers in clinical practice are known. They play important roles in the diagnostics of different human and animal diseases, severity assessment, risk assessment, prediction of drug effects and sensitivity to drugs and treatment response, the last function is a monitoring of diseases or influences [1, 2]. The primary results which give biomarkers or its panels correspond to data points, which are the basis of the Diagnostic Matrix. Two of them give the presence of a disease: true positive (t+) and false negative (f-). The other two values characterize the absence of a disease: true negative (t-) and false positive (f+). These values form the contingency table. Several diagnostic parameters which characterize the diagnostic value of investigated biomarkers are derived from these four values. Among them are the sensitivity (Se), specificity (Sp), accuracy (Ac), prevalence, predictive value [positive (PPV) and negative (NPV)], likelihood ratio [positive (LHR+) and negative (LHR-)], receiver-operating characteristics (ROC) curve and area under the ROC (AUC_{ROC} , named also the *c* index), diag-

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nostic odds ratio (OR), and others. Different investigators and different statistical approaches use different combinations of these parameters in the evaluation of biomarkers. But the required parameters of them are Se, Sp, Ac, and OR [1–3].

Nowadays, it is known several statistical tests, programs, and approaches which calculate some of these diagnostic parameters for one potential biomarker or a panel of biomarkers [4–6]. Many of them aren't free for researches; some of them are developed by bioinformatics centers for specific tasks and couldn't be used for other goals [7].

Last time, machine learning methods, like the MDR approach, are widely used for the modeling and evaluation of genetic, epigenetic, and pharmacogenomics interactions in multifactor diseases [8–10]. The MDR approach contributes to dichotomy data. It compares 2 sample groups and uses many statistical tests with combinatorial analysis of specified attributes. This configuration corresponds to a diagnostic matrix in biomarker searching methods [1]. We will develop an algorithm to adjust MDR analysis for the gene expression panel evaluation. As a result, we will show a possibility of the development of gene expression panels by the MDR approach.

Materials and Methods. *A collection of prostate tissues.* Samples of prostate cancer tissues and adenomas were collected, as described earlier [11–13].

Total RNA isolation and cDNA synthesis. 50–70 mg of frozen prostate tissues were used for total RNA isolation by TRI-reagent (SIGMA), according to manufacturer's protocol. All procedures of characterization of the total RNA quality and quantity, DNaseI treatment and cDNA synthesis were described earlier [11].

Quantitative PCR (qPCR). Relative gene expression (RE) levels of 57 transcripts were detected by qPCR, using 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 at 72 °C – 30 s. Primers for genes were selected from qPrimerDepot (<https://primerdepot.nci.nih.gov/>). Four reference genes *TBP*, *HPRT*, *ALAS1* and *TUBA1B* were used for the gene expression normalization [11, 12]. Two main models for RE levels calculation were used. There were the Livak method $2^{-\Delta Ct}$ and the $2^{-\Delta\Delta Ct}$ method representing relative quantities and fold changes, accordingly [14].

Statistical analysis. The Kolmogorov–Smirnov test was used to analyze the normality of a distribution. The Kruskal–Wallis test and the following Dunn–Bonferoni post hoc test were performed to determine RE differences by multiple comparisons between experimental groups [11, 12]. The Benjamini–Hochberg procedure with false discovery rate (FDR) 0.10–0.25 was used, when multiple comparisons were performed [15]. Classification methods in STATISTICA 10 software were used to find possible predictor genes and rules for sample groups' stratification. MDR 3.0.2 were used to develop gene expression panels and to analyze their diagnostic levels [8, 9].

Results and Discussion. We have detected RE of 57 transcripts from different groups: EMT-related genes [11], prostate cancer-related genes [12], CAF-associated genes, TAM-related genes, immune-associated genes (IAG) [13] in 37 prostate adenocarcinomas, paired conventional tissues (CNT), and 20 adenomas. Very high RE dispersion into prostate conventional normal tissue (CNT) groups from patients with different clinical characteristics couldn't give possibility to detect unchanged or normal RE levels. Therefore, RE of the adenoma group has been decided to use as a control group for the expression panels development, despite that prostate adenomas are the disease with its own mechanisms of development [11]. However, adeno-

mas are a conventional norm or control group in many studies to investigate changes in malignant prostate tumors [12].

To improve an algorithm of MDR analysis for the gene expression panel evaluation, it is necessary to do next steps: to choose a group of potential genes predictors, to identify threshold RE levels for dividing samples, to transfer continuous RE data to binary values. Then we will process the obtained data, by using the MDR approach to analyze possible variants to choose the most statistically significant variants of datasets, being the most appropriate for expression panels.

The first task of our work was to discover potential gene predictors, which could classify adenocarcinomas and adenomas into groups by RE levels.

First Method for stratification of prostate adenomas and adenocarcinomas that we used was discriminant-based univariate splits for categorical and ordered predictors. It chooses the most appropriate group predictors with high importance for group discrimination and constructs Classification trees. Examples of them are given below.

RE of 20 EMT transcripts (19 genes) was investigated. Among them, there are genes/transcripts with high predictor importance for T/A grouping: *HOTAIR*, *KRT18*, *PCA3*, *AR*, *PTEN*, *MMP9*.

Next group of genes/transcripts is prostate cancer-associated genes (14 transcripts). It has some genes with high predictor importance for the T/A group discrimination: *SRD5A2*, *PRLR*, *ESR1*.

Among 8 CAF genes, there are genes with high predictor importance for the T/A group discrimination: *CXCL12*, *CXCL14*, *THY1*.

Table 1. Types of gene RE changes to determine the threshold level for prostate adenocarcinoma group in comparison to adenoma group for MDR analysis

Gene group	Gene	RE changes	Gene group	Gene	RE changes		
EMT	CDH2	>2 times	CAF	CTGF	>2 times		
	FN1	>2 times		CXCL12	<2 times		
	KRT18	>2 times		CXCL14	>2 times		
	MMP9	>2 times		HIF1A	<2 times		
	PTEN	<2 times		FAP	>2 times		
	VIM	>2 times		S100A4	>2 times		
	PCA3	>2 times		THY1	>2 times		
	HOTAIR	>2 times		TAM	CD68	<2 times	
	SCHLAP1	>2 times			CD163	>2 times	
	ESR1	>2 times			CCL17	>2 times	
	GCR ins AG	>2 times			CCR4	<2 times	
	PrCa-associated	GCR ins B		>2 times	IAG	CTLA4	>2 times
		PRLR		<2 times		HLA-G	>2 times
		PRL		>2 times		IL1R1	<2 times
VDR		>2 times	MSMB	<2 times			
SRD5A2		<2 times					

Genes from TAM group (6 genes) have shown a high predictor importance for the T/A group discrimination for *CCRA*, *CCL22*, *CCL17*, *CD68*.

Discriminant-based univariate splits for the categorical and ordered predictors classification method found no predictors in IAG gene group (9 genes), but the other Classification and Regression tree models (C&RT) have shown the high predictor importance for all these genes in the classification for the T/A group discrimination. The highest predictor levels were shown by *IL1R1*, *CTLA4*, *MSMB*.

Classification RE analyses of investigated genes for group predictor identification have shown several genes (near 20 genes) with a high predictor importance to stratify T/A groups. All these genes have significant RE changes between adenocarcinoma and adenoma groups [11-13]. We took these genes for the further analysis. As a result, among 57 transcripts from 5 gene groups (EMT-related, PrCa-associated, CAF, TAM, IAG), we selected 30 genes (31 transcripts) with significant RE differences between A and T for expression panels development. Moreover, we considered RE differences in T group with different stages and GS.

The next task after the gene selection for panel development is to determine the threshold RE level, which divides samples with unchanged RE and changed RE for every gene. Based on our previous studies, we have found that the 2-time (up or down) RE change is significant to identify group differences [11, 12]. We chose it as the threshold RE level from adenomas as a control group to transfer continuous RE values to binary values. These data are shown in Table 1.

We have found 22 genes/transcripts with increased RE in T group in comparison with A, and 9 genes with decreased RE in T among 5 gene groups.

We have prepared RE data for MDR analysis to transfer data to binary values. Unchanged RE levels mean “0”, changed (up or down) RE levels – mean “1”. Prostate samples have 2 classes: T and A. We use the next parameters of an MDR program for analysis: Fisher’s exact test ($p < 0.05$), and the attribute count range – all genes in the investigated group, Ambiguous cell assignment – Class 1 (T), Search method of configuration – Exhausted and Random, Filter selection – Chi square.

4 different diagnostic values of biomarker/panel of biomarkers are known: excellent, good, poor, and no diagnostic value [1]. We are interested in excellent and good diagnostic values.

Table 2. EMT and PrCa-associated gene combinations with the highest statistical parameters of expression datasets

Gene panels	MDR Dataset Statistics
<i>KRT18</i> , <i>HOTAIR</i> , <i>ESR1</i>	Accuracy: 0.91 Sensitivity: 0.95 Specificity: 0.85 Odds Ratio: 99.17 X^2 : 37.01 ($p < 0.0001$)
<i>KRT18</i> , <i>MMP9</i> , <i>PCA3</i> , <i>HOTAIR</i> , <i>ESR1</i>	Accuracy: 0.91 Sensitivity: 0.95 Specificity: 0.85 Odds Ratio: 99.17 X^2 : 37.01 ($p < 0.0001$)

Table 3. CAF, TAM, IAG-associated gene combinations with the highest statistical parameters of expression datasets

Gene panels	MDR Dataset Statistics
<i>CXCL12</i> , <i>CXCL14</i> , <i>HIF1A</i>	Accuracy: 0.89 Sensitivity: 0.97 Specificity: 0.75 Odds Ratio: 108.0 X^2 : 33.61 ($p < 0.0001$)
<i>CXCL12</i> , <i>CXCL14</i> , <i>HIF1A</i> , <i>CD163</i>	Accuracy: 0.89 Sensitivity: 0.97 Specificity: 0.75 Odds Ratio: 108.0 X^2 : 33.6083 ($p < 0.0001$)

The highest one is the excellent diagnostic value, which has correspondence parameters of Accuracy >0.9, Sensitivity and Specificity (>0.9 both), and Odds Ratio (>100). The good diagnostic value has the next parameters: Accuracy >0.75–0.9, Sensitivity and Specificity >0.75–0.9, and Odds Ratio 25–100.

In MDR analysis, we analyzed each of the 5 gene groups separately (EMT, PrCa-associated, CAF, TAM, IAG), but we did not get high statistical values for expression panels. So, we decided to combine the groups of genes. The first group is tumor-associated genes (EMT, PrCa-associated) – 16 transcripts. The second group is a host organism-associated or tumor microenvironment-related genes (CAF, TAM, IAG) – 15 transcripts.

Combinatorial analysis between 16 (EMT and PrCa-associated) genes/transcripts has revealed the combinations of genes with high values of statistical parameters shown in Table 2.

Thus, the diagnostic parameters of datasets arose from one to three genes in datasets. The maximum of statistical data have panels with 3-5 genes. Their Accuracy and Sensitivity correspond to the excellent diagnostic value, but Specificity and Odds Ratio correspond to the good diagnostic value.

It is necessary to increase the statistical parameters to reach an excellent diagnostic value, by checking other genes with high predictor levels.

Combinatorial analysis of 15 genes (CAF, TAM, and IAG groups) has revealed the following combinations of genes with high values of datasets statistical parameters of potential expression panels (Table 3).

The highest levels of Accuracy, Sensitivity, Specificity, and Odds Ratio have datasets with 3-5 genes in panel in this gene group. Accuracy corresponds to the good diagnostic value, but Specificity is too low, whereas Sensitivity and Odds Ratio correspond to the excellent diagnostic value.

Table 4. Gene combinations from group of 31 transcripts with the highest statistical parameters in potential expression panels

Gene panels	MDR Dataset Statistics
<i>PCA3, HOTAIR, ESR1, IL1R1</i>	Accuracy: 0.9298 Sensitivity: 0.973 Specificity: 0.85 Odds Ratio: 204.0 X^2 : 40.695 ($p < 0.0001$)
<i>MMP9, PCA3, HOTAIR, ESR1, IL1R1</i>	Accuracy: 0.9298 Sensitivity: 0.973 Specificity: 0.85 Odds Ratio: 204.0 X^2 : 40.695 ($p < 0.0001$)
<i>CDH2, KRT18, PCA3, HOTAIR, ESR1, IL1R1</i>	Accuracy: 0.9298 Sensitivity: 1.0 Specificity: 0.8 Odds Ratio: ∞ (>500) X^2 : 41.1512 ($p < 0.0001$)

Increasing the attributes (genes) quantity in analyzed datasets by more than 5 times leads to decreasing the statistical parameters in this gene group.

According to our described results, we could suggest that MDR analysis into a large group of genes could give better statistical parameters. The last variant of MDR analysis was performed with 30 genes (31 transcripts) in one big group for T/A stratification. Table 4 shows gene panels and their statistical parameters for the best variants of combinatorial analysis.

In expression panels, more than 6 attributes (genes) in groups statistical parameters i.e. Accuracy, Sensitivity, Odds Ratio begin to decrease in comparing to panels with 4-6 attributes (genes). In addition, an increase in the number of genes in the panel overcomplicates the interpretation of the results (If-Then rules) and increases the cost of potential analysis.

Moreover, MDR is a machine learning approach, which divides analyzed samples of both T/A groups into two groups in random manner: training and testing sample groups. For them, it gives cross-validation statistics with all parameters as for the whole dataset (they are shown in tables above). The obtained data allow one to do the prognosis about a possible deviation in diagnostic values of investigated potential expression panels. It is worth to note that, all statistical parameters (Ac, Se, Sp, OR, Chi square) for both compared types of groups agree with parameters of their whole datasets.

We have improved an algorithm, which is appropriate for MDR analysis, and have found that expression panels which include genes from different cancer-associated gene groups have the highest statistical parameters.

We have analyzed RE of 57 transcripts in prostate tumors and have found several genes with high predictor levels for prostate cancer stratification. We have proposed an improved algorithm for gene expression data analysis to develop diagnostic panels based on relative gene expression levels. Moreover, we have developed an expression panel with high statistical parameters for prostate tumor stratification in a group of patients from the Ukrainian population. Our data require a detailed analysis and a larger cohort of patients with prostate tumors.

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РОЗРОБКА ЕКСПРЕСІЙНИХ ПАНЕЛЕЙ ДЛЯ ВИЗНАЧЕННЯ РАКУ ПЕРЕДМІХУРОВОЇ ЗАЛОЗИ

Досліджено адаптацію модифікованого алгоритму статистичного підходу для розробки експресійних панелей для детекції раку передміхурової залози. За даними моделей класифікації та регресії й статистично значущими відмінностями відносної експресії між групами аденокарцином та аденом, серед досліджуваних генів відібрано 31 транскрипт для MDR аналізу. Серед них 15 транскриптів з груп генів епітеліально-мезенхімального переходу та генів, асоційованих з раком передміхурової залози, і 16 транскриптів з груп генів пухлиноасоційованих фібробластів, пухлиноасоційованих макрофагів та імунасоційованих генів. З цих груп отримано ряд панелей з високими статистичними показниками. Найвищі показники діагностичних рівнів мали експресійні панелі, які розроблені з усіх п'яти груп генів: *PCA3, HOTAIR, ESR1, IL1R1* (Se = 0,97, Sp = 0,85, Ac = 0,93, OR = 204); *CDH2, KRT18, PCA3, HOTAIR, ESR1, IL1R1* (Se = 1,0, Sp = 0,8, Ac = 0,93, OR > 500). Запропоновано модифікований алгоритм для аналізу даних експресії генів, який може бути використаний для розробки діагностичних панелей з добрим та високим діагностичними рівнями для стратифікації раку передміхурової залози на групі пацієнтів з української популяції. Одержані дані потребують подальшого аналізу на більшій вибірці пацієнтів з раком передміхурової залози.

Ключові слова: пухлини передміхурової залози, MDR аналіз, експресійні панелі, пухлиноасоційовані гени.

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РАЗРАБОТКА ЭКСПРЕССИОННЫХ ПАНЕЛЕЙ ДЛЯ ВЫЯВЛЕНИЯ РАКА ПРОСТАТЫ

Исследована адаптация модифицированного алгоритма статистического подхода для разработки экспрессионных панелей для детекции рака простаты. Согласно данным моделей классификации и регрессии, а также статистически значимым отличиям относительной экспрессии между группами аденокарцином и аденом, среди исследуемых генов отобрано 31 транскрипт для MDR анализа. Среди них 15 транскриптов из групп генов эпителиально-мезенхимального перехода и генов, ассоциированных с раком простаты, и 16 транскриптов из групп генов опухолеассоциированных фибробластов, опухолеассоциированных макрофагов и иммуноассоциированных генов. Из этих групп было получено ряд панелей с высокими статистическими показателями. Самые высокие показатели диагностических уровней имели экспрессионные панели, полученные со всех пяти групп генов: *PCA3, HOTAIR, ESR1, IL1R1* (Se = 0,97, Sp = 0,85, Ac = 0,93, OR = 204); *CDH2, KRT18, PCA3, HOTAIR, ESR1, IL1R1* (Se = 1,0, Sp = 0,8, Ac = 0,93, OR > 500). Предложен модифицированный алгоритм для анализа данных экспрессии генов, который может быть использован для разработки диагностических панелей с хорошим и высоким диагностическими уровнями для стратификации рака простаты на группе пациентов из украинской популяции. Полученные данные требуют более детального анализа на большей выборке пациентов с раком простаты.

Ключевые слова: опухоли простаты, MDR анализ, экспрессионные панели, опухолеассоциированные гены.