Identification and Functional Profiling of Differentially Expressed Extracellular Vesicle-free MicroRNAs for Efficient Prostate Cancer Diagnosis

Mina Jahandideh, Ebrahim Barzegari*

Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran

Abstract

MicroRNAs are interesting as cancer diagnostic and prognostic biomarkers because of their unique tissue expression profiles, higher stability in the blood in comparison to mRNAs, and the possibility for reliable quantification. In the case of prostate cancer (PCa), it is currently emphasized to explore new biomarkers, particularly from microRNAs which are freely available in the bloodstream. In this study, the gene expression omnibus database (GEO), a repository of microarray data for PCa circulating extracellular vesicle-free microRNAs profiling, was analyzed for differentially expressed miRNAs (DE-miRs). Top 20 most differentially expressed miRs with significant (adjusted p-value < 0.01) high expression (fold change) levels were extracted by the simultaneous application of different filtering criteria. Then, microRNA-gene networks were constructed for the two sets of positively (n=20) or negatively (n=20) regulated miRNAs. Gene ontology annotations of the target gene sets were also extracted and analyzed. Results indicated that human miR-1587, miR-223-3p, miR-3125, and miR-642b-3p are highly significant DE-miRs in PCa. In addition, human miR-4459, miR-1273g, miR 642a-3p, and miR-642b-3p were identified as top-ranked hubs in the relevant miRNA-gene networks. FOXK1, PML, CD24, ATN1, BAZ2A, CDKN1A, NUFIP2, and HARNPU were identified as microRNA target genes with significant dysregulation. miR-4459, miR-1273g-3p, miR-3135b, miR-5001-5p, and miR-1587 were proposed as novel microRNAs with the potential to be utilized as diagnostic biomarkers of prostate cancer among circulating vesicle-free miRNAs.

Keywords: Prostate Cancer, Diagnosis, Biomarker, Vesicle-free microRNAs, Gene ontology

Introduction

Prostate cancer (PCa), as one of the most prevalent cancer types in males, is estimated to affect more than one million cases annually in the Western world (Bray et al., 2018). Benign forms of PCa can be cured through surgery or radio-therapy with a 5-year survival rate in nearly 100% of cases. However, metastatic cases are not generally treatable (5-year survival rate of less than 40%)(Hamdy et al., 2016; Helgstrand et al., 2018). For patients with advanced stage of the disease, over-treatment is used for long-term patient survival. Accordingly, it is critical to diagnose PCa in a timely and accurate manner.

Transrectal biopsy is the common diagnostic approach of PCa, usually performed following the observation of increased levels of prostate specific antigen (PSA) in the serum, and suspicious results from the low-sensitivity method of digital rectal examination. Although, this ultrasound-guided biopsy technique is erroneous (with false-positive rate 25%)(Bolla and van Poppel, 2012). Multi-parametric magnetic resonance imaging outperforms the standard transrectal biopsy method, but it is accompanied by false negatives and has limited availability (Boesen et al., 2018; Elkjaer et al., 2018). Considering these drawbacks, finding less invasive and more accurate strategies for prostate cancer diagnosis is imperative.

Liquid biopsy samples from blood and urine are convenient sources of possible nucleic acid markers associated with cancer initiation or progression. Circulating non-coding RNAs, specifically microRNAs (miRNAs, miRs), play a significant role in distinguishing various types of tumors, including PCa (Laursen et al., 2019; Lin and Gregory, 2015; Movahedpour et al., 2019; Schmidt et al., 2018; Urabe et al., 2019).

During recent years, miRNAs have brought about a micro-revolution in cancer research and diagnosis due to their contributions as oncogenes or tumor-suppressor genes (Ferracin et al., 2010). There are two forms of circulating cell-free microRNAs within the blood: I) vesicle-enclosed miRNAs (Huang et al., 2013; Valadi et al., 2007), and II) vesicle-free miRNAs (RNA binding proteins) (Wang et al.,

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*Corresponding author's e-mail address:
e.barzegari@kums.ac.ir

2010). As vesicle-free microRNAs are more available for extraction and performing downstream experiments, they could be considered as more appropriate candidates for development of novel detection methods based on the application of miR biomarkers.

Many studies focus on the applications of miRs as biomarkers in PCa detection. Even though, such data have been used fewer for understanding the basic functions of these regulatory elements in normal and cancerous tissues of the prostate. In the current study, we profile regulatory networks and functional targets of vesicle-free circulating microRNAs which are significantly dysregulated in PCa patients. By exploiting the non-coding RNA profiling data, this study aims to unveil the potentials of the new types of microRNAs to be utilized as biomarkers for PCa occurrence or prognosis.

Materials and Methods

Microarray data retrieval and analysis

The miRNA expression profiles of PCa patients vs. healthy individuals were obtained from the national center for biotechnology information (NCBI) gene expression omnibus (GEO) with accession number GSE113234 (Mello-Grand et al., 2019). These data were obtained by microarrays from 60 PCa patients and 27 healthy subjects. The microRNAs were hybridized on arrays with probes designed for a set of 2006 human miRs. During comparative studies (PCa vs. healthy subjects) using the last version of R (version 3.4.1), we retained differentially expressed miRNAs with fold-changes greater than 2.0. *p*-values were adjusted through Benjamini & Hochberg method. Shortlisted circulating microRNAs were then utilized in combination with the results from PSA test to develop an accurate and non-invasive prostate cancer detection strategy.

Identification of differentially expressed microRNAs

To identify the significantly up-regulated microRNAs in patients with PCa in comparison to healthy subjects, miRs with the highest fold change values were extracted from outputs. The same procedure was performed for the lowest values of logFC to identify the significantly down-regulated microRNAs. Different search filters including the existence of statistically significant differences and cut-off values for gene expression fold changes were applied simultaneously to identify top 20 differentially expressed miRs. These DE miRs with the highest or lowest fold changes were selected for

further analysis. The *p* values for these sets of miRNAs were less than 0.01 for both positively and negatively regulated microRNAs.

Target identification

MirWalk-database was used to identify the target genes of each DE-miRNA. For each miRNA, a set of the 100 most significantly related genes were determined. Thereby, totally 2000 genes were extracted as the targets of up-regulated and down-regulated miRs.

Functional enrichment analysis

Genes targeted by the most significantly up-regulated microRNAs in PCa were used as primary input to the Database for annotation, visualization and integrated discovery (DAVID, v6.8). This database provides clustering and annotations to the input genes list. Similar procedure was performed for the gene targets of down-regulated microRNAs. These analyses were designed to find enriched biological themes/ gene ontology (GO) terms related to the biological processes (BPs) encountered with the functions of DE-miRNA target genes. Top 20 of the significant GO terms were considered as representatives of the functional annotations for DE-miRNAs with key roles in prostate cancer.

A Visual Basic Script (VBScript) code was created in the Microsoft Excel program to identify the genes which are most frequently present in the collective sets of genes associated with the top BPs. It could be considered that these genes are involved in the pathogenesis or manifestation of PCa.

Network analyses

NetworkAnalyst 3.0 platform was used to extract the complete network of genes which are frequent among the miRs targets (Zhou et al., 2019). Moreover, miRNet 2.0 visualization web-tool was utilized to extract and analyze the miRNA-target gene interaction networks (Chang et al., 2020). The human microRNA disease database (HMDD, v3.2) was screened for evidences confirmed associations between miRs and PCa (Huang et al., 2019). Also, to find driver genes of prostate cancer, DisGeNet (version 7.0), a gene-disease association dataset, was administered. In order to visualized the functional interconnections of the target genes in addition to their significant GO terms, the GOnet server tool was applied using the threshold value $p < 1.94e-5$ as the relation significance (Pomaznoy et al., 2018).

Results

Gene expression value distributions

Cross-platform comparison of gene expression value distributions was performed to determine whether the expression profiles are comparable between PCa and healthy individuals in the corresponding GEO profile entries. As illustrated in Figure 1, both samples demonstrated symmetric distribution of gene expressions. This pattern indicates that the data are median-centered across samples, and cross-group comparison could be applied for further screenings.

MicroRNAs with differential expression levels

Table 1 shows a list of the top 20 miRNAs with significant positively or negatively altered gene expression levels ($p < 0.01$). Based on this analysis, hsa-miR-1587, hsa-miR-223-3p, hsa-miR-1915-3p, hsa-miR-6125, and hsa-miR-1273g-3p are the most significant differentially expressed miRNAs with higher expression levels in patients with prostate cancer vs. healthy controls. On the other hand, hsa-miR-3125, hsa-miR-642b-3p, hsa-miR-4505, hsa-miR-498, and hsa-miR-874 are top 5 down-regulated miRNAs.
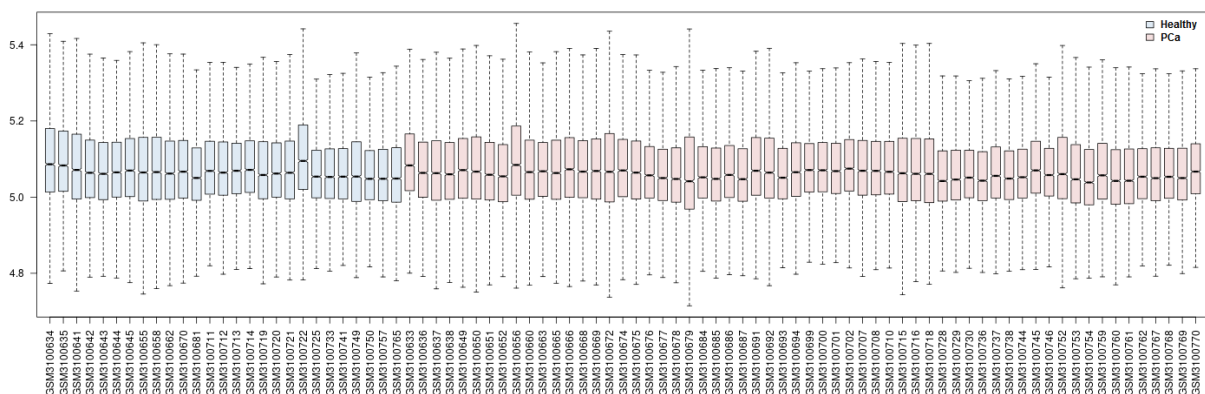

Figure 1. Gene expression value distributions are presented as a grouped boxplot for samples from healthy and PCa patients. GSM identifiers of the samples are shown on the horizontal axis, while, expression values are shown on the vertical axis. Data analysis was performed using built-in distribution analysis tool of the Gene Expression Omnibus database. Abbreviations: GSM, Gene Expression Omnibus Sample entry.

Table 1. Vesicle-free microRNAs differentially expressed in PCa vs. normal subjects with highest significance levels ($p < 0.01$).

Up-regulated miRNAs			Down-regulated miRNAs		
ID	**Fold Change**	***p* value**	**ID**	**Fold Change**	***p* value**
hsa-miR-1587	25.79288964	6.1e-11	hsa-miR-3125	0.002133045	1.8e-10
hsa-miR-223-3p	22.32543862	6.4e-05	hsa-miR-642b-3p	0.003146299	7.3e-13
hsa-miR-1915-3p	20.29551145	7.0e-13	hsa-miR-4505	0.005873541	1.0e-10
hsa-miR-6125	19.50742743	6.1e-11	hsa-miR-498	0.007903146	1.0e-11
hsa-miR-1273g-3p	12.49108029	2.0e-10	hsa-miR-874	0.009242724	3.7e-12
hsa-miR-3135b	10.76713107	2.2e-06	hsa-miR-4714-3p	0.011893235	1.6e-09
hsa-miR-6068	9.931160484	1.7e-08	hsa-miR-3177-5p	0.013058702	3.7e-12
hsa-miR-5001-5p	9.693932693	1.0e-08	hsa-miR-486-5p	0.035139858	3.5e-10
hsa-miR-4787-5p	9.57855506	1.7e-08	hsa-miR-4314	0.04792918	9.3e-11
hsa-miR-4687-3p	9.208735521	7.0e-10	hsa-miR-4516	0.050084115	7.3e-13
hsa-miR-4459	7.51622894	2.3e-03	hsa-miR-3141	0.056298912	4.8e-06
hsa-miR-6089	7.130171895	1.4e-04	hsa-miR-631	0.079323159	3.2e-10
hsa-miR-371b-5p	7.064802086	7.2e-07	hsa-miR-345-3p	0.0815455	3.4e-07
hsa-miR-4507	6.798294905	8.1e-08	hsa-miR-6088	0.090844785	5.0e-09
hsa-miR-23a-3p	6.768617929	5.6e-05	hsa-miR-4520b-3p	0.103252363	3.2e-10
hsa-miR-6724-5p	6.56447502	1.3e-08	hsa-miR-642a-3p	0.110994127	2.7e-08

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hsa-miR-4466	6.290714855	2.4e-07	hsa-miR-4304	0.113318287	3.3e-11
hsa-miR-638	5.81701254	2.4e-07	hsa-miR-1233-1-5p	0.115027055	2.8e-11
hsa-miR-15b-5p	5.093308711	2.9e-03	hsa-miR-4802-3p	0.120586864	6.1e-09
hsa-let-7f-5p	4.710857854	1.9e-03	hsa-miR-302d-5p	0.137088177	4.3e-09

MiRNAs target identification

The number of target genes which were identified for up-regulated and down-regulated DE-miRNAs in patients with prostate cancer vs. healthy individuals were 1326 and 1901, respectively. To verify that these genes are functionally interrelated, gene-gene interaction networks were extracted for PCa-associated genes (Figure 2). These networks indicated hub genes among the genes encode for positively or negatively regulated vesicle-free microRNAs in PCa. Full sets of dysregulated target genes were screened to detect functional profile of PCa DE-miRNAs. MiRNA-gene interaction networks were separately obtained for up- and down-regulated DE-miRNAs (Figure 3). Network analysis revealed the most contributing microRNAs and genes which are responsible for development of prostate cancer. Table 2 presents these network items with their relevant topological parameters.
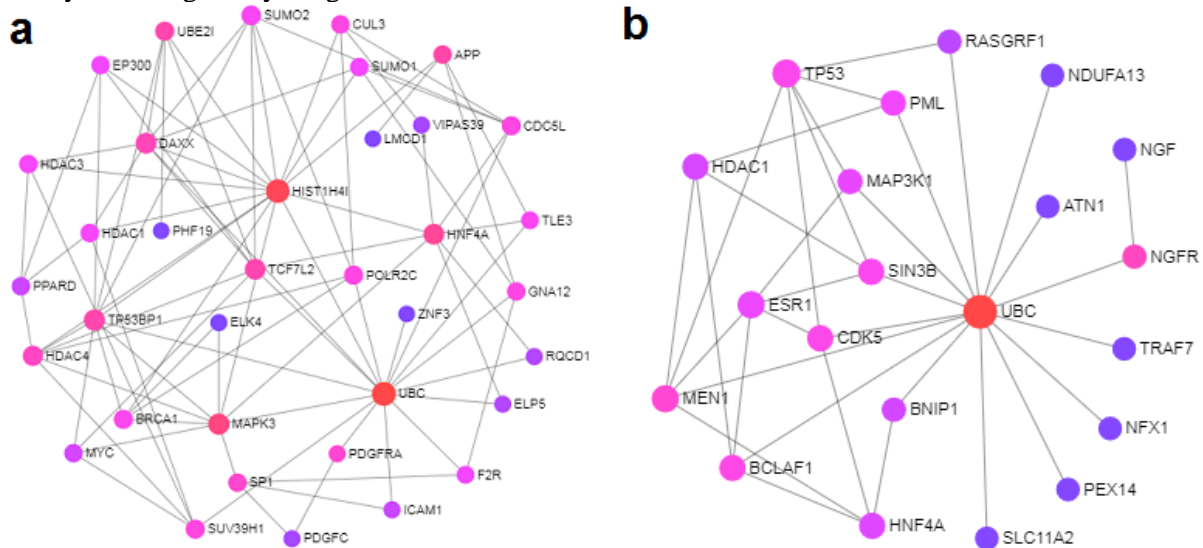

Figure 2. Interaction networks constructed for (a) positively, and (b) negatively regulated miRNA target genes in PCa samples. Data was obtained from MirWalk online tool.
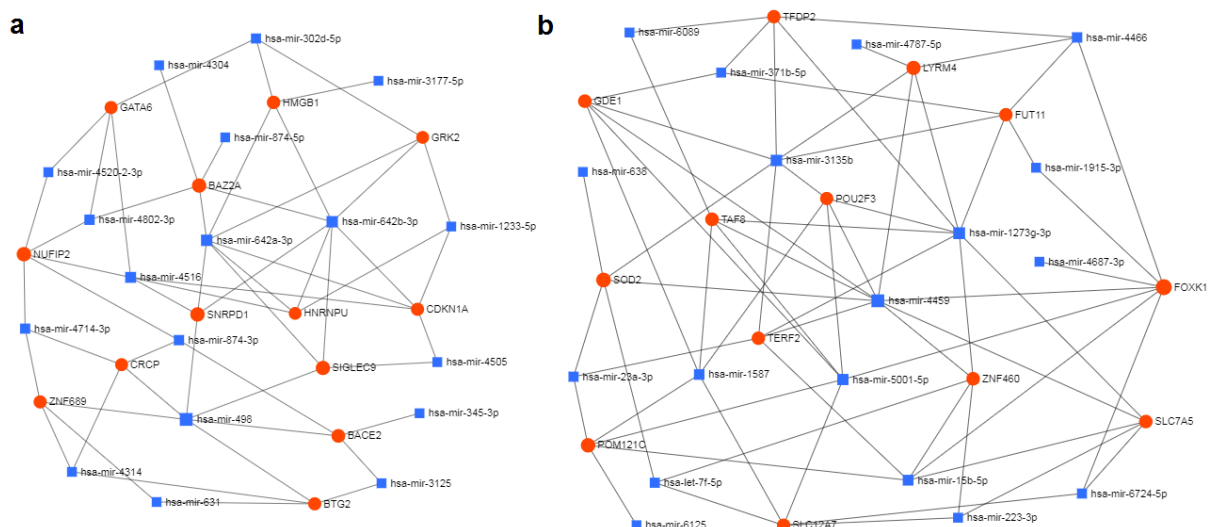

Figure 3. MiRNA-gene interaction networks which were built for (a) up-regulated, and (b) down-regulated differentially expressed microRNAs in prostate cancer samples are shown. miRNet 2.0 was utilized for extracting and analyzing the miRNA-gene networks.

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Table 2. MicroRNAs which are involved in the regulatory network of PCa development in addition to their target genes encode for these miRs are shown in the table.

Node item	Degree	Betweenness	Node item	Degree	Betweenness
Up-regulated miRs			**Down-regulated miRs**		
hsa-let-7f-5p	3	8.62873	hsa-miR-498	6	143.32320
hsa-miR-23a-3p	3	10.17321	hsa-miR-631	2	1.15202
hsa-miR-223-3p	3	3.53887	hsa-miR-302d-5p	3	12.16220
hsa-miR-15b-5p	5	27.67949	hsa-miR-3125	2	2.68535
hsa-miR-638	1	0	hsa-miR-4304	1	0
hsa-miR-1915-3p	2	2.88528	hsa-miR-4314	3	4.13384
hsa-miR-4459	9	82.29159	hsa-miR-642b-3p	7	76.55934
hsa-miR-3135b	7	44.17389	hsa-miR-4505	2	2.69919
hsa-miR-4466	4	15.77690	hsa-miR-4516	5	54.41083
hsa-miR-1587	5	21.67443	hsa-miR-3177-5p	1	0
hsa-miR-4687-3p	1	0	hsa-miR-4714-3p	3	20.77789
hsa-miR-371b-5p	3	4.10794	hsa-miR-4802-3p	3	28.51971
hsa-miR-4787-5p	1	0	hsa-miR-642a-3p	7	76.55934
hsa-miR-5001-5p	6	39.84926	hsa-miR-345-3p	1	0
hsa-miR-1273g-3p	8	55.44037	hsa-miR-874-3p	3	25.92176
hsa-miR-6089	2	1.58383	hsa-miR-4520-2-3p	2	4.81212
hsa-miR-6125	1	0	hsa-miR-1233-5p	3	2.28321
hsa-miR-6724-5p	3	7.19620	hsa-miR-874-5p	1	0
Target genes			**Target genes**		
SOD2	5	46.16845	SNRPD1	4	70.67985
SLC12A7	5	22.01802	BTG2	4	28.80109
ZNF460	5	25.13426	BACE2	4	48.91486
TERF2	5	21.99524	SIGLEC9	4	66.69920
POM121C	5	40.97833	CRCP	4	22.87670
SLC7A5	5	23.57412	ZNF689	4	29.71133
FOXK1	7	71.76947	GRK2	4	15.79189
FUT11	5	21.23700	GATA6	4	25.11072
POU2F3	5	16.91934	HMGB1	4	37.30794
GDE1	5	25.12142	BAZ2A	5	68.81256
LYRM4	5	36.66783	CDKN1A	5	28.53590
TAF8	5	27.21987	HNRNPU	4	15.30749
TFDP2	5	21.19667	NUFIP2	5	72.45046

Functional enrichment of target genes

We carried out functional annotation analysis based on the GO of BP components to investigate the roles of microRNAs target genes. Results confirmed that miRs with significantly changed expression levels between disease and healthy conditions could alter some key biological processes and thus, promote manifestation of the disease. Among various biological processes, target genes of up-regulated miRNAs were mostly enriched in the following pathways ($p < 0.01$): enzyme linked receptor protein signaling pathway, regulation of apoptosis, negative regulation of cytoskeleton organization, transmembrane receptor protein tyrosine kinase signaling pathway, regulation of cellular component size, negative regulation of protein complex disassembly, and transforming growth factor beta receptor signaling pathway. The most important prostate cancer related GO biological processes were considered as the ones with the most number of involved genes (Figure 4a). On the other hand, target genes of down-regulated

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miRNAs were shown to take part in negative regulation of megakaryocyte differentiation, telomere capping and organization, beta-catenin-TCF complex assembly, nucleosome assembly, chromatin silencing at rDNA, epigenetic regulation of gene expression, transcription initiation, double-strand break repair via nonhomologous end joining, protein hetero tetramerization, cellular protein metabolic process, gene silencing by RNA, semaphorin-plexin signaling pathway, histone H3 deacetylation, positive regulation of phosphatidylinositol 3-kinase signaling, and regulation of cell shape ($p < 0.01$ for all). The relative contributions of significant GO terms in negative regulation of PCa-associated miRs is shown in Figure 4b.

The most frequently represented genes which were determined based on the total count of the genes in the processes are: Promyelocytic leukemia (PML), Forkhead box K1 (FOXK1), Superoxide dismutase 2 (SOD2), Solute carrier family 12 member 7 (SLC12A7), POU class 2 homeobox 3 (POU2F3), Zinc finger protein 460 (ZNF460), Telomeric repeat binding factor 2 (TERF2), NADH: ubiquinone oxidoreductase subunit A13 (NDUFA13), Cyclin dependent kinase 5 (CDK5), Mitogen-activated protein kinase kinase kinase 1 (MAP3K1), and Maelstrom spermatogenic transposon silencer

(MAEL). Both highly represented genes and annotated genes of the miRNA-gene interaction network were used to draw a GO term-gene map for up-regulated microRNAs (Figure 5a).

In case of down-regulated genes, total counts of target genes in the processes revealed Histone H4 family members, High mobility group box 1 (HMGB1), Cell division cycle 5 like (CDC5L), Death domain associated protein (DAXX), Intercellular adhesion molecule 1 (ICAM1), Small nuclear ribonucleoprotein D1 polypeptide (SNRPD1), Zinc finger protein 689 (ZNF689), Heterogeneous nuclear ribonucleoprotein U (HNRNPU), Cyclin dependent kinase inhibitor 1A (CDKN1A), BTG anti-proliferation factor 2 (BTG2), Bromodomain adjacent to zinc finger domain 2A (BAZ2A), Platelet derived growth factor receptor alpha (PDGFRA), Peroxisome proliferator activated receptor delta (PPARD), Mitogen-activated protein kinase 3 (MAPK3), Tumor protein p53 binding protein 1 (TP53BP1), and zinc finger protein 3 (ZNF3) as the mostly represented genes. Both highly represented genes and annotated genes of the miRNA-gene interaction network were used to draw a GO term-gene map for down-regulated microRNAs (Figure 5b).
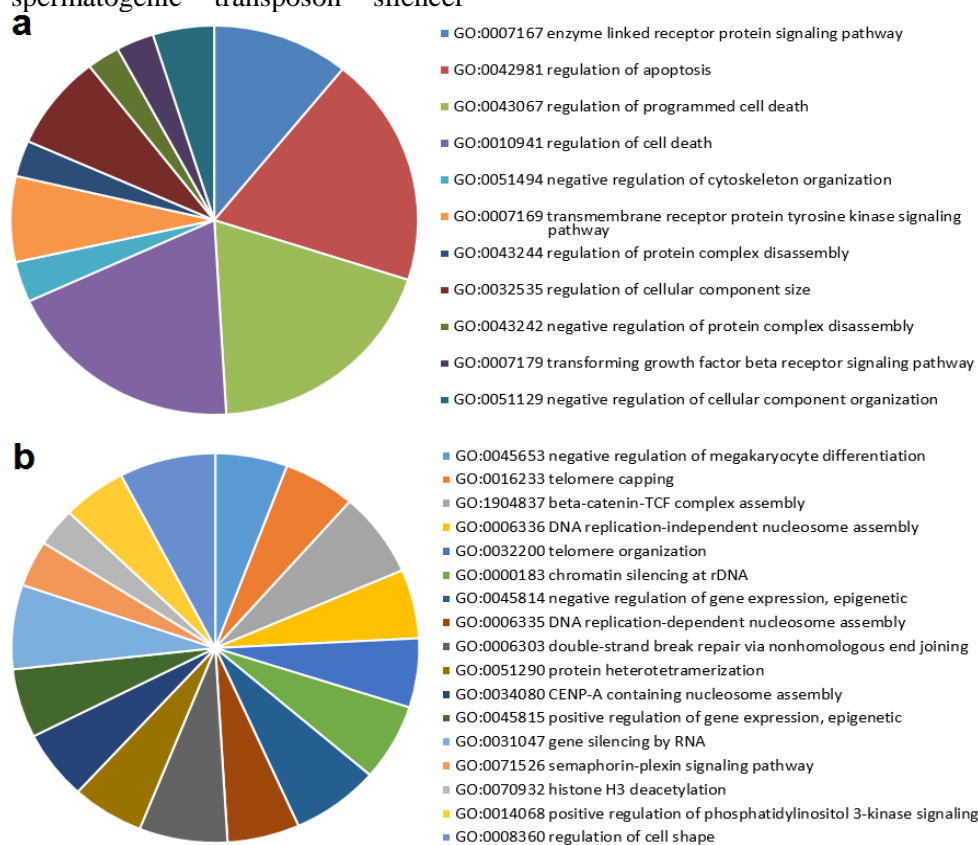

Figure 4. Pie charts indicated an overall view from biological processes which are associated with predicted miRs target genes, for (a) up-regulated, and (b) down-regulated microRNAs, respectively. Each sector size is proportional to the number of genes which are involved in the GO biological process ($p < 0.01$).
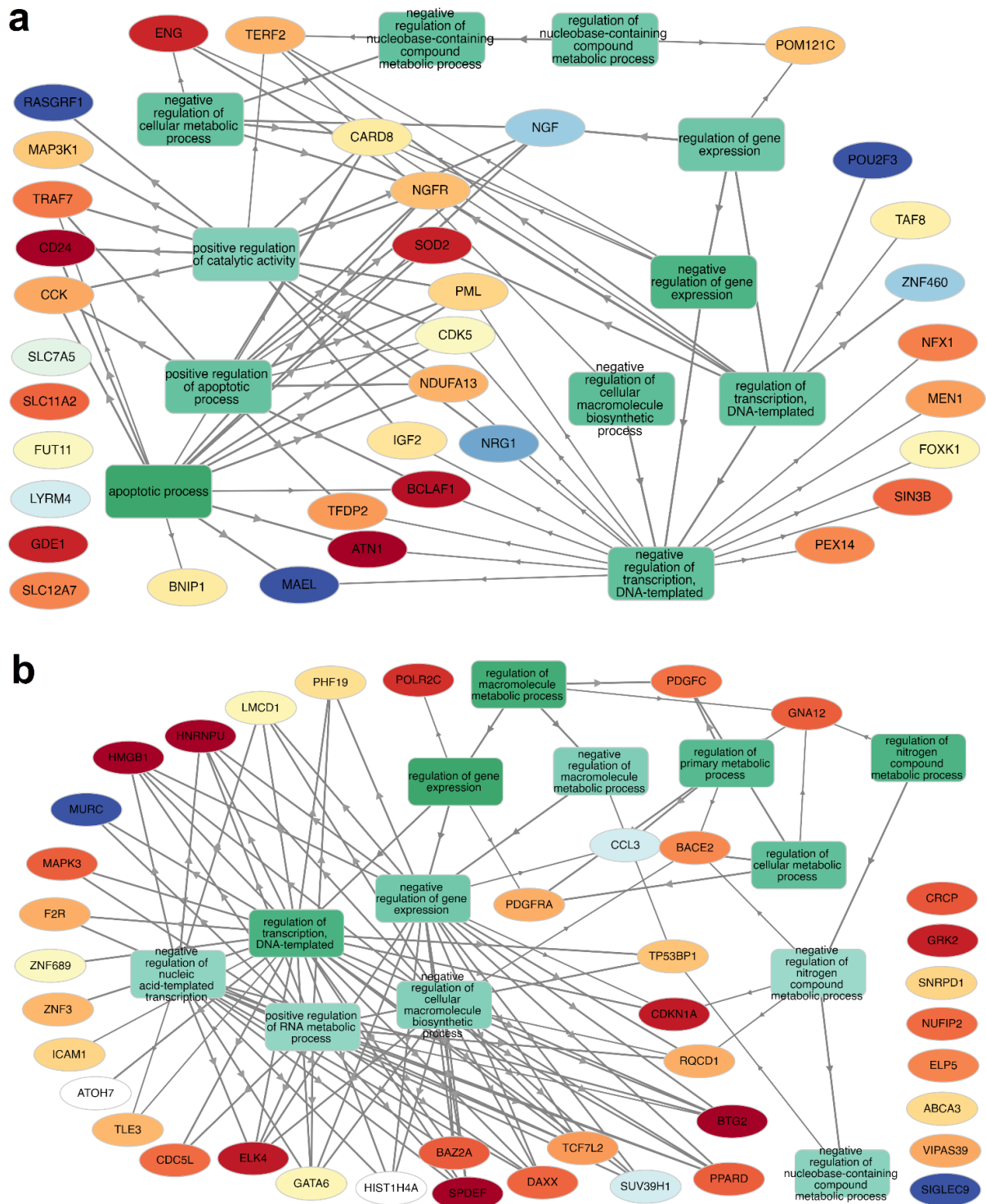
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Figure 5. Networks of significant gene targets of (a) up-regulated, and (b) down-regulated differentially expressed miRNAs. The intensity of GO term node colors indicates the significance of enrichment ($p < 0.0001$). Genes were colored due to their expression levels in the prostate gland tissue; blue represents the lowest level of gene expression, while, genes with the highest level of expression are shown with red color. To visualize the functional interconnection of target genes and their significant GO terms, the GOnet server tool was applied. In the server, p value threshold for relation significance was set 1.94e-5.

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Discussion

To date, numerous microRNAs have been identified in human prostate tissue, serum, plasma, and urine which are associated with localized and metastatic prostate cancers. Nevertheless, further experiments are required to evaluate the potential of miRNAs as biomarkers for the early diagnosis of PCa, particularly among men with increased risk for prostate cancer (McDonald et al., 2018). In the present study, we investigated whether blood circulating microRNAs demonstrate differential expressions according to the status of prostate cancer. As we were endeavored to find proper miRs with diagnostic value, vesicle-free microRNAs were selected due to their beneficial applicable properties in comparison to the vesicle-enclosed miRNAs. This kind of studies not only will help us to distinguish individuals with urgent need for performing prostate biopsy, but also may elucidate factors which are involved in the pathogenesis of the prostate cancer. Accordingly, the up-regulation of miR-375 and down-regulation of miR-146a-5p as extracellular vesicle-incorporated miRs with highly established roles in the pathogenesis of prostate cancer, were not the matter of our analyses (Endzelins et al., 2017). This approach will help us to avoid increased variability and more conveniently translate our findings to the clinical practices (Abramovic et al., 2020; Fabris et al., 2016; Fendler et al., 2016; Fredsoe et al., 2020).

Although we applied GEO series from the previous study performed by Mello-Grand et al. (2019), we only included PCa and healthy samples in the comparisons and omitted benign prostatic hyperplasia (BPH) samples from the analysis. In addition, we were stricter in the selection of the filters, as we considered p values <0.01 as the statistically significant differences. Moreover, despite the original research we did not assign any cut-off value during our analysis due to the below mentioned reasons: (1) we aimed to find miRs biomarkers that could discriminate PCa cases from healthy controls; (2) we aimed to find the responsible genes and their key interactions which are involved in the PCa pathogenesis using the corresponding GEO series accession. As an additional comparison, Mello-Grand et al. (2019) did not consider the target genes profiles for the miRs identified in their research. Also, they did not perform network and functional enrichment analyses.

The relationship between identified miRs and their target genes with prostate cancer has sufficient evidences from the literature. Significant up-regulation of the central miRNAs such as miR-223-3p (Bahtiyar et al., 2018; Cao et al., 2015; Feng et al., 2018; Jiang et al., 2005; Kurozumi et al., 2016; Liu et al., 2018; Mi et al., 2007; Triozzi et al., 2012; Volinia et al., 2006; Wei et al., 2014), miR-23a-3p (Aghaee-Bakhtiari et al., 2015; Cai et al., 2015; Jiang et al., 2005; Mi et al., 2007; Porkka et al., 2007; Wen et al., 2015), miR-15b-5p (Chen et al., 2018; Musumeci et al., 2011), miR-let-7f-5p (Ge et al., 2020; Jiang et al., 2005; Mello-Grand et al., 2019; Porkka et al., 2007), and miR-371 (HMDD v.3.2) in PCa tumors has been confirmed previously. Furthermore, miR-642b (HMDD v.3.2), miR-498 (Bendoraite et al., 2010; Porkka et al., 2007), miR-486 (Navon et al., 2009; Song et al., 2015), miR-4516 (HMDD v.3.2), miR-631 (HMDD v.3.2), miR-345 (Chen et al., 2016; Jiang et al., 2005; Porkka et al., 2007; Sayed et al., 2007; Tinay et al., 2018; Wang et al., 2014), miR-642a (HMDD v.3.2), and miR-302d (HMDD v.3.2) have been shown to be significantly down-regulated in PCa samples. These cell-free miRNAs could be considered as biomarkers for early detection of PCa (Zhang et al., 2014).

Combined analysis of the expression patterns and network topologies for identified DE-miRNAs in the present study allowed the identification of the miRs with high diagnostic potentials. Interestingly such potential markers can be found only among positively regulated miRs, including miR-4459, miR-1273g-3p, miR-3135b, miR-5001-5p, and miR-1587.

The miRNA-gene interaction networks constructed for DE-miRNAs confirmed most of the target genes which were predicted for these DE-microRNAs. Functional annotation of target genes indicated that the up-regulation of miRs in PCa is responsible for suppressed apoptotic process, enhanced nucleobase-containing metabolic process, and enhanced gene expressions which imply to augmented cell proliferation. The most important genes were FOXK1 (Chen et al., 2017), PML (Gurrieri et al., 2004), CD24 (Rouhrazi et al., 2018), ATN1 (Maugham et al., 2017), BCALF1, ENG (Liu et al., 2002), and SOD2 (Burri et al., 2008). In contrast, down-regulation of the identified miRs resulted in the negative regulation of gene expressions and macromolecule biosynthesis, implying that they are tumor suppressive microRNAs. The most important genes were BAZ2A (Gu et al., 2015), CDKN1A (Mitchell and El-Deiry, 1999), NUFIP2, HARNPU, HMGB1 (Wang et al., 2018), SPDEF (Findlay et al., 2008), BTG2 (Lim et al., 2008), and HIST1H4A (Yao et al., 2014).

Overall, we propose miR-4459, miR-1273g-3p, miR-3135b, miR-5001-5p, and miR-1587 as novel

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microRNAs with great potential to be utilized as diagnostic biomarkers for distinguishing prostate cancer patients from healthy individuals. Although, these *in silico* findings should be verified through experimental studies.

Conflict of interest

The authors declare that there is no conflict of interest.

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