ONCOLOGY

TP53 gene implications in prostate cancer evolution: potential role in tumor classification

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Abstract

Background and aims. Prostate adenocarcinoma (PRAD) is a complex disease that can be driven by alterations in both coding and noncoding genes. Recent research has identified coding and non-coding genes that are considered to play important roles in prostate cancer evolution and which may be used as biomarkers for disease diagnosis, prognosis, and treatment. TP53 is a critical hub gene in prostate cancer. Advanced studies have demonstrated the crosstalk between coding and non-coding RNAs, particularly microRNAs (miRNAs).

Methods. In this study, we investigated the roundabout of TP53 and their regulatory miRNAs (miR-15a-5p, miR-34a-5p, and miR-141-3p) based on the TCGA data set. We validated an additional patient cohort of 28 matched samples of patients with PRAD at tissue and plasma level.

Results. Therefore, using the UALCAN online database, we evaluated the expression level in PRAD of these genes revealing overexpression of TP53. qRT-PCR validation step endorsed the expression level for these genes. Additionally, we evaluated the expression level of the four key miRNAs (miR-15a-5p, miR-34a-5p, and miR-141-3p) interconnected as a network at tissue and plasma levels.

Conclusions. Through these results, we demonstrated the essential function of TP53 and its associated miRNAs that play a significant role in tumor control, highlighting miRNAs' potential as future therapeutic targets and biomarkers with important implications in managing prostate cancer.

Keywords: prostatic neoplasms, adenocarcinoma, microRNAs, biomarkers

Introduction

Prostate adenocarcinoma (PRAD) is the second most common cause of cancer death among men, particularly in Western countries [1,2]. While prostate-specific antigen (PSA) screening in combination with prostate biopsy has considerably improved the early diagnosis of prostate cancer, the sensitivity remains reduced, particularly for those expressing low PSA values. The prostate biopsy is also extremely invasive and might entail complications.

Advanced studies have demonstrated the crosstalk between coding and non-coding RNAs, particularly mRNA-microRNAs (miRNAs). miRNAs are short-length transcripts comprising 19-25 nucleotides that are highly stable in cells and do not degrade easily as the mRNAs play an essential role in cancer tumorigenesis and progression field [3-6]. Due to their short-conserved sequence, they can alter the physiological function of the coding genes by targeting the complementary mRNA sequences. This potential miRNA specificity to target coding genes will continue to generate interest for their potential clinical applications.

In cancer, miRNA alterations relate to the modulation of several signaling pathways involved in different hallmarks

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of cancer, from early carcinogenesis to late metastasis events. Among these, miRNAs' fine-tuning of the TP53 axis has been widely reported in the literature [7-9]. This is the case of miR-15a which focuses on multiple oncogenic targets [8]. TP53 is a critical hub gene in the prostate cancer [10-12]. Expression levels are correlated with the PRAD recurrence [10]. TP53 gene regulates cell proliferation and differentiation [5], and mutations in this gene occur in only a small percentage of cases. However, alterations in other genes and signaling pathways that are regulated by TP53 may also affect the evolution of prostate cancer [13]. miR-34 is a family of microRNAs involved in regulating cell proliferation, differentiation, and apoptosis. miR-34 is a downstream target of TP53 and is often dysregulated in cancer. In particular, miR-34a has been shown to be a tumor suppressor and is frequently downregulated in prostate cancer [14]. One study found that miR-141-3p promotes prostate cancer cell proliferation, migration, and invasion by targeting HKF9 [15]. In addition, miR-141-3p has been investigated as a potential biomarker for prostate cancer. Several studies have shown that miR-141-3p is present at higher levels in the plasma of patients with prostate cancer compared to healthy individuals or patients with benign prostatic hyperplasia (BPH). This suggests that miR-141-3p may have the potential as a non-invasive diagnostic and prognostic biomarker for prostate cancer. Overall, miR-141-3p appears to play a role in prostate cancer development and progression and may have the potential as a biomarker and therapeutic target for the disease [16,17].

This study investigated the relationship between TP53 and key regulatory miRNAs (miR-15a-5p, miR-34a-5p and miR-141-3p) identified on bioinformatics analysis and then validated on an additional patient cohort, tissue and plasma (Figures 1a and 1b).

Methods

***In silico* analysis based on TCGA dataset-mining analysis in PRAD**

We performed a bioinformatic analysis based on the TCGA databases. TCGA is a user-friendly web resource for analyzing cancer data, furnishing data to analyze directly on the platform graphs and plots outlining gene expression and survival curves. The UALCAN and STARBASE databases generated data on miRNAs and their target genes for subgroup analysis of clinical and pathological features (Gleason score 7-9) [18,19].

mRNA-miRNA interactions

To assess the mRNA-miRNA interactions, we used miRNet, a valuable online tool that permits visual exploration of target interaction in a biological network context [20,21].

qRT-PCR data validation of TCGA data analysis

Patients and sample collection (Table I). To validate the bioinformatic analysis, we initially collected samples from 71 patients with confirmed PRAD between 2018 - 2020 (Figure 1b). A database with clinical, pathological and molecular characteristics was created from all the patients. From each patient, tumor tissue and adjacent tissue were collected after the initial check by a pathologist. Additionally, blood, serum and plasma were collected. All the patients were selected from the Urological Surgery Department at the "Prof. Ion Chiricuta" Oncology Institute, Cluj-Napoca.

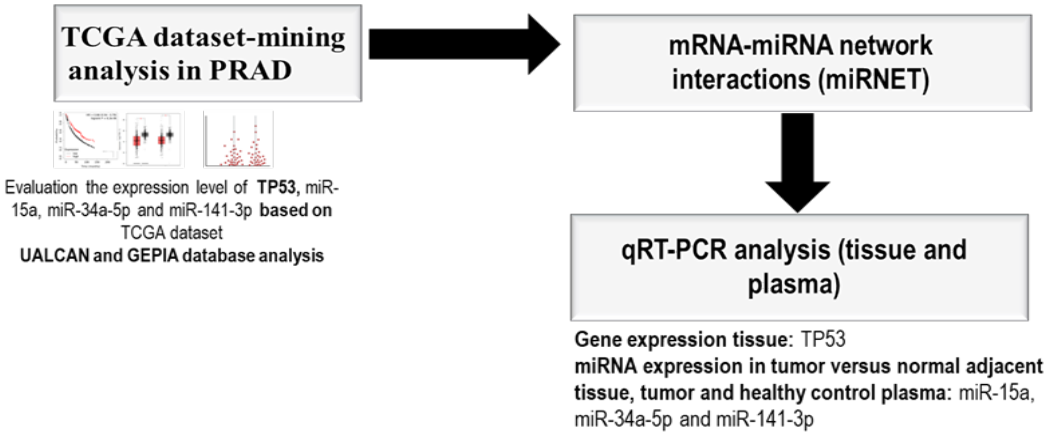

Figure 1a. Flow chart of the study.

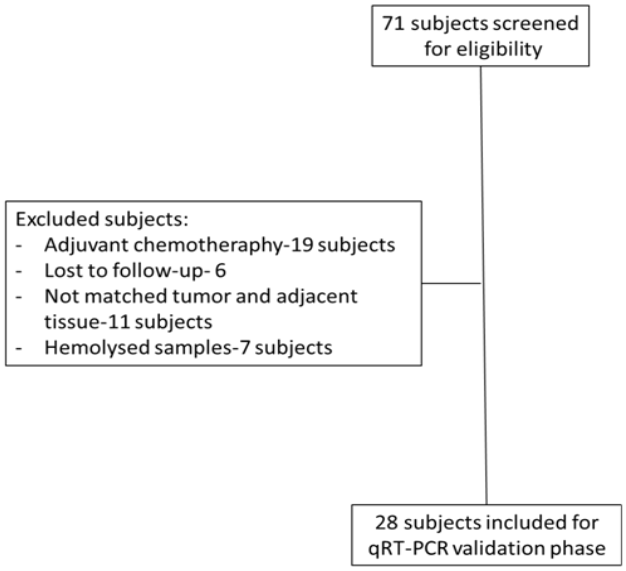

Figure 1b. Flow chart for the patients included in the study.

The tissues were collected immediately after the pathological examination and stored in liquid nitrogen at -170ºC. A trained pathologist selected the tissues to be further analyzed for molecular investigations, not to alter the pathological diagnosis of the tumors.

Two 5 ml vacutainers for peripheral blood collection (one with EDTA and one without anticoagulant), one for TriReagent and one for serum and plasma, were collected from all patients. All methods were performed to limit the blood hemolysis and exclusion from the cohort to be analyzed. The blood was prepared with TriReagent to conserve the mRNA and miRNA sequences. Coagulated blood was centrifuged at 3000 rpm for 10 minutes to obtain serum and plasma. We used RNAse-free tubes for serum and plasma storage at -80ºC. Repeated thawing of the frozen samples was avoided to limit the degradation of the biological material.

Ethical conditions. The present study followed all the regulations of the Declaration of Helsinki regarding human rights and informed consent for molecular biology research studies. Based on the data explained by the principal investigator, the "Prof. Ion Chiricuta" Institute of Oncology Ethical Committee approved the study with data anonymization for all results to be published. The approval number is 5991/ 26.06.2019.

A total of 28 patients were included in the qRT-PCR study; their data are shown in table I. The median age of the patients was 66.2±5.1 years old. All patients included were stage II and III, with a Gleason score between 7 and 9.

RNA isolation from tissue. The RNA isolation from tissue samples (tumor and normal adjacent) for genes and miRNAs was done using the TriReagent-based method, followed by the Nanodrop quality control step.

Table I. Clinical characteristics of PRAD patients included in the qRT-PCR clinical study (tissue and plasma).

Patient no.	Age	PSA	cTNM	L	V	R	Gleason Score
1	75	5.5	3aXX	0	0	0	7(3+4)
2	63	7	3a0X	0	0	1	7(3+4)
3	68	13	30X	0	0	0	7(3+4)
4	64	6	2c0C	0	0	0	7(4+3)
5	64	9.25	2XX	0	0	0	7(4+3)
6	64	47	3b0X	0	1	1	7(4+3)
7	64	7.45	2b0X	0	0	0	7(3+4)
8	60	9	2c0X	0	0	0	7(3+4)
9	65	7	20X	0	0	0	7(3+4)
10	76	5.53	2cXX	0	0	0	7(3+4)
11	68	15	3b1X	1	0	1	7(4+3)
12	65	78	2c0X	0	0	0	7(4+3)
13	64	4.9	3a0X	0	0	1	7(3+4)
14	70	19	3b1X	1	0	1	7(3+4)
15	66	4.26	2cXX	0	0	0	7(3+4)
16	65	8.3	3aXX	0	0	0	7(3+4)
17	71	25	3a0X	0	0	1	7(4+3)
18	65	5.75	2c0x	0	0	0	7(3+4)
19	63	4.49	3aXX	0	0	0	7(3+4)
20	76	4.5	2XX	0	0	1	7(3+4)
21	69	8.9	3a00	0	0	1	7(3+4)
22	58	8.09	3bXX	1	0	0	7(4+3)
23	70	1.9	3a0X	1	0	1	7(3+4)
24	74	7	20x	1	0	0	7(4+3)
25	54	19	3b1X	1	1	0	8(4+4)
26	63	19.71	3b0X	0	0	1	9(4+5)
27	68	46	3b001	0	0	1	9(5+4)
28	64	9.5	20X	1	0	0	9(4+5)

cTNM: clinical TNM classification, T: Tumor classification, N: Node classification, M: Metastatic classification, L: lymph involvement; V: Vessel involvement.

RNA isolation from plasma. For the RNA isolation from plasma samples (patients with PRAD and healthy subjects), we used Plasma/Serum Circulating and Exosomal RNA Purification kit (Slurry Format, cat no.42800, Norgene).

Gene expression quantification by qRT-PCR. The RNA was reverse-transcribed into cDNA for gene expression evaluation using a High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Subsequently, an amplification step has been performed using SYBR Select Master Mix (Applied Biosystems) on ViiA™7 System and specific primers for the target genes. The primer sequences are displayed in table II.

The expression levels' relative quantification was done using the $2^{-\Delta\Delta CT}$ method [3], and graphical representation was done using GraphPad Prism (version 9).

Table II. Primer sequence and assay ID used for genes' quantification.

Gene	Primer Sequences	
	FW	RW
B2M	CACCCCCACTGAAAAAGATGAG	CCTCCATGATGCTGCTTACATG
TP53	CACCCCCACTGAAAAAGATGAG	CCTCCATGATGCTGCTTACATG
TAZ	GCTGCAGACATCTGCTTCAC	TTCCCCTCATTCTCTGCTTG
MALAT1	AACTGCAGAGAGTTTGAGTGGTTTT	TGTCCTTATAGGCTGGCCATT

Table III. Primer sequence and assay ID used for miRNAs' quantification.

miRNA Assay	Sequence	Assay ID
miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG	000389
miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU	000426
miR-141-3p	UAACACUGUCUGGUAAAGAUGG	000463
U6 snRNA	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCTGCGCAA GGATGACACGCAAATTCGTGAAGCGTTCCATATTTT	001973
RNU48	GATGACCCCAGGTAACTCTGAGTGTGTCGCTGATGCCATCACCGCAGCGCTCTGACC	001006

Tumor tissue/normal adjacent tissue and plasma miRNAs' expression profiling. For miRNA quantification from tissue (tumoral versus adjacent normal tissue) and plasma (PRAD and healthy subjects miRNAs), we used 50 ng RNA for cDNA synthesis performed with TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) and TaqMan Fast Advanced Master Mix (Applied Biosystems) on the same instrument, primers used for miRNA quantification are displayed in table III. The miRNAs were evaluated on the same patient cohort, and for normalization, U6 and RNU48 with the same method and graphical representation as for gene expression.

Results

TP53 expression levels in PRAD. Using the UALCAN online tool, the analysis revealed that the mRNA expression level of TP53 has been overexpressed in PRAD versus normal adjacent tissue (Figure 2).

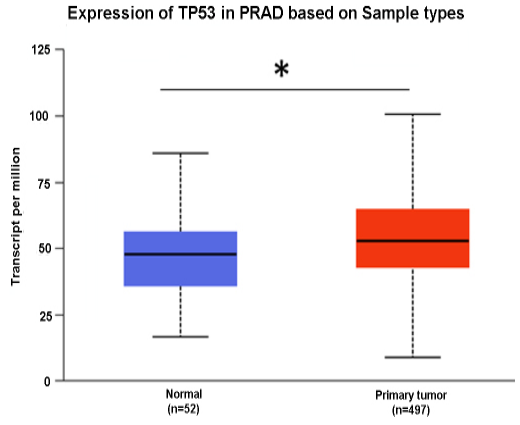

Figure 2. TP53 expression level in PRAD. (A) The expression level of TP53, generated using the UALCAN online tool, graphical representation as "primary tumor" and normal adjacent tissue displayed as "normal" (* $p \leq 0.05$).

Validation of TP53 gene by qRT-PCR. To validate the observed alteration of the TP53 genes in PRAD, qRT-PCR was performed, wherein B2M was used as an endogenous control for the normalization of qRT-PCR data using the $2^{-\Delta\Delta CT}$ method. These transcripts were validated using 28 matched pairs of tumor and adjacent normal tissues of PRAD with a Gleason score between 7-9, revealing the overexpression of the TP53 gene (Figure 3).

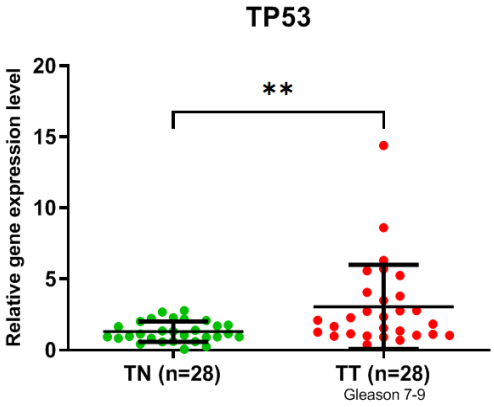

Figure 3. TP53 expression levels in PRAD tumor tissue (TT, n=28) versus normal adjacent tissue (NT, n=28). Scatter plots demonstrate the overexpression of TP53 in tumor tissues versus normal adjacent tissues. To normalize the gene expression data, B2M data were analyzed using the $2^{-\Delta\Delta CT}$ method (**$p \leq 0.01$).

TP53 interactions network with key miRNAs in prostate cancer. TP53 was interconnected with key regulatory miRNAs, miR-15a-5p, miR-25-3p miR-34a-5p (Figure 4), via BCL2 genes, network interaction generated using miRNET online tool. miR-141-is interconnected indirectly to with TP53 via BCL2 genes.

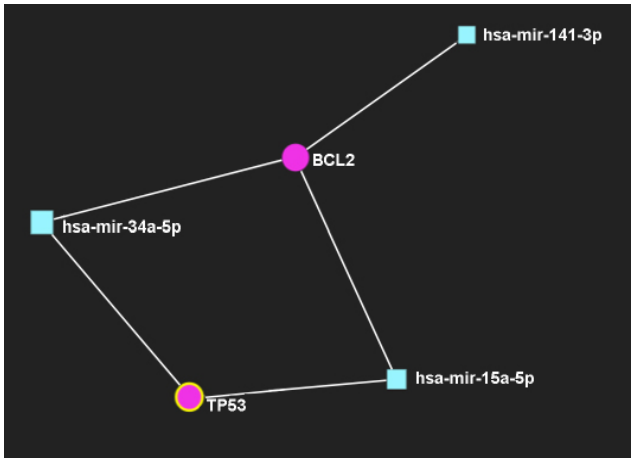

Figure 4. TP53-miRNA interaction network. mRNA-miRNA interaction network for TP53, a key suppressor gene revealed direct interconnection with miR-15a-5p, miR-34a-5p, indirectly via BCL2 with miR-141-3p.

Evaluation of the expression level of miR-15a, miR-34a-5p and miR-141-3p. We evaluated the expression level for miR-15a, miR-34a-5p and miR-141-3p, revealing an increased level in tumor tissue versus normal adjacent tissue in the TCGA patient cohort, with emphasis on no important alteration considering the Gleason score (Figure 5). Then the expression levels for these transcripts were validated in our patient cohort (Figure 6), data being in agreement with those from TCGA.

To validate the selected miRNAs, we investigated the expression level in plasma of the same patients' cohort versus healthy controls, confirming the increased expression in PRAD plasma versus healthy controls (Figure 7). Furthermore, these findings suggest that these miRNAs are useful both as biomarkers and therapeutic targets in PRAD.

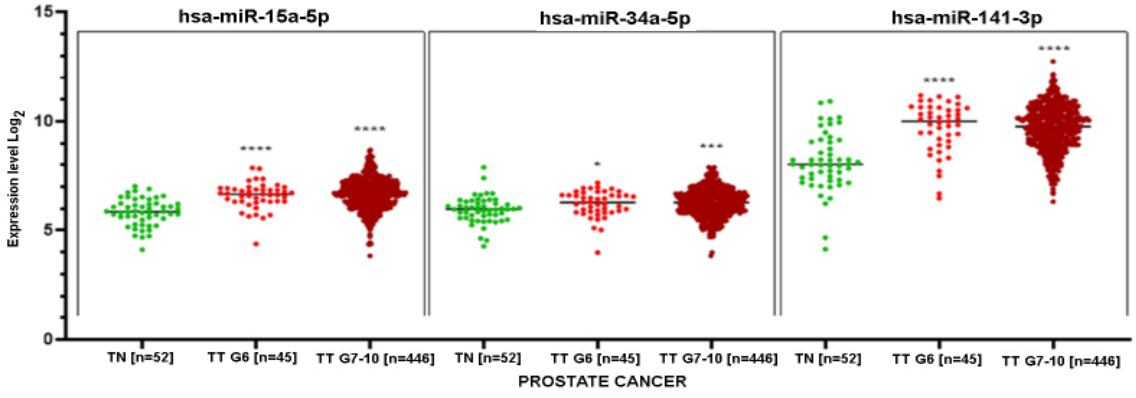

Figure 5. miR-15a-5p, miR-34a-5p, and miR-141-3p expression level considering Gleason score in TCGA patient cohort, revealing overexpression of these transcripts for the cases with Gleason 6 and Gleason 7-1.

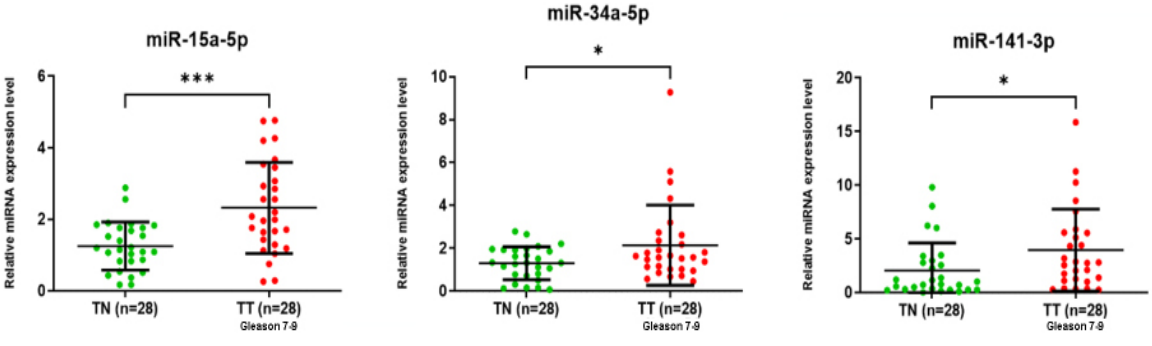

Figure 6. miR-15a-5p, miR-34a-5p and miR-141-3p expression levels in tumor tissues (TT) versus normal adjacent tissues (TN) for cases with Gleason score 7-10. Scatter plots demonstrate the overexpression of miR-15a-5p, miR-34a-5p, and miR-141-3p in tumor tissues (TT) versus normal adjacent tissues (TN). For normalization of the gene expression data, U6 and RNU48 were analyzed based on the $2^{-\Delta\Delta CT}$ method (* $p \leq 0.05$ and *** $p \leq 0.001$).

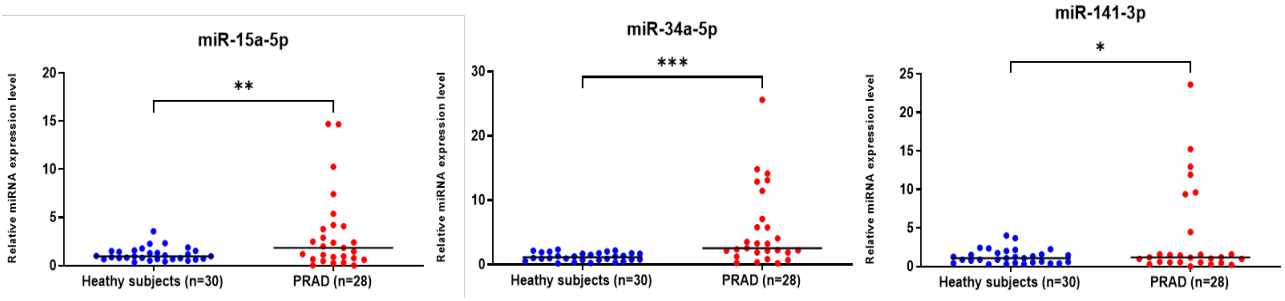

Figure 7. miR-15a-5p, miR-34a-5p and miR-141-3p expression levels at plasma levels in PRAD (n=28) versus healthy controls (n=30) by qRT-PCR. For normalization of the miRNA expression data, U6 and RNU4 were analyzed based on the $2^{-\Delta\Delta CT}$ method (* $p \leq 0.05$, **$p \leq 0.01$**** $p \leq 0.0001$).

Discussion

Although earlier studies have reported progress in elucidating the potential molecular mechanism in prostate cancer development, the fundamental knowledge of the TP53 network signaling remains undeciphered regarding its prognostic value and related biological processes in PRAD. Most of the studies are related to the mutation status of TP53 [10,22,23], with less information related to the expression level for this gene, as in the present study. However, further research is needed to fully understand the role of this axis in prostate cancer and develop effective therapeutic strategies.

Therefore, miR-141-3p has been proposed as a potential prostate cancer diagnosis, prognosis, and treatment biomarker. Its detection in blood or urine samples could be used as a non-invasive diagnostic or prognostic tool, and its inhibition may be a potential therapeutic strategy for prostate cancer treatment [15].

miR-15a-5p is presented in the literature as an important transcript related to the androgen signaling [24], representing an important therapeutic target for this pathology [25]. miR-15a is involved in multiple biological processes, overexpression of miR-15a [26,27] along with miR-16 inhibiting the TGFβ signaling pathway [28], regulated cell proliferation and invasion by Wnt/β-Catenin signaling pathway [29], or immune invasion and malignant progression of PRAD via up-regulating PD-L1 [30].

TP53 transcriptionally controls miR-34a expression, often altered in cancer [31]. miR-34a-5p transcript is overexpressed in PRAD. It correlated with STK4 (gene correlated with disease-free survival) [32,33]. miR-34a is transcriptionally regulated via TP63, TP73, and other transcription factors, such as STAT3, and MYC are involved [34]. The expression level may dynamically change in EMT, hypoxia, and inflammation [34]. The overexpression of this transcript mainly in high-grade tumors [26]. Meanwhile, other studies reveal the downregulation of this transcript [27,35].

Another key element is represented by miR-141 with prognostic value in PRAD [36]. Literature data report it to be related to outcomes and aggressive tumor characteristics in PRAD [16,37]. The high expression of this transcript at tissue and plasma levels is related to disease progression and metastatic disease [37]. MiR-141 was significantly higher in the plasma of patients with advanced PRAD than in the matched controls [38]. miR-141 was significantly up-regulated in the serum samples of metastatic tumors versus localized tumor samples [39]. Another study demonstrated the prognostic role of miR-141 along with miR-21 and miR-375 in the PCa diagnosis [40]. Despite this, the role of this transcript remains controversial in PRAD, considering the interconnection with epigenetic factors [41]. Overall, these findings suggest that the dysregulation of BCL2 and miR-141 may contribute to the development and progression of prostate cancer and that targeting these molecules may be a potential therapeutic strategy for the disease, but no study investigated the relationship between miR-141 and Bcl2 in prostate cancer as yet. Additional investigation needs to be done to validate this.

Conclusions

Although various gene expression studies have already been performed, there have not been any detailed analyses of TP53 and their targeting transcripts (miR-15a, miR-34a-5p, and miR-141-3p) that can be used for prognostics for overall survival in PRAD. The validation of these miRNAs in tumor tissues and liquid biopsy opens a new field of investigation in the near future, focusing on the genes targeted by these miRNAs and their potential role in the disease evolution and how this can be controlled. While at the beginning of their validation in liquid biopsies from cancer patients, these short noncoding sequences gain more territory to become concrete molecules as biomarkers. Overall, these findings

suggest that a panel of coding and noncoding genes may be used as biomarkers for prostate cancer evolution and may help guide personalized treatment strategies for patients with this disease. Further research is needed to validate these findings and to develop effective diagnostic and therapeutic approaches based on this gene panel.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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