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Altered expression of lncRNAs DANCR, MIR4435-2HG, and HULC following radiotherapy and their association with radioresistance in prostate cancer

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► ***Original article***

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ABSTRACT

Background: Radioresistance in prostate cancer (PCa) patients is a challenge that has necessitated identifying standard markers to predict patients' response to radiotherapy (RT). This study was designed to explore the potential of DANCR and MIR4435-2HG, related to the transforming growth factor-beta (TGF-β) pathway, as well as HULC, whose role in radioresistance has been previously examined, in predicting radiation response in PCa patients. ***Materials and Methods:*** Plasma samples were collected from 30 healthy men and 30 PCa patients. RT-qPCR was conducted to detect DANCR, MIR4435-2HG, HULC, and mir-542, mir-424, and mir-372 expressions. Expression levels of TGF-β and TGF-β1 were determined using the enzyme-linked immunosorbent assay (ELISA) and western blot assay. Patients were followed for 11–14 months after RT, and their prostate-specific antigen (PSA) was recorded at 3 and 6 months. ***Results:*** Examination of MIR4435-2HG, DANCR, and HULC in plasma samples showed that in radioresistant patients, MIR4435-2HG (0.04 ± 1.47), DANCR (0.08 ± 1.84), and HULC (0.10 ± 1.51) were significantly increased, while mir-424 (0.07 ± 0.49), mir-372 (0.15 ± 0.31), and mir-542 (0.06 ± 0.62) were decreased. Worse pathological features were significantly associated with higher lncRNA expressions. Elevated TGF-β levels were detected in plasma samples from patients who received RT. ***Conclusion:*** The significant increase of DANCR, MIR4435-2HG, and HULC in plasma samples of radiation-resistant patients suggests a potential link to radioresistance in PCa. These biomarkers hold promise for personalized medicine, offering new avenues for improving patient management and treatment outcomes.

INTRODUCTION

Prostate cancer (PCa) is recognized as commonly diagnosed as solid malignancies and is the fifth major cause of cancer-related deaths among men worldwide [1]. Among various treatment options, including surgery, chemotherapy, hormone therapy, and radiotherapy (RT), RT is incorporated into the treatment plans of more than half of all cancer patients [2, 3]. Despite its widespread use, RT faces a significant limitation. Approximately 30-40% of PCa patients exhibit resistance to radiation, which compromises treatment efficacy and outcomes [4]. Monitoring treatment response in PCa patients often relies on prostate-specific antigen (PSA) levels. Although PSA is a widely accepted biomarker, its application is limited following RT due to the heterogeneous kinetics of PSA decline, the absence of a definitive nadir level, and the long interval often required to reach that nadir. These limitations make PSA an unreliable and non-specific indicator of treatment response in many cases [5, 6]. Given the molecular heterogeneity and subclonal diversity of PCa, there is growing interest in identifying alternative blood-based biomarkers to enhance personalized treatment strategies [7, 8]. Advances in genomic profiling and personalized medicine offer promising avenues to improve the management and outcomes of PCa patients [9, 10]. Long non-coding RNAs (lncRNAs), RNA sequences that exceed 200 nucleotides in length, usually transcribed by RNA polymerase II or I - have risen to prominence among the array of novel molecular biomarkers [11, 12].

Evidence indicates that lncRNAs are often aberrantly expressed in diverse cancers and play pivotal roles in tumor growth, invasion, and metastasis [13]. Importantly, lncRNAs have been implicated in modulating the cellular response to

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radiation, making them potential biomarkers for radiosensitivity or radioresistance [14]. Several specific lncRNAs have been associated with PCa biology and radiation response. For instance, *MIR4435-2HG*, located on chromosome 2q13, has been shown to contribute to carcinogenesis and tumor proliferation in multiple malignancies [15]. Overexpression of this lncRNA appears to facilitate PCa invasion and metastatic progression driven by stimulation of the TGF-β signaling cascade [16]. Similarly, the differentiation-inhibiting non-coding RNA *(DANCR or ANCR)*, encoded on chromosome 4, has been implicated in tumor aggressiveness across cancer types, including PCa. *DANCR* enhances proliferation and invasion by targeting *miR-214* and modulating *TGF-β* activity [17, 18]. Another notable lncRNA, hepatocellular carcinoma upregulated lncRNA (*HULC*), initially identified in liver tumors, is also involved in metastatic processes in PCa and other cancers [19, 20]. Collectively, these observations indicate that lncRNAs not only contribute to tumorigenesis but also may serve as predictive biomarkers for radiation response [21].

Along with lncRNAs, microRNAs (miRNAs) modulate gene expression by binding to target mRNA sequences, which can result in either inhibited translation or mRNA breakdown [22]. Notably, lncRNAs and miRNAs often interact through competitive endogenous RNA (ceRNA) networks, in which lncRNAs sponge miRNAs and modulate their regulatory functions [23]. This interplay has particular relevance in PCa, where it can influence key signaling pathways, including the *TGF-β* pathway. In the context of PCa, *TGF-β* is one of the most influential pathways in both tumor progression and resistance to therapy [24]. The tumor microenvironment (TME), heavily influenced by *TGF-β* signaling, is known to modulate the outcomes of RT [25]. Furthermore, various non-coding RNAs (ncRNAs) have been identified as upstream regulators of the *TGF-β* pathway [26]. The upregulation of this molecule has been associated with radioresistance via multiple mechanisms, including epithelial-mesenchymal transition (EMT), DNA damage repair, and immunosuppression [27-29].

This study represents the first to investigate the potential association between changes in the expression levels of lncRNAs *DANCR, MIR4435-2HG,* and *HULC* and radiation resistance in prostate cancer, using plasma samples from patients. Findings highlight the potential of these lncRNAs as non-invasive biomarkers of radiation response. Moreover, this study analyzes the relationship between the pathoclinical characteristics of PCa patients and the expression levels of *TGF-β*, *DANCR, MIR4435-2HG* and *HULC*. By identifying lncRNA signatures linked to radiation resistance, this study contributes to the advancement of personalized medicine strategies in PCa, particularly in RT approaches based on individual molecular profiles.

MATERIALS AND METHODS

Study participants

Samples of blood were collected from 30 PCa patients aged 40 to 80 years before and after RT. The selected patients did not undergo surgery, RT, or chemotherapy before sampling. Additionally, 30 healthy individuals without a history of malignancy and RT, who matched the patients in terms of age and gender, were enrolled as the regular group or the control group (Ctrl) in the same period. Inclusion criteria for this purpose were the following: 1) definite diagnosis of PCa via biopsy and the Gleason score (GS) system, and 2) an age group of 40 to 80. The exclusion criteria, on the other hand, were: 1) infectious diseases, 2) history of other malignancies, 3) history of any treatment, and 4) organic severe or systemic disease. All participants, including patients and volunteers, provided written informed consent to take part in the experiment. The Ethics (IR.IUMS.REC.1401.491) Iran University of Medical Sciences Ethics Committee (IUMS) evaluated and granted approval for this study.

Table 1. Patients' demographic and clinical parameters.

Age		P value
<69.93	10	<0.0001
³69.93	20	
Weight		
<75.9	15	<0.0001
³75.9	15	
PSA (Prostate Specific Antigen)		
<18.66	22	<0.0001
³18.66	8	
GS (Gleason Score)		
<8	12	
³8	18	
Grade		
2-3	12	
4-5	18	
Smoke		
Yes	11	
No	19	
Underlying diseases (diabetes, blood pressure, etc.)		
Yes	22	
No	8	

Radiation therapy (RT)

Half of the patients participating were treated by tomotherapy (Radixact tomotherapy machine, USA) and the other half by IMRT (Electa Synergy platform, Sweden) with a total dose of 70 Gy, energy of 6 MV, five daily fractions per week, 1.8 to 2.5 Gy per fraction, and a dose rate of 600 cGy/min. Figure 1 illustrates the CT image of the patient, the plan based on the CT image, and the dose table for the patient. Table 2 reports the average dose of the prostate (which receives the highest dose) and the rectum and bladder (which usually receive the highest dose among the usual organs of all patients).

Sampling and plasma isolation

About 5 mL of peripheral whole Blood collection

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was performed using ethylene diamine tetraacetic acid (EDTA) tubes (Tajhizat Pezeshki Baran, Iran) from each patient and Ctrl subject. Next, necessary measures were taken to prevent blood hemolysis. One sample of blood was obtained from the patients before the first fraction and another after receiving the last fraction of RT. To obtain plasma from blood samples, after centrifugation (Pole Ideal Tajhiz, Iran) for 10 min at 2500 g and 4°C and sedimentation of blood cells, the supernatant was transferred to 2-mL microtubes (Notarkib Co., Iran). Then, they were centrifuged again (12,000 g for 15 min at 4°C) to remove cellular components. Finally, the obtained plasma was slowly and carefully collected and transferred to other microtubes. Plasma samples were kept at -80°C until use.

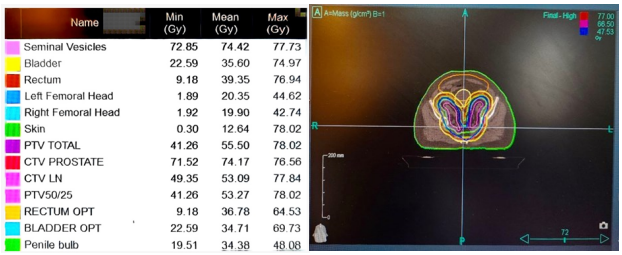

Figure 1. Treatment plan A. Table of dose B. Treatment plan (AccuracyPrecision 2.0.1.1) of the PTV (purple), bladder (yellow), and rectum (orange).

Table 2. The treatment plan doses.

Dose (Prostate)	
Mean (Gy)	69.77
Dose (Bladder)	
Mean (Gy)	39.35
Dose (Rectum)	
Mean (Gy)	39.85
Energy	
Total (%)	6 Mev (100)

RNA isolation

Total RNA was obtained from plasma samples following the RNA isolation kit manufacturer's guidelines (Norex, New Remix Co., Iran). Initially, 500 µL of each plasma sample was combined with 1000 µL of Norex lysis buffer from the kit and incubated at room temperature for 5 min. In the next step, 200 mL of chloroform (Merck KGaA, Germany) was added to the microtubes. After vortexing, the mixtures were kept at room temperature for five minutes. Post-centrifugation (12,000 g and 4°C for 15 min), three phases were formed in each microtube. Afterward, the upper phase containing RNA was carefully separated and transferred to other microtubes. Subsequently, an equal volume of isopropanol was added (Merck KGaA, Germany) to each microtube and kept overnight at -20°C. After two centrifugations and washing with 75% alcohol, the microtubes were drained and dried. Finally, after adding 30 µL of diethylpyrocarbonate (DEPC) water, the samples were kept at -80°C until use.

cDNA synthesis

cDNA was synthesized using the cDNA synthesis kit (Notescript, Notarkib Co., Iran). All synthesis steps were performed on ice. About 5 µL of total RNA (500 ng), 1 µL of reverse transcription enzyme, and 4 µL of a mixture of primers and buffer were added to each microtube and mixed (total volume: 10 µL). Synthesis was conducted using a thermal cycler (PEQLAB, Germany). The thermal cycling protocol consisted of 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C, followed by storage at −20°C.

Real-time quantitative polymerase chain reaction (qRT-PCR)

A Notarkib assay kit (Notarkib Co., Iran) was used to measure the expression levels of *miR-424, miR-372, miR-542*, *HULC, DANCR*, and *MIR4435-2HG* by RT-qPCR in plasma samples. All procedures were performed on ice. Briefly, 1 µL of cDNA solution, 3 µL of nuclease-free water, 5 µL of SYBR Green Master Mix, and 1 µL of the primer mix were added to each qRT-PCR strip tube (Roche, Germany) (total volume of 10 µL). In this study, U6 RNA was used as an internal control for lncRNAs and miRNAs. qRT-PCR was performed using the Corbett Research Rotor-Gene Real-Time PCR System (RG-6000, CORBETT, Australia). The amplification program was as follows: 10 min at 95°C (1 cycle), followed by 40 cycles of 15 s at 95°C and 40 s at 60°C. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, where Ct denotes the threshold cycle, and ΔCt = mean Ct of ncRNA − mean Ct of the *U6* control.

All gene-specific primers are shown in table 3. Primer efficiencies were calculated using calibration curves prepared from serial dilutions of cDNA. All primer pairs demonstrated efficiencies between 90% −110% and correlation coefficients (R^2) > 0.99. Furthermore, specificity was confirmed by melt curve analysis.

Table 3. The sequences of primers used in the RT-qPCR assay.

Name		Product length (bp)	Sequences
Hsa-424-5P	F	63	ATAGAGCTCTCCTGTTACGT
Hsa-542-3p	F	70	AAGCGTGTGACAGATTGA-TAACTG
Hsa-372-3p	F	68	AAAGTGCTGCGACATTT-GAGCGT
Hsa-R-universal	R	55	CAGTGCAGGGTCCGAGGTA
Lnc-DANCR	R	73	GTTTCTCATGACCTCCCCCG
	F		AGGCGCTATAGCAAGGTCAC
Lnc-HULC	R	93	CTCTGTGGGAGGTTGCACTC
	F		ATTCGACCTGTTTCAGTGCT
Lnc-MIR4435-2HG	R	90	CAAGTCTCACACATCCGGG
	F		CGGAGCATGGAACTCGACA
Lnc-RNU6	R	78	AACGCTTCACGAATTTGCGT
	F		CTCGCTTCGGCAGCACA

Enzyme-linked immunosorbent assay (ELISA)

A human *TGF-β* ELISA kit (cat. no. KPG-MTGF-B; KPgene, Iran) was used to measure *TGF-β* levels in plasma samples of PCa patients and the Ctrl group. The measurement was conducted according to the kit's protocol, and finally, the detection wavelength of

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450 nm was used for the OD reading.

Western blot assay

To evaluate *TGF-β*1 levels using the western blot assay, the protein content was extracted from plasma samples of PCa patients (before and after RT) and the Ctrl group. A total of 30 µg of protein was loaded, resolved on 10% sodium dodecyl sulfate – polyacrylamide gels, and transferred onto a polyvinylidene fluoride (PVDF) membranes. Next, the membranes were blocked for 2 h in 5% nonfat milk dissolved in 0.1% Tris-buffered saline (TBS). Membranes were rinsed with phosphate-buffered saline (PBS) (Zist Mavad Pharmed, Iran), incubated with primary antibodies, anti-*TGF-β*1 antibody (1:1000, cat# MBS9212175, mybiosource.com), and albumin (1:1000, Cat# NB-22-52254-100, clinisciences.com) at 4°C overnight. Following rinsing, membranes were incubated with secondary antibody at 37 °C for one hour. This study normalized relative protein expression levels with albumin as an internal control. Protein expression levels were quantified using ImageJ software (V1.34, National Institutes of Health, USA).

Follow-up

The patients in the present study were followed up for 11 to 14 months after RT. Serum PSA levels were measured at 3- and 6-months post-treatment. According to the findings, patients were categorized into two groups: sensitive and resistant. Refractory patients were selected based on the Phoenix criteria (2nd Consensus Conference by ASTRO and Radiation Oncology Group in Phoenix) [30] and consultation with two oncologists. Patients with an increase of 2 ng/mL or more above the PSA nadir were considered resistant groups. The frequent decrease or stability of PSA nadir during follow-up was considered a response to treatment.

Statistical analysis

Experimental results are presented as mean ± SD and were evaluated using SPSS 25 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 10 (GraphPad software, USA). Comparisons among groups were performed with Student's t-test, one-way ANOVA, or paired t-test; P<0.05 was considered significant.

RESULTS

Upregulation of lncRNAs in plasma samples following RT

As shown in figure 2, to investigate whether lncRNAs *DANCR*, *MIR4435-2HG*, and *HULC* are effective non-invasive biomarkers for predicting the response to RT in PCa patients, RT-qPCR was performed to measure the expression levels of these lncRNAs in plasma samples of PCa patients (before and after RT) and the Ctrl group.

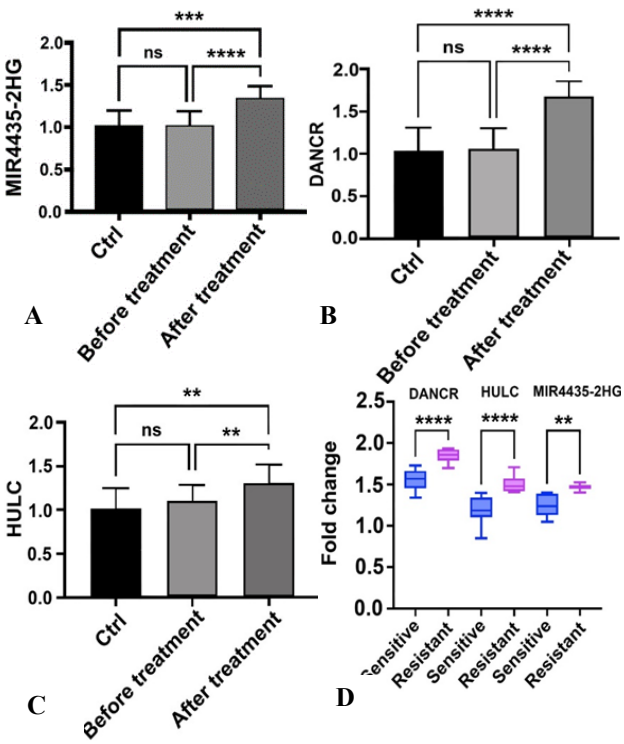

Figure 2. The expression levels of **A)** MIR4435-2HG, **B)** DANCR, and **C)** HULC in the plasma samples of patients with PCa (n = 30) and the Ctrl group (n = 30), and the expression levels of **D)** MIR4435-2HG, DANCR, and HULC in plasma samples of radiosensitive patients (n = 9) and radioresistant patients (n = 21). Differences between the groups were calculated using one-way analysis (*P < 0.05, **P < 0.01, and ***P < 0.001).

The results indicated that the expression levels of *DANCR*, *MIR4435-2HG*, and *HULC* in plasma samples of PCa patients were increased. Furthermore, *MIR4435-2HG* (1.33 ± 0.14) (P = 0.0005; figure 2A), *DANCR* (1.68 ± 0.17) (P = 0.002, figure 2B), and *HULC* (1.31 ± 0.20; *P* = 0.0001; figure 2C) levels were markedly higher in post-RT plasma samples compared with pre-RT samples. Notably, the *DANCR* (1.84 ± 0.08) (P < 0.001; figure 2D), *HULC* (1.51 ± 0.10) (P < 0.001; figure 2D), and *MIR4435-2HG* (1.47 ± 0.04) (P < 0.001; figure 2D) levels were significantly elevated in radiation-resistant patients (n = 9) relative to radiation-sensitive patients (n = 21). The association between the expression changes of *DANCR*, *MIR4435-2HG*, and *HULC* in the plasma of PCa patients and the clinical pathology and demographic information was evaluated. To assess this relationship, the data were classified into two groups, and the expression levels of *DANCR*, *MIR4435-2HG*, and *HULC* were subsequently compared in these two groups. As summarized in table 4, the high *DANCR* expression showed a significant relationship with GS (P = 0.04), grade (P = 0.04), underlying disease (P = 0.04), PSA (P = 0.02), and smoking (P = 0.03). Moreover, *MIR4435-2HG* and *HULC* demonstrated a significant relationship with GS, grade, background of diseases, and smoking. However, there were no statistically significant correlations between the expressions of any of the investigated genes and the

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variables of age and weight (table 4). This can indicate the effect of *MIR4435-2HG*, *DANCR* and *HULC* in PCa carcinogenesis and invasion.

Downregulation of miRNAs in plasma samples following RT

As shown in figure 3, in the present study, *miR-372, miR-424,* and *miR-542* were selected based on their association with *HULC*, *MIR4435-2HG*, *DANCR*, and *TGF-β*. Then, RT-qPCR was conducted to assess *miR-542, miR-424*, and *miR-372* expression in 30 PCa patients and 30 healthy controls (figure 3).

The results of examining the levels of *miR-372, miR-424,* and *miR-542* in plasma samples are shown in figure 3. It was determined that *miR-542* (0.77 ± 0.18), *miR-424* (0.65 ± 0.16) and *miR-372* (0.55 ± 0.21) were downregulated in PCa patients compared with the Ctrl group (no statistically significant differences were observed) ($P > 0.05$), whereas the expression levels of *miR-542* (0.55 ± 0.21) ($P = 0.0001$; figure 3A), *miR-424* (0.65 ± 0.16) ($P = 0.0001$; figure 3B) and *miR-372* (0.77 ± 0.18) ($P = 0.004$; figure 3C) were significantly decreased in plasma samples of patients after RT. Additionally, the expression levels of *miR-542* (0.62 ± 0.06), *miR-424* (0.49 ± 0.07) and *miR-372* (0.31 ± 0.15) ($P < 0.001$; Figure 3D) were significantly reduced in radiation-resistant patients (n = 9) compared with radiation-sensitive patients (n = 21) (figure 3D).

Upregulation of TGF-β/β1 in plasma samples following RT

Plasma levels of *TGF-β* and *TGF-β*1 in 30 PCa patients (before and after RT) and the Ctrl group were evaluated using ELISA and the western blot assay, respectively. Plasma levels of *TGF-β* (figure 4A) and *TGF-β*1 (figure 4B) were upregulated in patients with PCa compared with the Ctrl group. The expression levels of *TGF-β* and *TGF-β*1 were increased in plasma samples after RT. The relationship between *TGF-β* levels and the pathological information of PCa patients was analyzed using the χ^2 test. This study showed that the plasma levels of *TGF-β* are unrelated to the PSA levels of PCa patients (table 5).

Table 4. Association of the expression levels of lncRNAs DANCR, MIR4435-2HG, and HULC with clinicopathologic variables of patients with PCa. Differences between groups were calculated using Student's t-test (*P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001).

Clinical factor	Scale	*MIR4435-2HG*	P value	*DANCR*	P value	*HULC*	P value
Age	£69	1.39±0.03	0.98	1.29±0.17	0.81	1.67±0.20	0.50
	> 69	1.38±0.17		1.29±0.21		1.70±0.12	
PSA	<18.9	1.26±0.14	0.05	1.20±0.13	0.02	1.57±0.13	0.02
	³18.9	1.46±0.04		1.51±0.10		1.85±0.08	
GS	£7	1.16±0.09	0.04	1.11±0.11	0.04	1.51±0.09	0.01
	>7	1.43±0.04		1.45±0.10		1.81±0.09	
Grade	4-5	1.43±0.04	0.01	1.45±0.10	0.04	1.81±0.09	0.04
	1-3	1.16±0.09		1.11±0.11		1.51±0.09	
N stage	N0	1.45±0.04	0.02	1.14±0.13	0.02	1.54±0.08	0.05
	N1	1.24±0.12		1.47±0.1		1.78±0.16	
Underlying disease	YES	1.44±0.04	0.05	1.42±0.12	0.04	1.78±0.11	0.05
	NO	1.12±0.06		1.08±0.10		1.48±0.08	
Smoking	YES	1.33±0.15	0.02	1.44±0.1	0.03	1.78±0.17	0.05
	NO	1.23±0.12		1.15±0.20		1.58±0.11	
Weight	£76.8	1.39±0.03	0.91	1.27±0.15	0.85	1.69±0.11	0.52
	>76.8	1.37±0.18		1.31±0.23		1.67±0.20	

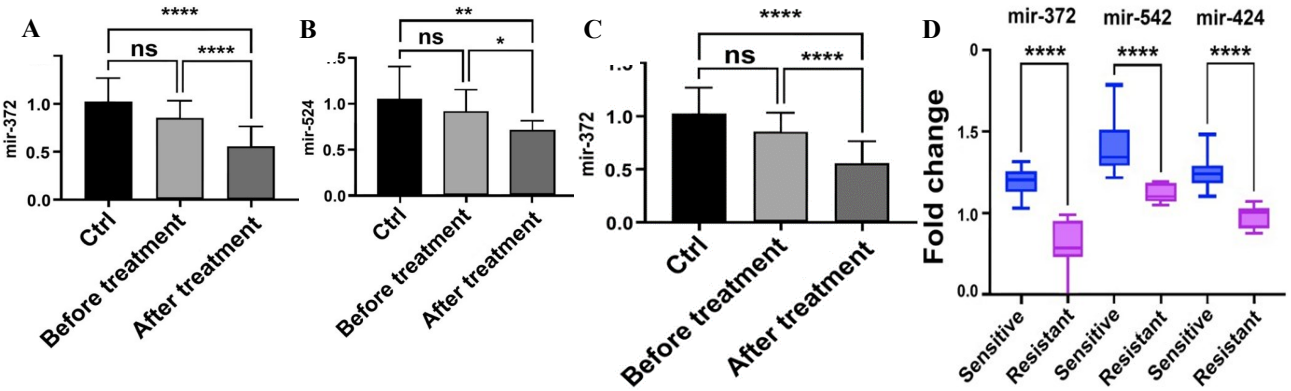

Figure 3. The expression levels of **A)** miR-424, **B)** miR-542, and **C)** miR-372 in plasma samples of patients with PCa (n = 30) and the Ctrl group (n = 30), and the expression levels of **D)** miR-372, miR-424, and miR-542 in plasma samples of radiosensitive patients (n = 9) and radioresistant patients (n = 21). Differences between the groups were calculated using one-way analysis (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001).

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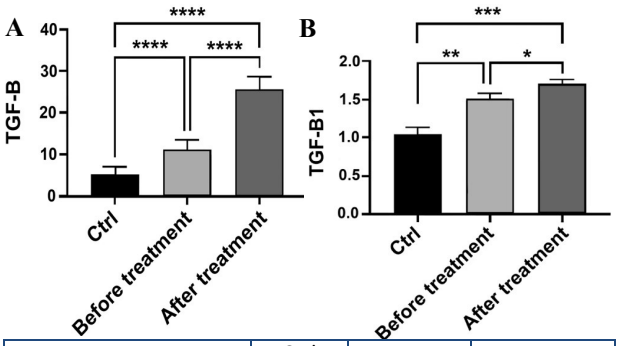

	Ctrl	Pre RT	Post RT
Albumin			
Fold change	1.00	1.54	1.73
TGF-β1			

Figure 4. The ELISA assay was performed to detect the expression of **A)** TGF-β, and the western blot assay was performed to detect the expression of **B)** TGF-β1 in plasma samples of 30 PCa patients and 30 healthy men (Ctrl). Differences between the groups were calculated using one-way analysis (*P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001).

Table 5. Association of TGF-β expression with clinicopathologic information of PCa patients (P<0.05).

Clinical factor		*TGF-β*	P value
PSA	<18.9	30.85± 0.78	0.94
	³18.9	29.82±1.9	
GS	£7	31.86±2.3	0.002
	>7	25.43±0.95	
Grade	4-5	25.43±0.95	0.002
	1-3	31.86±2.3	

As shown in figure 4A, it was found that *TGF-β* levels were significantly increased in PCa patients after RT (28.39 ± 3.63) (P = 0.0004) compared with before RT. The results demonstrated that *TGF-β*1 in the plasma samples of PCa patients was significantly higher than in the Ctrl group (P = 0.0006). In addition, an increase in the expression of *TGF-β*1 was observed in the plasma of patients after RT compared to the plasma of patients before RT (P = 0.0052; figure 4B). This relationship was investigated in the present study to establish the association between *TGF-β* expression, PCa pathology, and progression. Median *TGF-β* levels were significantly higher (P = 0.002) among patients with a GS higher than 7 compared with those with a GS lower than 7. Furthermore, *TGF-β* expression was greater in grade 3–5 PCa cases (P = 0.002) compared with grade 1-2 PCa patients. When the results were evaluated according to primary PSA values, the median *TGF-β* expression levels were also similar (P = 0.94) between patients with PSA levels higher than 18.9 ng/mL and patients with PSA levels lower than 18.9 ng/mL (table 5).

Pre- and post-treatment PSA levels in PCa patients

Figure 5 illustrates serum PSA levels recorded at diagnosis and during follow-up (3 and 6 months).

The analysis of serum PSA levels in PCa patients is shown in figure 5. PSA results of PCa patients were recorded at 3 months (0.87 ± 2.07) and 6 months (1.29 ± 0.82) after treatment (P = 0.58). A significant decrease in serum PSA levels (less than 1 ng/mL) was observed in 70% of patients during follow-up (P<0.0001). However, this decreasing trend was not observed in 30% of patients in the sixth-month follow-up, and the PSA serum level in these patients increased to more than 2 ng/mL (figure 5).

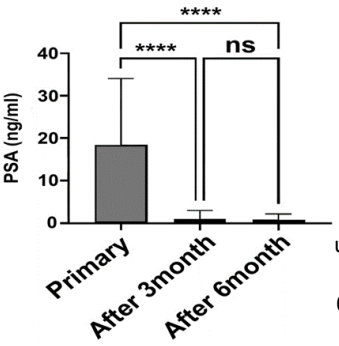

Figure 5. Serum PSA levels in PCa patients were recorded at the time of diagnosis and during the treatment follow-up. Differences between the groups were calculated using one-way analysis (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001).

DISCUSSION

PCa remains a major health concern, causing over 359,000 deaths annually [31]. While RT is a standard treatment, the radioresistant nature of some prostate tumors limits its effectiveness [32]. Conventional response markers such as PSA levels and imaging-based morphology often fail to detect resistance early [33]. This has led to increasing interest in non-invasive, sensitive biomarkers to better predict treatment outcomes and identify resistant cases [34]. This study found that plasma levels of the lncRNAs *DANCR, MIR4435-2HG* and *HULC* were significantly higher in radioresistant PCa patients compared to radiosensitive ones. Elevated expression of these lncRNAs was also associated with more aggressive clinical features, which may contribute to poor responses to RT (table 4). Given their stability and regulatory roles, lncRNAs are emerging as promising biomarkers [35]. In the present study, the plasma expression of these lncRNAs was quantified in PCa patients and healthy Ctrl using RT-qPCR. Our findings showed significantly elevated plasma levels of *MIR4435-2HG*, *DANCR* and *HULC* in PCa patients compared to healthy Ctrl. Notably, the expression levels of these lncRNAs further increased following RT, with *MIR4435-2HG* rising 1.3-fold, *DANCR* 1.6-fold, and *HULC* 1.2-fold (figure 2A-C). Previous studies support these findings. Xing *et al.* reported that the inhibition of *MIR4435-2HG* in the PC-3 cell line reduced cell migration and invasion [36]. Similarly, *DANCR* has been proposed as a biomarker for PCa progression and was shown by Jia *et al.* to promote invasion [37]. *HULC* overexpression has also been correlated with metastasis and unfavorable prognosis in PCa [38]. This study examined the

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relationship between *MIR4435-2HG, DANCR*, and *HULC* expression and clinicopathological features in PCa patients. Consistent with previous reports, no significant correlation was found between these lncRNAs and demographic variables such as age or weight. However, their expression levels were significantly elevated in patients with higher tumor grade, GS, and PSA levels (table 4), suggesting a potential role in PCa progression and aggressiveness [16, 39, 40].

lncRNAs can regulate gene expression by acting as molecular sponges for miRNAs, thereby influencing their mRNA targets [41]. Based on miRDB and DIANA TOOLS predictions, *HULC*, *MIR4435-2HG*, and *DANCR* are likely to interact with *miR-372, miR-424*, and *miR-542*, respectively - all of which are known to target the *TGF-β* signaling pathway. To investigate this regulatory axis, the expression of these miRNAs was examined in plasma samples from PCa patients.

Our findings revealed a significant post-RT reduction in *miR-372, miR-424,* and *miR-542* levels (figure 3A - C). This aligns with previous reports linking reduced *miR-424* to more aggressive PCa phenotypes [42]. Additionally, *miR-542* downregulation has been associated with enhanced metastasis via *NOP2* [43], and *miR-372* has been shown to regulate PCa progression in vitro by targeting *PRDM16* [44]. The dysregulation of ncRNAs is known to influence radiation response in various cancers [45]. In this study, the expression levels of *MIR4435-2HG*, *DANCR, HULC*, and their associated miRNAs were compared between radiation-resistant and radiation-sensitive PCa patients using a χ^2 test. The results revealed that *MIR4435-2HG, DANCR*, and *HULC* were significantly upregulated (by 1.1-, 1.3-, and 1.3-fold, respectively). Conversely, *miR-542, miR-424,* and *miR-372* were downregulated (by 1.4-, 1.5-, and 2.2-fold, respectively) in radiation-resistant patients (figure 2D and 3D). These findings suggest a regulatory network influencing radiosensitivity. Consistent with our results, Chen *et al.* previously reported elevated *HULC* levels in radiation-resistant PCa cell lines, where its silencing enhanced radiosensitivity by promoting cell cycle arrest and apoptosis [19]. This competitive binding could disrupt miRNA-mediated repression of key genes, such as those in the *TGF-β* pathway, contributing to therapy resistance in PCa. Nevertheless, further mechanistic studies are needed to validate this regulatory axis.

Numerous studies have highlighted a bidirectional regulatory relationship between *TGF-β* signaling and lncRNAs [46]. For example, Zhang *et al.* demonstrated that *MIR4435-2HG* promotes migration and metastasis in PCa by upregulating *TGF-β* signaling [47]. Similarly, Deng *et al.* showed that *DANCR* enhances invasion and metastasis by suppressing *miR-214* and activating the *TGF-β* pathway [17]. *TGF-β*1 is also implicated in tumor radioresistance. Wu *et al.* reported that inhibiting *TGF-β*1 sensitizes PCa cells to radiation by inducing DNA damage [48]. Motivated by this, the plasma levels of *TGF-β* and *TGF-β*1 in PCa patients and Ctrls were measured in the present study. *TGF-β* and *TGF-β*1 were significantly elevated in PCa patients compared to the Ctrl group and increased further after RT (figure 4A–B). Consistent with the results of the study by Reis *et al.* [49], The results indicated a statistically significant association between *TGF-β* expression and higher GS, but no association with serum PSA. It was also observed that *TGF-β* levels were increased in patients with advanced tumor grades (table 5). These findings further support the role of *TGF-β* in PCa progression and radioresistance [26].

PSA monitoring is a standard follow-up practice for PCa patients worldwide; however, optimal timing and frequency of testing remain undefined [50], and post-RT PSA levels have been shown to correlate well with treatment outcomes [51]. In our cohort, PSA levels generally decreased after treatment but rose in 30% of patients by six months post-treatment, with a mean level of 2.78 ng/mL. These patients exhibited more aggressive clinical features (figure 5).

A limitation of this study is the limited sample size and brief follow-up duration, which may influence generalizability of the results. Further large-scale, longitudinal studies are necessary to validate the applicability of these lncRNAs as clinical biomarkers for PCa RT response.

CONCLUSION

In the present study, *MIR4435-2HG*, *DANCR*, and *HULC* were significantly upregulated in radiation-resistant PCa patients. Along with the downregulation of the related miRNAs and the upregulation of *TGF-β*, particularly *TGF-β*1, these molecules may serve as potential biomarkers for predicting radiation response. Further studies are warranted to validate these findings and elucidate their molecular mechanisms, supporting the development of personalized treatment strategies in PCa.

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***Author contribution*:** S.M.T., Z.H-M., and A.N-R. conceived and designed the study. M.T. collected the data and wrote the first draft of the manuscript. M.B. contributed to the supervision and Real-time data

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collection. B.M. and M.T. followed up with patients and assessed their conditions. S.T. examined the progress of treatment planning. Z.A. analyzed the results.

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