Microrna 138 Upregulation is Associated with Decreasing Levels of CCND1 Gene Expression and Promoting Cell Death in Human Prostate Cancer Cell Lines

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Abstract
Background: This research intended to discover the significance of miR-138 (microRNA 138) on the expression profile, proliferation, and the associated regulatory mechanisms in prostate cancer (PCa).
Methods: Thirty-five specimens of prostate were studied to evaluate the expression level of miR-138 by RT-qPCR (Quantitative reverse transcription polymerase chain reaction). Bioinformatics analysis was performed to search for the target genes of miR-138, and ABL1 (ABL proto-oncogene 1, non-receptor tyrosine kinase), CCND1 (cyclin D1), CCND3 (cyclin D3), VIM (vimentin), TWIST1 (twist family bHLH transcription factor 1), HIF1A (hypoxia-inducible factor 1 subunit alpha), and TERT (telomerase reverse transcriptase) genes were selected. Then, the biological role of miR-138 and CCND1 in the progression of PCa was investigated using RT-qPCR and luciferase reporter gene assay. Finally, overexpression of miR-138 on the proliferation in PCa cell lines was analyzed using the MTT (3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide, Sigma, Germany) assay.
Results: RT-qPCR showed that the expression of miR-138 downregulated in PCa tissues and cell lines. Bioinformatics analysis and RT-qPCR assay demonstrated that CCND1 expression level was negatively correlated with miR-138 in PCa tissues and the PC3 cell line. Moreover, CCND1 was predicted to be the target gene of miR138 in the PC3 cell line based on the results of luciferase reporter gene assay. Substantially, over-expression of miR138-5p mimic could inhibit the expression level of CCND1 gene in PC3 cell lines. Lastly, over-expression of miR-138 inhibited the proliferative capacities in PC3 and DU-145 cells.
Conclusion: Our research introduces miR-138 as a negative regulator of CCND1 in the progression of PCa with an inhibitory impact on the proliferation rate of PCa cell lines. This regulatory mechanism could be utilized for the design and target selection of remedial miRNA-based approaches.

Introduction
The second most predominant type of malignant cancer is Prostate cancer (PCa). It is also the fifth leading cause of cancer-related death in men worldwide. Currently, the two most widely used PCa screening tools are serum prostate-specific antigen (PSA) levels and digital rectal examination. Although, serum PSA facilitated the detection of PCa at early stages but suffers from a high false-positive. Hence, more specific PCa biomarker would be valuable in improving PCa screening. Other biomarkers, including PCA3 score, Prostate Health Index (PHI), and 4Kscore, were recommended to improve the predictive value of PSA. Moreover, cancer-related miRNAs (microRNAs) appeared as promising candidates in the diagnosis, prognosis, and treatment of cancer. MiRNAs are single-stranded noncoding RNA molecules (18-24 nucleotides) that bind to the 3'-untranslated region (3'-UTR) of target mRNA to repress gene expression post-transcriptionally. MiRNA expression signatures are dissimilar between cancer and normal tissue and distinct cancer subtypes. More importantly, well-provided evidence has emphasized the anomalous expression of miRNAs in the malignancy of cancers. Therefore, miRNAs as cancer-specific biomarkers can be used to alleviate diagnosis, prognosis, and outcome of the therapy. MiR-138 (MicroRNA 138) were attended as a tumor
suppressor in numerous malignancies, including prostate, pancreatic, nasopharyngeal, colorectal, osteosarcoma, non-small cell lung cancers, and other malignant tumors.\textsuperscript{10-15} MiR-138 has the tumor suppressor function via diverse mechanisms such as apoptosis motivation, suppression of proliferation, metastasis, invasion, and modification of chemosensitivity in tumor cells. Distinct mechanisms imply heterogeneous targets of miR-138 in different cancer types.\textsuperscript{16-18} Considering various functions and heterogenous targets in different types of cancers, miR-138 could be regarded as a propitious remedial strategy for cancer.\textsuperscript{19} MiR-138 negatively regulated FOXC1 to suppress the malignant progression of PCa.\textsuperscript{20,21} MiR-138 was also incorporated in the suppression of the Wnt/β-catenin pathway in PCa.\textsuperscript{22} Furthermore, miR-138 and CCND1 (cyclin D1) expression exhibited a reverse correlation in nasopharyngeal carcinoma (NPC), but this association has not been examined in PCa and other cancer types.\textsuperscript{23} High CCND1 expression was also correlated with progressive malignancy in PCa.\textsuperscript{23,24} CCND1 (located on chromosome 11q13) functions as an essential coordinator in cell cycle progression from G1 (growth) to the S phase (synthesis).\textsuperscript{25} Overexpression of CCND1 was discovered in several types of cancer as an oncogene. This oncogenic behavior might be through diverse mechanisms such as disturbing cell cycle, proliferation, and neoplastic cell transformation.\textsuperscript{26-30} In the present research, we intended to discover the target genes for miR-138 and corroborate the importance of miR-138 in tumorigenesis in PCa. Bioinformatics analysis was carried out to explore the expression of miR-138 and discover their expression pattern. We assigned seven target genes for miR-138: ABL1 (ABL proto-oncogene 1, non-receptor tyrosine kinase), CCND1, CCND3 (cyclin D3), VIM (vimentin), TWIST1 (twist family bHLH transcription factor 1), HIF1A (hypoxia-inducible factor 1 subunit alpha), and TERT (telomerase reverse transcriptase). Among them, CCND1 emerged as a possible contributor to miR-138-regulated tumorigenesis due to its higher expression in PCa. As such, we characterized the expression profile of miR-138 in PCa cell lines, a non-cancer cell line, and PCa clinical samples. Moreover, the expression profile of CCND1 was evaluated in PC3 and DU145 as PCa cell lines and Huvec as a non-cancer cell line. The inverse regulatory function of miR-138 on CCND1 was examined by luciferase assay. We also investigated the impact of transient overexpression of miR-138 on the cell proliferation and expression of CCND1 in PCa cell lines to exhibit whether miR-138 is capable of hindering the proliferation of PCa cells via moderating CCND1.

Methods

**MiRNA prediction**

MiR-138 complementary sequences located within the 3′-UTR of target mRNAs were regarded as target sequences. PicTar (http://pictar.mdc-berlin.de), TargetScan (www.targetscan.org), Mirtargetlink2 (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2) and mirdb (http://www.mirdb.org/) were used for this purpose. These programs combine seed matches, conservation analysis, the thermodynamic stability of miRNA-mRNA duplexes, and site accessibility, among other characteristics, to maximize the target prediction specificity.\textsuperscript{31} Total scores computed by each tool were calculated, and genes with the maximum score were considered as target genes. The number of target sites, GC content of the seed site, and nearness of any base pairing to promote the accessibility of miRNA to the mRNA response element were examined manually in the computational programs to predict the best-presumed miRNAs. Based on previous reports, genes that were substantially upregulated in the other cancer types were also considered.

**Samples**

Thirty-five specimens of PCa were taken during radical prostatectomies. Clinical samples were provided by the Department of Pathology, Hashemi Nejad Hospital (Tehran, Iran) between 2014 and 2015. All volunteers were informed and filled out the consent form. The research was approved by the Research and Ethical Committees of Pasteur Institute of Iran (#825, #1972, and IR.PII.REC.1397.59). Gleason score, pathological stage, and histological prognosis were evaluated based on the guidelines of the Union for International Cancer Control.\textsuperscript{32} PCa tissues were collected following radical prostatectomy from untreated patients with PCa who were 48-80 years old (mean, 65.66 years). Within 1 h of prostatectomy, the specimens were dissected by a uropathologist (Hashemi Nejad Clinical Research Developing Center, Tehran, Iran) and were stored at -80 °C before RNA extraction. These samples were used in our previous work, where we described the patient demographics and clinicopathological features in detail.\textsuperscript{33}

**Cell culture**

PC3 and DU145 as human PCa cell lines were supplied by Leibniz-Institute DSMZ (Germany). DU145 cells were grown in RPMI 1640 medium reinforced with 10% FBS (Gibco, USA). PC3 cells were maintained in a 1:1 mixture of Ham’s F12 and RPMI 1640 medium (10% FBS). HUVEC (Human umbilical vein endothelial cell line) was provided by the National Cell Bank of Iran and was maintained in a 1:1 mixture of Ham’s F12 and DMEM medium (20% FBS). The cells were cultured in 5% CO\textsubscript{2} and 37 °C.\textsuperscript{34}

**RNA extraction and reverse transcription**

RNA extraction of PC3, DU145, HUVEC cell lines, and clinical samples was conducted based on previously described protocols.\textsuperscript{35} The ratio of absorbance at 260 nm and 280 nm was considered as the purity index of the extracted RNA. After that, 1.5 µg of each extracted RNA was applied for cDNA synthesis by PrimeScript RT reagent kit based on the manufacturer’s instruction (Takara Bio, Japan). Reverse transcription of miRNA was the same as
mRNA except for designed stem-loop RT primers which substituted for oligo dT primers.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)
The expression of different mRNA was studied by a Rotor-Gene 6000 system (Corbett Life Science; Qiagen, Germany). The mixture for RT-qPCR consisted of 10 μl SYBR green Master Mix (Takara, Japan), 0.5 pMol of the specific primers (Table 1), and 1 μl of cDNA in final volume of 20 μl. Water is added as needed to make a final volume of 20 μl. The program of RT-qPCR was as follows: initial denaturation at 95 °C for 30 seconds, followed by 40 cycles of denaturation at 95 °C for 5 seconds and 60 °C for 30 seconds. The mixture for Real-Time PCR with the total volume of 20 μl was comprised of 10 μl qPCR Master mix, 1 μl universal reverse-primer, 1 μl specific forward primer, and 1 μl cDNA. The thermocycling protocol consisted of an initial denaturation at 95 °C for 15 minutes, 40 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds, and 70 °C for 30 seconds. The expression of mRNA was normalized with GAPDH. The SNORD47 (U47) and U6 miRNAs were applied to normalize miRNA expression. The normalization of the target gene with the reference standard was performed using REST® (relative expression software tool). The relative expression was founded upon the expression ratio of the target genes in contrast with the reference gene. The ratio of mRNAs to miRNAs was calculated using the ΔΔCt method. The average Ct values of the target gene were subtracted from the Ct of the housekeeping gene to calculate ΔCt. The results were analyzed in GraphPad. The proliferation efficiency of RT-qPCR was evaluated using serial dilutions of 10^1, 10^2, and 10^3 of a pooled cDNA sample (15 samples) for every primer set, besides normalizing against references.33

| Table 1. Specific primers were designed for seven target genes predicted by computational tools. |
|-----------------|-----------------|
| **Primers**     | **Gene**        |
| AACGGGAAGCCTTGTCATCAATGGGAA | GAPDH |
| GCATACGAGAGGGGCCAGGG | ABL1 |
| GCTGGTTATCTGGAAGAAGCTCT | CCND1 |
| GCAAGCAAGAAAGTTGCGGC | CCND3 |
| GACCTCGTTGCCCCGTGTG | CCND3 |
| GGAGCGTATGAAAGGAGGA | CTCCCAAAAGGGGCCTC |
| CCAAGACAGGATACGCACA | CCND3 |
| GATAATCTTTGGTGACCTC | VIM |
| TTAAGGGCATTCCCCTTACAGG | HIF1A |
| CTCAGTACGCTTTCTCCGCT | TERT |
| CGAATGTCCATCCAAATTCAC | HIF1A |
| TCCAAGAAGCTCTAAGGGTGT | TERT |
| ATGTTCCATTTTCAATCTGCTGTA | HIF1A |
| GTGCTACGGCCACATGCGGA | TERT |
| GGGCATTGCTAGGAGGAAGTT | TERT |

Luciferase assay
The possibly paired sequences of 3′-UTR of the CCND1 gene were found from miRTarBase, which sums up the interaction sites reported in previous studies and predicts specific interactions.14 Three interactional regions in 3′-UTR of CCND1 predicted by miRTarBase, including regions 757-776, 3096-3118, and 1457-1497 (NM_053056.3), were synthesized (Biomatik, Canada), and subcloned into the Xhol/NotI restriction sites of psiCHECK™-2 Vector (Promega, USA) consecutively with 20 nucleotides in between. The final construct was named psiCHECK2-CCND1-3′UTR. Syn-cel-miR-39-3p (negative control, abm, Canada) and miR-138-5p (miR-138 mimic) sequence vectors were purchased from Qiagen, Germany. Initially, about 105 PC3 cells were grown in a 96-well plate. After 12-16 hours, psiCHECK2-CCND1-3′, miR-138-5p-mimic+psiCHECK2-CCND1-3′UTR, and Syn-cel-miR-39-3p+psiCHECK2-CCND1-3′UTR were co-transfected into the PC3 cells in triplicates. Dual-Luciferase Reporter Assay System (Promega, USA) was adopted to evaluate the luciferase activity 24 hours after transfection, based on the manufacturer’s instruction as previously elucidated.44 The multi-well plate luminometer Renilla luciferase activity was normalized against firefly luciferase.

Cell proliferation analysis
The viability of transfected cells with miR-138 was examined using MTT (3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide, Sigma, Germany) assay. Briefly, about 10^4 PC3 and DU145 cells were grown in every well of a 96-well plate and transfected with miR-138-5p-mimic and Syn-cel-miR-39-3p. The cells that had only been incubated with Lipofectamine were used as control. The optical density was read at 540 nm against 630 nm as the reference. Cell proliferation was interpreted respective to non-transfected cells.

Statistical analysis
Statistical analysis was conducted utilizing the mean ± standard error of mean (SEM) in the GraphPad Prism version 8.01 (GraphPad, USA). The difference among the various categories was calculated using Analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test. P values ≤ 0.05 were deemed as noteworthy. All assays were conducted at least in triplicate.

Results

Identification of miR-138 candidate target genes in PCa
As indicated in Table 2, a total of seven genes were selected based on previously validated genes in papers, and the highest scores in Target Scan, Microcosm, PicTar, and miRanda: ABL1, CCND1, CCND3, VIM, TWIST1, HIF1A, and TERT genes. MiR-138 expression diminished in PC3 and DU145 in comparison with HUVEC
The expression of miR-138 was examined in PCa cell lines.
Table 2. Selection of miR-138 target genes using four computational algorithms and considering previously validated genes in papers.

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*1: MiR-138 targets a specific gene, 0: MiR-138 does not target a specific gene.

Figure 1. Results of quantitative RT-PCR on PCa and HUVEC cell lines: relative expression (2−ΔΔCT) of miR-138 in HUVEC cells compared to PC3 and DU145. U6 or U47 housekeeping genes were applied as the normalizers.

Figure 2. Results of quantitative RT-PCR on PCa tissue samples compared with U6 or U47 housekeeping genes as the normalizers.

Figure 3. Results of quantitative RT-PCR on CCND1 gene in PC3 and DU145 cell lines. Substantial overexpression of CCND1 in comparison with GAPDH as a housekeeping gene in PC3 cells was observed. The expression of CCND1 exhibited an insignificant rise in the DU145 cell line.

The expression of miR-138 diminished in the PCa clinical samples

The expression of miR-138 in PCa clinical samples was examined via RT-qPCR. U6 and U47 genes functioned as normalizers. The expression of miR-138 evidently diminished in PCa clinical samples in comparison U6 and U47 as normalizers (Table S1). Results of quantitative RT-PCR on miR-138 in clinical samples in comparison U6 and U47 as normalizers is demonstrated in Figure 2.

Among seven candidate genes, only CCND1 enhanced in PCa cell lines

To evaluate the influence of reduced amounts of miR-138 on PC3 and DU145 cancer cells, the expression profile of seven selected target genes, including ABL1, CCND1, CCND3, VIM, TWIST1, HIF1A, and TERT genes was examined utilizing RT-qPCR. PCR efficiency of every sample was between 95% to 105%, based on the slope of the standard curve (Figure S1). Moreover, the relative expression of ABL1, CCND1, CCND3, VIM, TWIST1, HIF1A, and TERT genes was compared to GAPDH as the control group (These target genes were previously normalized to GAPDH to quantify their relative expression). As elucidated in Figure 3, among seven evaluated target genes, only CCND1 was expressed at a significantly higher level (p-value <0.05) in comparison with GAPDH as the housekeeping gene in PC3 cells. The expression of CCND1 insignificantly raised in the DU145 cell line compared to the control group. The expression of other candidate genes, including ABL, CCND3, VIM, TWIST1, HIF1A, and TERT, displayed no substantial change in comparison with GAPDH in PC3.
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and DU145 cell lines. Results of quantitative RT-PCR on all candidate genes in PC3 and DU145 cell lines is demonstrated in Table S2.

**CCND1 was corroborated as the target of miR-138 in the PC3 cell line by luciferase assay**

MiRanda was utilized to analyze and predict 3′UTR sequences of the CCND1 gene that could be targeted by miR-138. MiR-138-complementary sites located on the 3′ noncoding region of CCND1 transcripts in miRTarBase are represented in Figure 4. To examine and corroborate the impact of miR-138 on CCND1, the predicted 3′UTR sequences of CCND1 were cloned downstream of luciferase in the psiCHECKTM-2 vector. PC3 cells were transfected in three groups: psiCHECK2-CCND1-3′UTR plus miR-138-5p-mimic, psiCHECK2-CCND1-3′UTR plus Syn-cel-miR-39-3p, and psiCHECK2-CCND1-3′UTR alone. As elucidated in Figure 5, miR-138-5p-mimic diminished the efficacy of luciferase compared to psiCHECK2-CCND1-3′UTR plus Syn-cel-miR-39-3p, and psiCHECK2-CCND1-3′UTR alone. While no substantial variation was noted between the psiCHECK2-CCND1-3′UTR plus Syn-cel-miR-39-3p transfected cells, and the psiCHECK2-CCND1-3′UTR transfected cells. The data of the luciferase assay evidenced that transfection of miR-138-5p-mimic considerably diminished Renilla luciferase activity, and it turned out that miR-138 could modulate the CCND1 expression through acting on its 3′-UTR in the PC3 cell line.

**MiR-138-5p-mimic diminished the expression of CCND1 in the transient transfected PCA cell lines**

RT-qPCR was utilized to evaluate the expression of CCND1 in the miR-138-5p-mimic transfected PC3 and DU145 cells to realize whether miR-138 could modulate CCND1 by acting on its 3′-UTR in PCA cell lines. As displayed in Figure 6, the expression of CCND1 insignificantly diminished in comparison with GAPDH following transfection with miR-138-5p-mimic in DU145 cells, while CCND1 mRNA level significantly decreased in comparison with GAPDH following transfection in PC3 cells. Results of quantitative RT-PCR on CCND1 gene in comparison with GAPDH in PC3 and DU145 cell lines is demonstrated in Table S3.

In conclusion, CCND1 expression revealed a tendency to decrease in DU145 cell lines and a substantial decline in PC3 cells. Diminished expression of CCND1 in miR-138 transfected PC3 and DU145 cell lines implies the suppressive influence of miR-138 on the expression of CCND1 in the PCA cell lines.

**MiR-138-5p-mimic could have a pivotal function in the inhibition of cell proliferation in the transient transfected PCA cell lines**

The cell viability test by MTT was carried out to understand the functional activity of overexpression of miR-138 in the proliferation of PCA cell lines. Cells receiving only Lipofectamine (blank) were utilized as the negative control,
and cells with no transfection functioned as the external control group. PC3 and DU145 cells were transfected in three categories containing miR-138-5p-mimic and Syn-cel-miR-39-3p in addition to the cells incubated only with Lipofectamine. The outcome of the MTT assay implied that the proliferation of PC3 cells 24 hours subsequent to transfection with miR-138-5p-mimic significantly declined compared to Syn-cel-miR-39-3p transfected cells and Lipofectamine incubated cells. Furthermore, miR-138-5p-mimic transfected DU145 cells substantially evidenced a diminished proliferation rate compared with Lipofectamine incubated cells, while miR-138-5p-mimic and Syn-cel-miR-39-3p transfected cells expressed insubstantial variations (Figure 7).

In conclusion, the proliferation of all miR-138-5p-mimic transfected cell lines considerably diminished compared to non-transfected cell lines.

Discussion
During the first phases of cancer diagnosis, miRNAs are being used more and more. They are also utilized for the therapy and prognosis of this disease. Dysregulation of miRNAs in cancer cells are involved in cancer growth and progression. As elucidated before, expression of the microRNAs inclusive of miR-26a, miR-138, miR-1266, miR-185, and miR-30c diminished in PCa tissues and cell lines. Moreover, miRNA expression profiles showed widespread dysregulation in primary PCa compared to normal prostate tissue. As expounded earlier, miR-138 arrested the malignant advancement of several human cancers, including pancreatic cancer, colorectal cancer, and other cancer types.

Here, distinctive expression of miR-138 was scrutinized in PCa tissues, and PCa cell lines in comparison with non-cancerous cell. We corroborated that the expression of miR-138 considerably lessened in PCa cell lines in comparison with the HUVEC cell line. Additionally, miR-138 obviously diminished in PCa tissues. These findings were in line with previous studies where miR-138 evidently lessened in PCa tissues and cell lines.

Here, according to bioinformatic analysis with online software such as Target Scan, Microcosm, PicTar, and miRanda, it was assumed that ABL1, CCND1, CCND3, VIM, TWIST1, HIF1A, and TERT might be the target gene of miR-138. In accordance with RT-qPCR findings, the expression of CCND1 substantially augmented in PCa tissue and PC3 cell line and had an insignificant rise in the DU145 cell line. Our data proposed that CCND1 showed a reverse correlation with the miR-138 in the PCa cell lines. Additionally, dual-luciferase reporter assay was utilized to explore the associations between miR-138 and 3' UTR of CCND1 and to examine whether this relationship is influential in the PCa progress. In accordance with the results of the luciferase reporter assay, overexpression of miR-138 prohibited the expression of CCND1 in PC3 cell lines. It was inferred that miR-138 could negatively regulate the expression of CCND1 in PCa cell lines. As pointed out previously, a reverse correlation was distinguished between miR-138 and the expression of CCND1 in nasopharyngeal carcinoma. CCND1 has also been associated with miRNAs in other tumorigenesis pathways in PCa. In a new signaling pathway comprising LOX1-AS1, miR-541-3p, and CCND1, which modulates the cell cycle and proliferation of PCa cells, CCND1 exposed as the target of miR-541. Additionally, owing to the post-transcriptional regulatory function of the miR17 family, CCND1 evidently elevated when the miR17 family was suppressed in PCa cell lines.

Subsequently, we scrutinized the influence of miR-138 overexpression on the expression profile of CCND1 in the transiently transfected PC3 and DU145 cell lines. Conclusively, overexpression of miR-138 could repress CCND1 expression in PC3 cells, while the expression level of the CCND1 gene exhibited an insignificant reduction in the DU145 cell line. MiR-138 has also shown other negative regulatory influences on PCa. As previously elucidated, overexpression of miR-138 substantially arrested the capability of the Wnt/β-catenin pathway in C4-2B and PC3 cells. Accordingly, miR-138 reversely modulates β-catenin in PCa cells.

MTT assay was utilized to appraise the inhibitory function of miR-138 on the proliferation scale of PCa cell lines. Our findings revealed that miRNA-138 could precipitate an anti-proliferative effect on PCa cells, which was in line with preceding research that miR-138 motivated anti-proliferative influence on several cancers such as clear cell renal cell carcinoma, squamous cell carcinoma, tongue squamous cell carcinoma, and head and neck squamous cell carcinomas.

Consonant with the earlier investigation, miR-138 and its target CCND1 were implicated in the modulation of the nasopharyngeal carcinoma progress. Overexpression of
MiR-138 also induced arrest in the G1 cell cycle, repression of cell proliferation in vitro, and inhibitory effects on the tumorigenicity of mice xenografts. Moreover, it was previously declared that overexpression of miR-138 hindered the metastasis and proliferation of PCa through targeting and downregulating FOXC1. Furthermore, earlier experiments revealed that elevated expression of miR-17 family members contributed to the lessening of CCND1 which resulted in motivated apoptosis, suppressed proliferation capability, and colony forming potential. MiR-138 and PD-L1 also exhibited a negative correlation in colorectal cancer. The diminished proliferation rate in miR-138-5p mimic transfected HCT116 and SW620 cells was evidenced by MTT assay through regulating PD-L1 as the target gene. In the current research, the potential of miR-138 to suppress cell proliferation indicated its function as a tumor suppressor in PCa, which might be partial via regulating CCND1 as the target gene. Our results were in line with previous research that miR-138 repressed proliferation and motivated apoptosis through modulating multiple targets. MiR-138 also functioned in the modulation of enhancer of zeste homolog 2 (EZH2) to repress proliferation efficiency, motivate apoptosis and arrest G0/G1 cell cycle as a tumor suppressor in non-small cell lung cancer cells.

Therefore, miR-138 elucidated assorted biological functions through modulating the CCND1 gene in PCa. It implies the reverse modulatory impact of miR-138 on the expression of CCND1 in PCa. However, the anti-proliferative effects of miR-138 could be applied through the mechanisms other than CCND1 regulation in the DU145 cell line. Further studies are required to identify the cooperative genes. Distinguishing these functional targets might have clinical importance hereafter. Our findings need more complementary studies, such as evaluating the repressive influence of miR-138 on the malignant advancement of PCa in vivo as well as clinicopathological features of PCa patients through analysis of the biological function of CCND1.

Here, to the best of our knowledge, we evidenced the direct association of miR-138 with the CCND1 gene for the first time in PCa. The overexpression of CCND1 was also negatively correlated with miR-138 in PCa cell lines. Our findings implied that miR-138 could modulate the enhanced expression of CCND1 to some extent. Conclusively, miR-138 functions as a tumor suppressor through the moderation of the CCND1 gene in the malignant advancement of PCa.

**Conclusion**

Our findings revealed an etiological relationship between miR-138 and CCND1 gene as its target in PCa. We also corroborated the functionality of this correlation in the growth and proliferation of PCa that could function in miRNA-based diagnostic and therapeutic approaches. However, further comprehensive evaluation of the modulatory pathways and molecular mechanisms in PCa would highly facilitate the promotion of design and selection of targets for therapeutic procedures. While our findings provide valuable insights into the role of miR-138 and CCND1 in PCa, the generalizability of these results to a larger population remains to be confirmed. Moreover, our research focused exclusively on PCa, and it is essential to evaluate the role of miR-138 and its target gene in other types of cancers. The heterogeneity of cancer types and their underlying molecular mechanisms may limit the applicability of our findings beyond PCa. Additional approaches could provide further insights into their modulatory pathways and molecular mechanisms in PCa. Incorporating complementary evaluation techniques, such as gene expression profiling, proteomics, or functional assays, would strengthen the robustness of our findings. These advancements would enhance the design and selection of targets for more effective therapeutic interventions in PCa and potentially other cancers as well.

**Ethical Issues**

The study was carried out in accordance with the guidelines of the Research and Ethical Committees of Pasteur Institute of Iran (#825, #1972, and IR.PII.REC.1397.59). Informed consent was obtained from all subjects involved in the study.

**Author Contributions**


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**Conflict of Interest**

The authors report no conflicts of interest.

**Supplementary Data**

Supplementary data, Figure S1, Table S1-S3, are available at https://doi.org/10.34172/PS.2023.17.

**References**

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30. Alao JP. The regulation of cyclin D1 degradation: Roles