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Original Article

miR-let-7a suppress cell invasion and migration via the depressing PKM2 in prostatic cancer

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Abstract: Objective: Some cancer reports found that miR-let-7a overexpression could suppress cancer cell biological activities M2 splice isoform of pyruvate kinase (PKM2) down-regulation, however, the correlation between miR-let-7a and PKM2 was unclear in prostatic cancer. The aim of this study was to study the effect and mechanism of miR-let-7a in prostatic cancer. Methods: To evaluate the PKM2 protein expression in adjacent and cancer tissues from 30 prostatic cancer patients by IHC assay and RT-PCR, the and analyzed the correlation between PKM2 and miR-let-7a. In the cell culture experiments, the DU-145 and PC-3 cells which were two representativeness kinds of prostatic cancer cell lines were divided into 3 groups: NC (un-treated) group; BL (the cell were transfected with blank vector) group and miR-let-7a (the cell were transfected with miR-let-7a mimics). The invasion and migration abilities of difference groups were evaluated by transwell and wound healing assay, and measured the relative protein expression (PKM2 and heterogeneous-nuclear ribonucleoprotein A (hnRNPA1) by WB assay. Results: The PKM2 protein expression of cancer tissues were stronger than that of adjacent normal tissues; meanwhile, The miR-let-7a and PKM2 gene expressions were significantly differences between two tissues (*P<0.05, respectively*), and the miR-let-7a was negatively correlation with PKM2 mRNA expression in cancer tissues (r=-5.3105). In the cell experiments, the invasion cell number of miR-let-7a group was significantly decreased compared with BL group (*P<0.05, respectively*) and the wound healing rate of miR-let-7a group was significantly down-regulation compared with BL group (*P<0.05, respectively*). By the WB assay, the PKM2 and hnRNPA1 which were closely correlated with miR-let-7a proteins expression were significantly differences among three groups (*P<0.05, respectively*). Conclusion: miR-let-7a was known as a supper factor in prostate cancer, miR-let-7a overexpression inhibited the cell invasion and migration abilities via regulation PKM2 and hnRNPA1.

Keywords: miR-let-7a, DU-145, PC-3, PKM2, hnRNPA1

Introduction

At present, the prostatic cancer incidence rate has continued to rise and the death rate became a threat to the health of older men the most common malignant tumors [1, 2]. The metabolism of tumor cells is aerobic glycolysis instead of oxidative phosphorylation (Warburg effect), pyruvate kinase M2 (PKM2) is the key enzyme in the Warburg effect. PKM2 can regulate the proliferation of gastric cancer cells by regulating Bcl-xl, and regulate the phosphorylation of histone H3 to promote the proliferation of glial cells [3, 4]. At the same time, PKM2 can also regulate epithelial mesenchymal transition (EMT) by regulating epithelial growth factor in hepatocellular carcinoma cell [5]. However, the study PKM2 associated with invasion and metastasis of prostate cancer has been relatively limited. microRNA (miRNA) disorders in a variety of tumors and involved in tumor generation and development process, through regulating cell proliferation, apoptosis, invasion, migration and other biological behavior, play oncogenes or tumor suppressor in tumor [6, 7]. However, It has been unclear that the effects and mechanisms of miR-let-7a in the development of prostate cancer. The previous studies found miR-let-7a was low expression in cancer tissues with PKM2 up-regulation. However, it has been unclear that the effects and mechanisms of miR-let-7a in prostate cancer.

In our present study, we firstly measured the PKM2 gene and protein expression and miR-let-

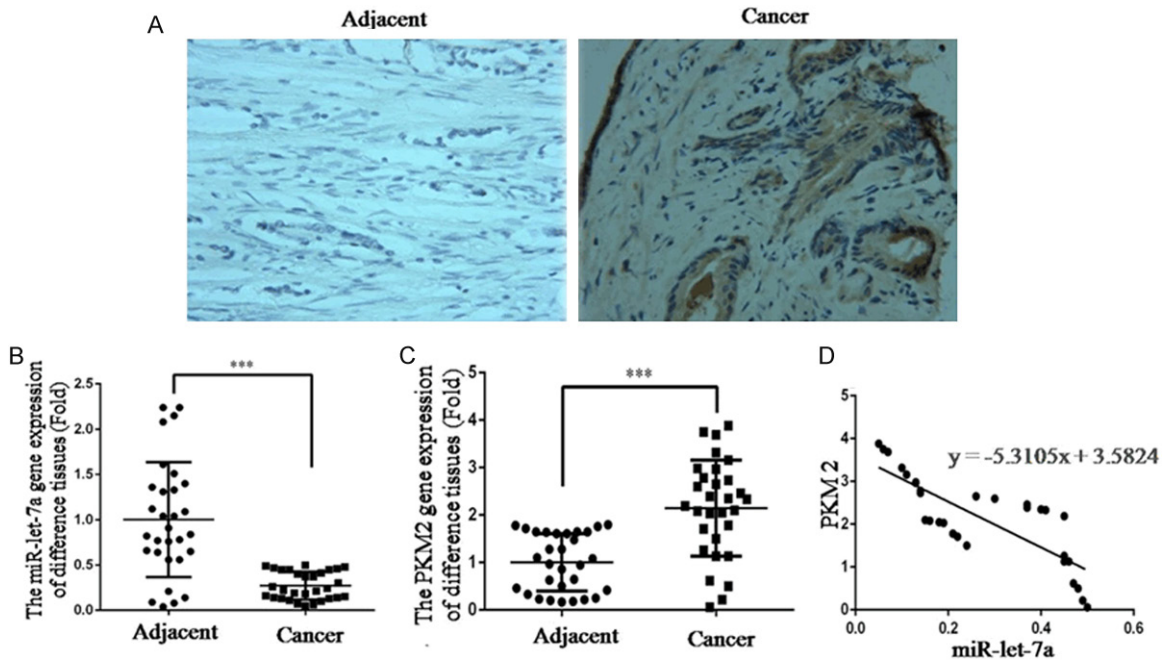

Figure 1. The miR-let-7a and PKM2 expression and correlation in clinical. A. The PKM2 protein expression in adjacent and cancer tissues, the PKM2 protein of cancer tissues was +++; PKM2 protein of adjacent tissues was. B. The miR-let-7a gene expression of adjancent and cancer tissues, $P<0.05$. C. The PKM2 gene expression of adjancent and cancer tissues, $P<0.05$. D. The correlation between miR-let-7a gene and PKM2 gene. The miR-let-7a gene expression was negatively correlation with PKM2 gene expression.

7a gene expression by RT-PCR or/and Immunohistochemistry (IHC) in adjacent normal and cancer tissues, and analysised the correlation between PKM2 and miR-let-7a in cancer tissue. In the cell experiments, we evaluated the miR-let-7a over-expression had effects to suppress the cell invasion and migration abilities in DU-145 and PC-3.

Materials and methods

Human tissue samples

Thirty pairs of prostatic cancer specimens including primary prostatic cancer tissues and paired adjacent normal tissues were derived from the prostatic cancer patients who treated in our hospital. The prostatic cancer patients were 40-60 years old including I-II stage prostatic cancer patients (n=19) and III-IV stage prostatic cancer patients (n=11). All tissues were divided into two parts: one part was kept in liquid nitrogen and stored at -80°C until using and another was stored in the 10% polyoxymethylene to IHC staining.

IHC assay

Thirty pairs of prostate cancer adjacent normal tissue and cancer tissue by 10% neutral formalin fixed, paraffin embedded, sliced paraffin sections of 4 tested m, immunohistochemical staining method comprises the following steps: dewaxing, hydration, 3% hydrogen peroxide inactivation of endogenous peroxidase, citric acid, antigen repair, adding the PKM2 anti-body to incubated overnight at 4°C in the dark, and added horseradish peroxidase labeled streptavidin second antibody solution, DAB staining, hematoxylin staining, dehydration, neutral resin sheet. Specific steps refer to the immunohistochemical Kit (ZSGB-BIO).

RT-PCR assay

The total RNA were extracted from adjacent and cancer tissues with Trizol reagent (In-vitrogen, USA) depending on manufacturer's instruction. To measure the miR-let-7a and PKM2 gene expression, we used Taqman miRNA reverse transcription Kit (Invitrogen,

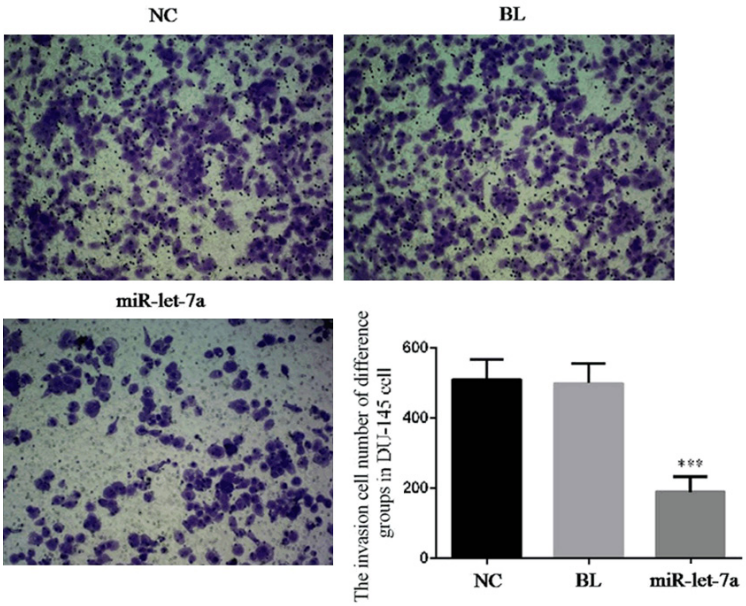

Figure 2. The invasion DU-145 cell number of difference groups by transwell assay. NC: Normal control group; BL: Transfection with empty vector; miR-let-7a group: Transfection with miR-let-7a. Compared with NC group, the invasion DU-145 cell number of miR-let-7a group was significantly suppressed, and the invasion DU-145 cell number of BL group was no significantly. ***: Compared with NC group, P<0.05.

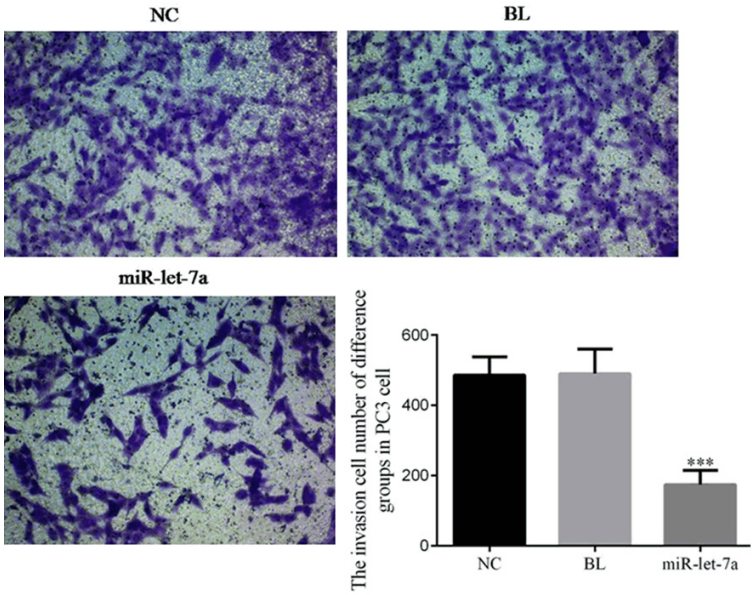

Figure 3. The invasion PC-3 cell number of difference groups by transwell assay. NC: Normal control group; BL: Transfection with empty vector; miR-let-7a group: Transfection with miR-let-7a. ***: Compared with NC group, P<0.05.

USA) or SYBP Green Master Mix Kit (Roche, USA) to get cDNA.U6 was used for normalization. The specific primers were as follows: miR-let-7a, forward: 5'-GGTGAGGTAGTAGGTTGTATAGTT-3'; reverse: 5'-CTCGCTTCGGCAGCACATATA-3'; PKM2, forward: 5'-CTGTGGACTTGCCTGCTGTG-3'; reverse: 5'-TGCCTTGCGGATGAATGACG-3'; β-actin, forward: 5'-AGAAAATCTGGCACCACACC-3'; reverse: 5'-TAGCACAGCCTGGATAGCAA-3'. The β-actin was used as reference in this study. The relative quantification of miR-let-7a and PKM2 was calculated by $2^{-\Delta\Delta CT}$ method.

Cell lines and cell culture

Prostate cancer cell lines DU-145 and PC-3 (ATCC, USA) were cultured in RMPI 1640 (Hyclone, USA) medium contained 10% feral bovine serum (FBS) (Gbico, USA). The DU-145 and PC-3 were maintained in incubator which contained 5% CO_2 at 37°C.

Cell transfection

Collect the logarithmic phase of DU-145 and PC-3 cells plated at the density of 1×10^6 cells/hole in 6 well plates, cell fusion degree reached 90%, according to the Lipofectamine™ 2000 specification miR-let-7a transfected with the recombinant plasmid mimic prepartion. miR-let-7a: 5'-UGAGGUAGUAGGUUGUAUAGUU-3' and BL: 5'-UUCUCCGAACGUGUCACGUTT-3'.

The cell invasion by transwell assay

The cells of difference groups were added in serum free medium, and palced in the transwell upper chamber, the RPMI 1640 medium contained 10% FBS were added in transwell bedroom, after 24 h, removing the chamber, the negative cells were fixed with methanol for 20 min, and

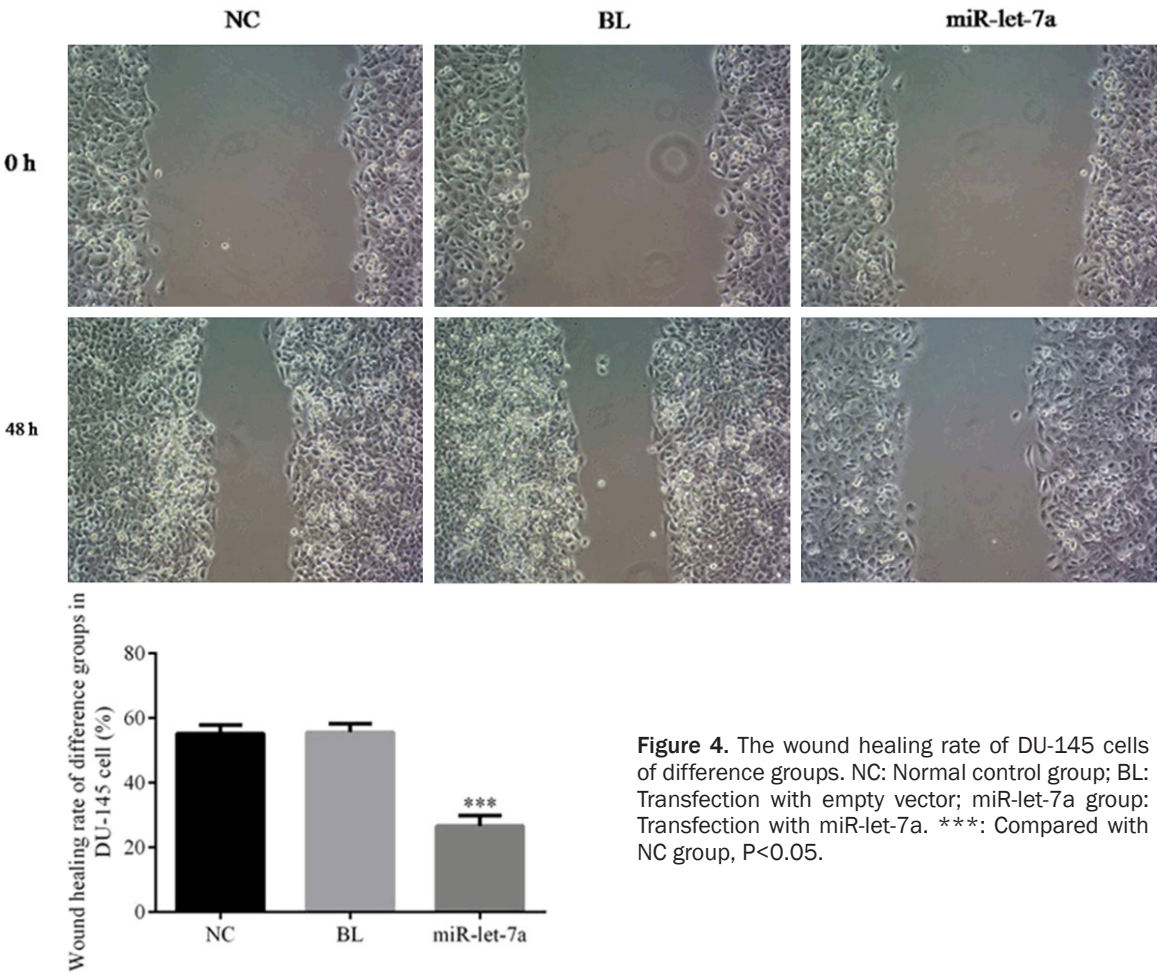

Figure 4. The wound healing rate of DU-145 cells of difference groups. NC: Normal control group; BL: Transfection with empty vector; miR-let-7a group: Transfection with miR-let-7a. ***: Compared with NC group, P<0.05.

placed in liquid crystal violet for 15 min. Collecting 3 random visual fields to calculate the penetrating cell number under light microscope.

Measuring the cell migration ability by wound healing assay

The cells of each group in the logarithmic growth phase were digested and inoculated in 6 holes, the density of which was 3×10^5 cells/hole, and the culture medium was replaced by the anti culture medium for second days. After treated with 6 h, the cells were replaced with fresh culture medium. 12 h after using the liquid gun head vertical in the 6 hole board draw a straight line, PBS cleaning 2 times, the suspension cell discard, add 1.5% FBS medium, micro photo. After 48 h cultures, cell migration was observed.

Evaluating the relative protein expression by WB assay

After 48 h digestion, the cells were collected and washed with PBS for 2 times. The supernatant was centrifuged and the supernatant was removed. The 130 mL cell lysate was added to each tube, and the lysis cells were mixed with an oscillating apparatus. The cells were lyse-d by 3 min on ice and centrifuged at 4°C for 15 min by 125000×g. Determination of protein concentration by BCA protein concentration assay kit (Azure sky, China). The cell lysates were mixed with 5× loading buffer at a volume ratio of 4:1, Denaturation at 100°C for 8 min, and the total protein of 60 g was detected by Western blot. PKM2 and hnRNPA1 antibodies were purchased from Abcam (Cambridge, UK). The development of ECL color liquid and gel imaging system was carried out (BioRad, USA),

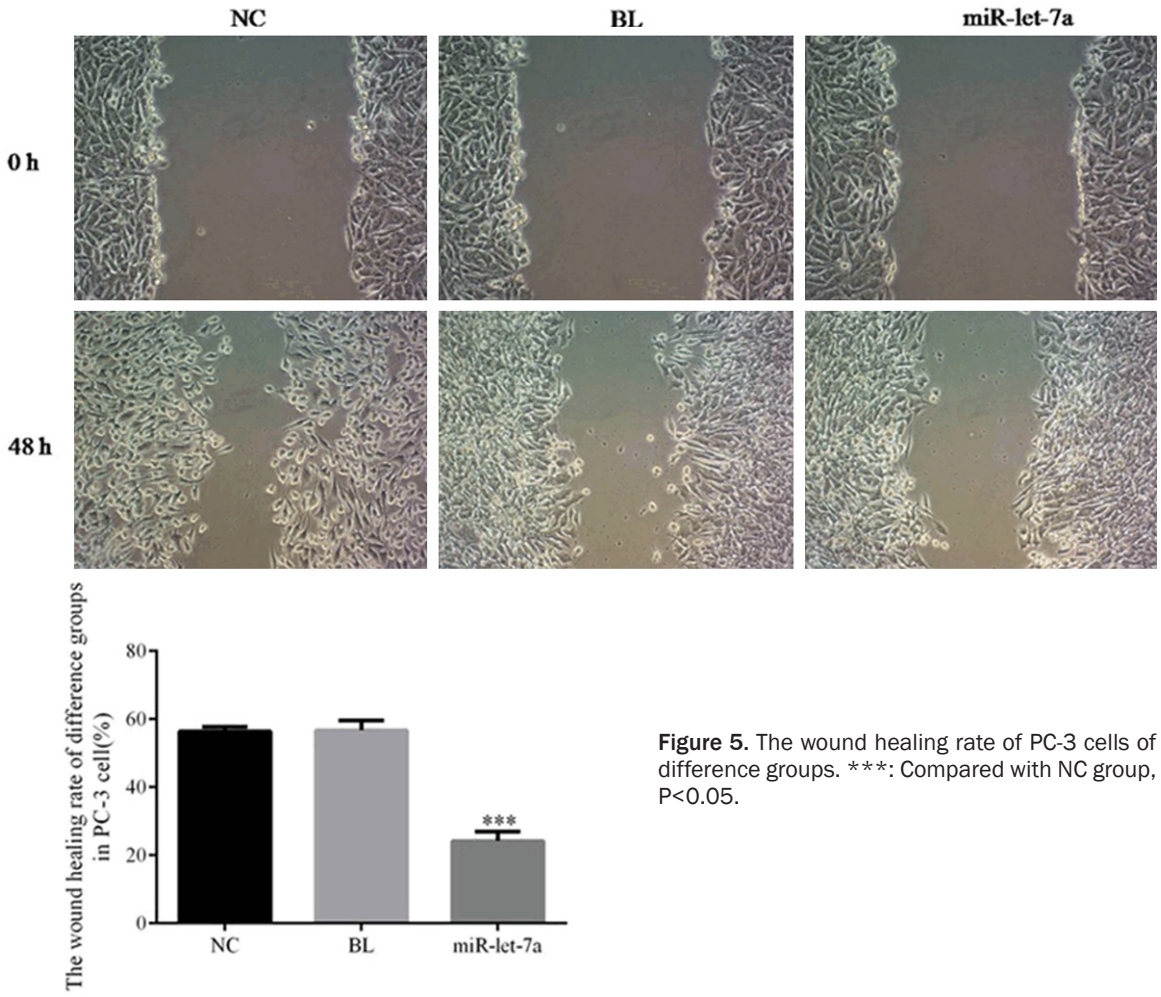

Figure 5. The wound healing rate of PC-3 cells of difference groups. ***: Compared with NC group, P<0.05.

and Quantity one software was used to quantify the gray level.

Statistical analysis

All experiments were repeated more than 3 times. The relative data were performed using SPSS 19.0 and express as mean ± standard deviation (SD). The relative data were analyzed using *One-way ANO*VA test and *t* test. *P≤0.05* was considered as statistically significant.

Results

The relative clinical data

To evaluate the PKM2 protein expression in adjacent normal and prostatic cancer tissues from 30 pairs of prostatic cancer patients by IHC assay. As shown in **Figure 1A**, The PKM2 protein expression of cancer tissues was stronger (+++) than that of adjacent tissues (-). Discuss the miR-let-7a and PKM2 gene expressions and their correlation. Measuring the miR-let-7a and PKM2 gene expression by RT-PCR and analyzed the correlation between them. The results were shown that miR-let-7a of cancer tissues were significantly down-regulation compared with adjacent tissues (*P<0.05,* **Figure 1B**), however, the PKM2 gene expression of cancer tiss-ues were significantly reduced (*P<0.05,***Figure 1C**). The miR-let-7a levels was negatively correlation with PKM2 mRNA levels in cancer tissues (r=-5.3105, **Figure 1D**).

miR-let-7a over-expression su-ppress the cell invasion ability in DU-145 and PC-3 ells by transwell assay

The invasion ability of DU-145 cell was significantly suppressed miR-let-7a group (miR-let-7a over-expression) compared with that

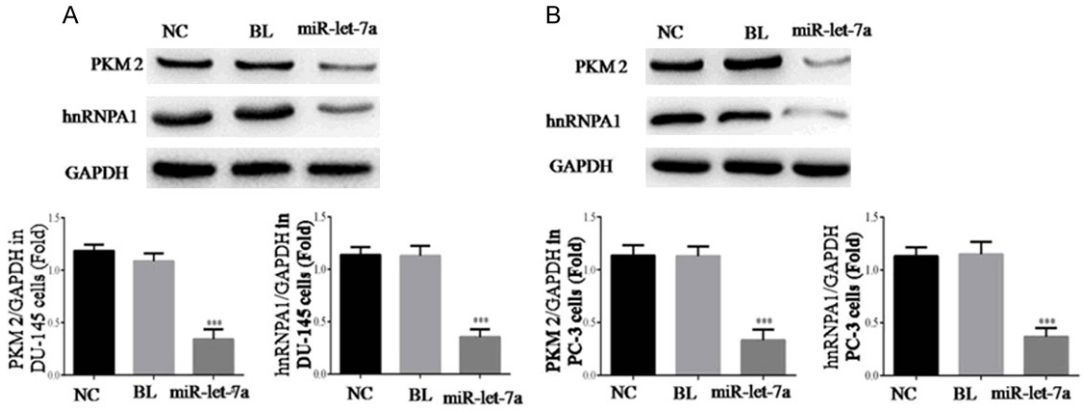

Figure 6. To evaluate relative protein expression by WB assay NC: Normal control group; BL: Transfection with empty vector; miR-let-7a group: Transfection with miR-let-7a. A. The PKM1 and hnRNPA1 protein expression of difference groups in DU-145 cells, P<0.05. B. The PKM1 and hnRNPA1 protein expression of difference groups in PC-3 cells, P<0.05.

in NC (untreated DU-145) or BL (DU-145 transfected with blank vector) (*P<0.05*, **Figure 2**). Meanwhile, The invasion ability of PC-3 cell was significantly suppressed miR-let-7a group (miR-let-7a over-expression) compared with that in NC (un-treated PC-3) or BL (PC-3 transfected with blank vector) (*P<0.05*, **Figure 3**).

miR-let-7a over-expression suppress the cell migration ability in DU-145 and PC-3 cells by wound healing assay

The migration ability of DU-145 cell was significantly inhibited miR-let-7a group (miR-let-7a over-expression) compared with that in NC (untreated DU-145) or BL (DU-145 transfected with blank vector) (*P<0.05*, **Figure 4**). Meanwhile, The migration ability of PC-3 cell was significantly inhibited miR-let-7a group (miR-let-7a over-expression) compared with that in NC (un-treated PC-3) or BL (PC-3 transfected with blank vector) (*P<0.05*, **Figure 5**).

Measuring the relative protein expression by WB assay

The PKM2 and hnRNPA1 proteins expression of miR-let-7a groups (miR-let-7a over-expression) were significantly decrease compared with NC (un-treated) and BL (cell transfected with blank) groups (*P<0.05, respectively*). The data were shown in **Figure 6**.

Discussion

miRNA is a kind of non coding short chain RNA, which has been found in recent years. It plays the role of oncogenes and tumor suppressor genes in the development of many tumors [8, 10]. According to the principle of complementary pairing of miRNA, the target is bound to mRNA, which can regulate the expression of genes by degrading the target mRNA or inhibiting the translation process [11]. In our study, we firstly found miR-let-7a gene expression was significantly down-regulation in prostatic cancer tissues compared with adjacent normal tissues. Secondly, we found that miR-let-7a overexpression had effects to suppress cell invasion and migration abilities in prostatic cancer cell lines DU-145 and PC-3 cells. Lastly, to understand the mechanims of miR-let-7a in prostatic cancer, we measured the relative protein expression in difference groups.

Pyruvate kinase (PK) is a key enzyme in cell metabolism, plays a key role in the glycolytic pathway, and is closely related to cell differentiation, proliferation and apoptosis. PKM2 is a kind of Isozyme of PK, which is mainly in the form of four mer in normal cells. However, in malignant tumor cells, PKM2 was present in the presence of two, and its expression was increased [12]. Recently, PKM2 was found to be highly expressed in peripheral blood and tumor tissues of patients with ovarian cancer,

colorectal cancer and lung cancer [13-15]. Yang et al [16] study found that silencing PKM2 expression could inhibit the proliferation and tumorigenesis of glioma cells. Kwon et al [17] found that PKM2 could promote the proliferation of colorectal cancer cells and regulate the expression of Bcl-xl gene. Heterogeneous-nuclear ribonucleoprotein A1 (hnRNP A1) is one of the hnRNP A/B subfamily members, is important in RNA binding protein, can freely shuttle between the nucleus and cytoplasm, through regulating pre-mRNA and mRNA transcription and post transcription regulation process, and can maintain the stability of certain genes, and through the combination of different and different gene activation the signal pathway, thus affecting cellular functions, play in the mature and biological function of many cell molecules, protein and tumor, plays an important role in the development process [18, 22]. Related studies confirmed that PKM2 could positively regulate hnRNP A1 [23, 24]. In our present study, PKM2 and hnRNP A1 proteins expression were decreased with miR-let-7a overexpression in prostatic cancer cell lines DU-145 and PC-3 cells.

In conclusion, miR-let-7a was a suppressive factor in prostatic cancer cell lines DU-145 and PC-3 cells' invasion and migration via regulation PKM2 and hnRNPA1 expressions.

Disclosure of conflict of interest

None.

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