Up-regulation of miR-124 inhibits invasion and proliferation of prostate cancer cells through mediating JAK-STAT3 signaling pathway

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Abstract. – OBJECTIVE: Signal transducer and activator of transcription 3 (STAT3) is an important protein in Janus kinase (JAK)-STAT signaling pathway, and can facilitate expression of Bcl-2 and Cyclin D1 gene, thus playing a role in tumor pathogenesis. Bioinformatics analysis revealed targeted binding sites between microRNA-124 (miR-124) and 3'-UTR of STAT3 mRNA. This study aims to investigate the role of miR-124 in regulating STAT3 expression and proliferation, cycle, apoptosis and invasion of prostate cancer cells.

MATERIALS AND METHODS: Dual luciferase reporter gene assay demonstrated targeted correlation between miR-124 and STAT3. Expression of miR-124, STAT3, p-STAT3, Bcl-2 and Cyclin D1 was compared between normal human prostate epithelial cell RWPE-1 and prostate cancer cell DU145. In vitro cultured DU145 cells were treated with miR-124 mimic and/or si-STAT3, to compare expression of STAT3, phosphorylated STAT3 (p-STAT3), B-cell lymphoma-2 (Bcl-2) and Cyclin D1. Flow cytometry detected cell apoptosis and cycle, followed by clonal formation and transwell assay to test malignant proliferation and cell invasion.

RESULTS: Targeted regulation existed between miR-124 and STAT3. Comparing to RWPE-1, DU145 cells had lower miR-124 expression, G0/G1 phase ratio, or cell apoptosis, plus higher expression of STAT3, p-STAT3, Bcl-2 and Cyclin D1, ratio of S or G2/M phase. Transfection of miR-124 mimic and/or si-STAT3 remarkably decreased gene expression, weakened clonal formation, cell invasion, ratio of S and G2/M phase, cell apoptosis and increased G0/G1 ratio.

CONCLUSIONS: miR-124 up-regulation significantly suppresses STAT3, pSTAT3 and downstream Bcl-2 and Cyclin D1 expression, weakens cell invasion or malignant proliferation potency, induces G0/G1 phase arrest, and facilitates cell apoptosis.

Key Words: miR-124, STAT3, Cell apoptosis, Cell cycle, Invasion, Proliferation, Prostate cancer.
of target gene mRNA to degrade mRNA or inhibit mRNA translation, modulating cell proliferation, differentiation and migration. The role of abnormal expression of function of mRNA in tumor onset has drawn increasing research focus. Studies showed significantly decreased miR-124 expression in PCa tissues/cells, suggesting its potential role as tumor suppressor gene in PCa occurrence. Bioinformatics analysis showed the existence of complementary binding sites between miR-124 and 3'-UTR of STAT3. This study investigated the role of miR-124 in regulating STAT3 and downstream target genes Bcl-2 and Cyclin D, and in affecting proliferation, cycle, apoptosis and invasion of PCa cells.

**Materials and Methods**

**Major Reagent and Materials**

Human prostate cancer cell line DU145 and normal prostate epithelial cell line RWPE-1 were purchased from Shengbo Biomed (Zhanjiang, China). Dulbecco’s Modified Eagle’s medium (DMEM), Keratinocyte-serum-free media (SFM) culture medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Gibco (Rockville, MD, USA). Trizol and Lipofectamine 2000 were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Hilden, Germany). miR-124 mimic and miR-NC nucleotide fragments were designed and synthesized by Ruibo Bio-Technology Co. Ltd. (Guangzhou, China). siRNA sequence and negative control sequence were synthesized by GE Pharmacon (Milpitas, CA, USA). Rabbit anti-Bcl-2 and Cyclin D1 were purchased from GeneTex Inc. (Irvine, CA, USA). Transwell chamber was purchased from Greiner Bio-One (Frickenhausen, Germany). Matrigel was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Dual luciferase activity assay kit was used to test dual luciferase activity. Nucleotide sequences were: miR-NC, 5’-ACUACUGAGU GACAGUAGA-3’; miR-124 mimic, 5’-GGCAUCACCUCGUGCUCUUAA-3’; miR-124 inhibitor, 5’-UAAGGCACGC GGUGAAUGCC-3’.

**Cell Transfection and Grouping**

In vitro cultured DU145 cells were divided into five groups: miR-NC transfection group, miR-124 mimic transfection group, si-NC transfection group, si-STAT3 group, and miR-124 mimic + si-STAT3 transfection group. 72 h after transfection, cells were collected for gene and protein expression assay. Nucleotide sequences for transfection were: si-STAT3 sense, 5’-CAUCUGCAUCUAGCUAGA-3’; si-STAT3 anti-sense: 5’-UAGCCGAUCUGAGUAGA-3’; si-NC sense: 5’-UUUCUGACAAACGUAGGC-3’; si-NC anti-sense, 5’-ACGUAGUACACGUGAGCAAGGA-3’.

**qRT-PCR for Gene Expression**

Trizol reagent kit was used to extract RNA following manual instruction. QuantiTest SYBR Green RT-PCR Kit was used to test gene expression by one-step qRT-PCR. In a 20 μL qRT-PCR system, there were 10 μL 2×QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL of forward and reverse primer (0.5 μm/L), 2 μg template RNA, 0.5 μL QuantiTest RT Mix, and ddH2O. Primer sequences used were: miR-124P: 5’-CGGTA AGGCACGGCCA CGCGTGTA-3’; miR-124P: 5’-AGTGCAAGACTGTGGCATG3’; U6P: 5’-ATTTGGAACGGATACAGAGAAGATT3’; U6P: 5’-GGAAC
GCTTC ACGAA TTTG-3'; STAT3PF: 5'-ATCAC GCCTT CTACA GACTG C-3'; STAT3PR: 5'-CA- TCC TGGAG ATTCT CTACC ACT-3'; Bel-2PF: 5'-GGTGG GGTC A TGTGT GTGGG-3'; Bel-2PR: 5'-CGGTT CAGGT ACTCA GTCAT CC-3'; CyclinD1PF: 5'-CAATG ACCCC GCACG ATT -TC-3'; CyclinD1PR: 5'-CATGG AGGGC GGATT GGAA-3'; β-actinP F: 5'-GAACC CTAAG GC -CAA C-3'; β-actinP R: 5'-TGTCA CGCAC GATTT CC-3'. PCR conditions were: 95°C pre-denature for 15 min, followed by 40 cycles each containing 94°C denature for 15 s, 60°C annealing for 30 s, and 72°C elongation for 30 s. Gene expression was examined on ABI ViiATM 7 fluorescent quantitative polymerase chain reaction (PCR) cycler.

**Western Blot**

Radioimmunoprecipitation assay (RIPA) lysis buffer was used to extract protein. A total of 40 μg samples was separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and was then transferred to polyvinylidene fluoride (PVDF) membrane, which was blocked in 5% defatted milk powder at room temperature incubation. Primary antibody (STAT3 at 1:300, p-STAT3 at 1:100, Bcl-2 at 1:200, Cyclin D1 at 1:200, β-actin at 1:800) was added for 4°C overnight incubation. After phosphate buffered saline tween-20 (PBST) rinsing, horse radish peroxidase (HRP) conjugated secondary antibody (1:5000 dilution) was added for 60 min of incubation. The membrane was rinsed in phosphate buffered saline (PBS) and incubated using Enhanced Chemiluminescence (ECL, Amersham Biosciences, Little Chalfont, UK) method. After dark exposure and development, the film was scanned and analyzed.

**Clonal Formation Assay for Malignant Growth Potency**

Cells from all transfection groups were inoculated into 10 cm diameter culture dish at 100 cells density. Cells were incubated for 14-21 weeks. After that, cells were fixed in paraformaldehyde for staining with Giemsa dye. Clones were counted under low-magnification microscope. Clonal formation rate = (clone number/inoculate cell number) ×100%.

**Transwell Assay for Cell Invasion Potency**

Transwell assay was employed to test cell invasion potency. In brief, 1×10⁵ cells were inoculated into the upper chamber containing Matrigel and serum-free Dulbecco’s Modified Eagle Medium (DMEM). DMEM containing 10% fetal bovine serum (FBS) was added to the bottom chamber. After 48 h, un-penetrated cells were removed. Chambers were then fixed in methanol and stained with 0.1% crystal violet. Cell number was counted under five high-magnification fields.

**Cell Apoptosis Assay**

Cells were collected and digested with trypsin, and were re-suspended in binding buffer. 5 μL Annexin V-fluorescein isothiocyanate (FITC) and 5 μL propidium iodide (PI) staining buffer were sequentially added. Flow cytometry was used to test cell apoptosis.

**PI Staining for Cell Cycle**

Cells were digested with trypsin and rinsed in phosphate buffered saline (PBS). After 70% ethanol fixation overnight and phosphate buffered saline (PBS) washing, PI was added for staining in the dark at 37°C for 30 min. Flow cytometry was used to detect cell cycle.

**Statistical Analysis**

SPSS18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Student t-test was used to compare measurement data between groups. Statistical significance was defined when \( p<0.05 \).

**Results**

**miR-124 Targeted and Inhibited STAT3 Expression**

Bioinformatics analysis showed the existence of complementary binding sites between miR-124 and 3’-UTR of STAT3 mRNA (Figure 1A). Dual luciferase gene reporter assay showed that transfection of miR-124 mimic and miR-124 inhibitor remarkably decreased or increased relative luciferase activity in HEK293T cells, respectively (Figure 1B), suggesting that miR-124 could target 3’-UTR of STAT3 mRNA and inhibited its expression. qRT-PCR results showed that transfection of miR-124 mimic and miR-124 inhibitor remarkably decreased and potentiated STAT3 mRNA expression in DU145 cells, respectively (Figure 1C).

**MiR-124 Down-Regulation and STAT3 up-Regulation in DU145 Cells**

qRT-PCR results showed that, compared to RWPE-1 cells, DU145 cells had significant-
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Decreased miR-124 expression, plus lower expressions of STAT3, Bcl-2, and Cyclin D1 mRNA (Figure 2A). Western blot results showed significantly higher protein expression of STAT3, p-STAT3, Bcl-2, and Cyclin D1 in DU145 cells (Figure 2B), weakened cell clonal formation ability (Figure 2B), decreased cell invasion potency (Figure 2C), and potentiated cell apoptosis (Figure 2D). Flow cytometry results showed significantly lower basal apoptotic rate of DU145 cells compared to RWPE-1 cells (Figure 2D).

MiR-124 up-Regulation Inhibited STAT3 Expression, Cell invasion or Proliferation, Induced cell Apoptosis and Cycle Arrest

Transfection of miR-124 mimic and/or si-STAT3 significantly decreased expression of STAT3, p-STAT3, Bcl-2, and Cyclin D1 in DU145 cells (Figure 3A), weakened cell clonal formation ability (Figure 3B), decreased cell invasion potency (Figure 3C), and potentiated cell apoptosis (Figure 3D) or G0/G1 phase arrest (Figure 3E).

Discussion

JAK-STAT signal transduction pathway can respond to multiple extracellular growth factors and mitogen stimuli. Under the existence of activator of JAK-STAT signal pathway, member receptor may undergo dimerization, which can further phosphorylate and activate JAK kinase to phosphorylate receptor tyrosine, facilitating the recruitment of STAT onto tyrosine phosphorylation site of receptor complex via SH2 domain. Under this scenario, JAK kinase can phosphorylate and activate STAT protein with spatial proximity, separating it from receptor complex to form dimer, which is transported from cytoplasm to nucleus, where it can facilitate transcription and expression of genes related to cell proliferation, cycle, and apoptotic regulation. STAT3 is the most important member of STAT protein family (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6). Previous studies attributed anti-apoptotic factor B-cell lymphoma-2 (Bcl-2) and Cyclin D1 as important target genes under regulation by STAT3 transcriptional factor. By enhancing gene transcription and expression, STAT3 participates in facilitating cell proliferation and cycle progression, and modulating/antagonizing cell apoptosis, making it one STAT protein with closest correlation with human tumor pathogenesis. A previous study showed elevated STAT3 expression in PCa patient tumor tissues, indicating its tumor-facilitating role in PCa. Reports also indicated significantly lower miR-124 expression in PCa tumor tissues/cells, indicating its possible role as tumor suppressor gene in PCa pathogenesis. Bioinformatics analysis showed complementary binding sites between miR-124 and STAT3. This work thus investigated if miR-124
played a role in mediating STAT3 expression and affecting proliferation, cycler, apoptosis and invasion of PCa cells. Dual luciferase gene reporter assay showed that transfection of miR-124 mimic significantly depressed relative luciferase activity, whilst miR-124 inhibitor elevated relative luciferase activity, indicating targeted regulatory correlation between miR-124 and STAT3. Transfection of miR-124 mimic and miR-124 inhibitor remarkably increased and decreased STAT3 mRNA expression in DU145 cells, respectively, further demonstrating targeted regulation between miR-124 and STAT3. G0/G1 phase ratio of DU145 cells was significantly lower than that of RWPE-1 cells, whilst ratios of S phase and G2/M phase were higher, indicating rapid cell cycle progression. Flow cytometry results showed that comparing to RWPE-1 cells, DU145 cells had significantly lowered apoptotic rate. Further assay found decreased miR-124 expression in DU145 cells, whilst expression of STAT3, p-STAT3, Bcl-2 and Cyclin D1 was elevated. These findings showed possible role of miR-124 down-regulation up-regulating STAT3 and p-STAT3, facilitating downstream gene Bcl-2 and Cyclin D1 expression, and facilitating proliferation of PCa cells for suppressing their apoptosis. Chu et al\(^1\) showed hyper-methylation of miR-124 gene promoter region in PCa cells, leading to suppression of miR-124 expression. Shi et al\(^{15}\) found that comparing to prostate epithelium RWPE-1, PCa cell lines including 22Rv1, LNCaP, LAPC-4, cdS2 and C4-2B, all had decreased miR-124 expression. Moreover, miR-124 expression level in PCa tissues was also lower than benign prostate tissue hyperplasia\(^\text{19}\). Falzarano et al\(^{19}\) observed significantly elevated miR-124 expression in PCa patients after treatment, indicating that miR-124 expression was one important mechanism governing PCa pathogenesis. In this study, miR-124 was down-regulated in prostate cells, indicating its role in PCa pathogenesis, as consistent with Chu et al\(^{18}\), Shi et al\(^{15}\), and Falzarano et al\(^{19}\). Abdulghani et al\(^{17}\) showed significantly elevated STAT3 expression in PCa tumor tissues, with higher expression level in those with bone or lung metastasis, indicating the correlation betwee-
STAT3 expression and both onset and distal metastasis potency of PCa. In this study STAT3 expression was significantly higher than normal human prostate epithelium, indicating the role of STAT3 up-regulation in PCa pathogenesis, as similar with Abdulghani et al\textsuperscript{17}. Further researches found that transfection of miR-124 mimic and/or si-STAT3 significantly down-regulated STAT3, p-STAT3 and downstream Bcl-2 and Cyclin D1 expression in DU145 cells, thus weakening cell invasion and malignant proliferation potency, inducing cell cycle arrest at G0/G1 phase, and facilitating cell apoptosis. Shi et al\textsuperscript{15} found that over-expression of miR-124 remarkably weakened proliferation potency of PCa cells. Chu et al\textsuperscript{18} showed that miR-124 up-regulation may suppress in vitro proliferation and invasion of PCa cells via targeted inhibition on androgen receptor expression, and weakening the tumor formation potency in recipient animals. Kang et al\textsuperscript{20} revealed that miR-124 could target and inhibit PACE4 expression to exert anti-cancer effects for antagonizing PCa cell proliferation. Qin et al\textsuperscript{21} showed that miR-124 up-regulation can inhibit PCa cell motility, migration and invasion via targeted inhibition on Slug gene expression to weaken the epithelial-mesenchymal transition (EMT) process. Shi et al\textsuperscript{22} found that over-expression of miR-124 may suppress PCa cell proliferation, and potentiate their drug sensitivity towards enzalutamide induction. Moreover, intravenous injection of miR-124 significantly inhibited in vivo growth of PCa tissues and facilitated tumor cell apoptosis\textsuperscript{23}. Abdulghani et al\textsuperscript{17} demonstrated that over-expression of STAT3 significantly enhanced in vitro DU145 cell motility and distal metastasis in vivo. After

Figure 3. Elevated miR-124 expression inhibited STAT3 expression, cell invasion or proliferation, and induced cell apoptosis or cycle arrest. (A) Western blot for protein expression; (B) Clonal formation assay; (C) Transwell assay for cell invasion potency; (D) Flow cytometry for cell apoptosis; (E) Flow cytometry for cell cycle. a, p<0.05 comparing between miR-124 and miR-NC group; b, p<0.05 comparing between si-STAT3 and si-NC group; c, p<0.05 comparing between miR-124 mimic + si-STAT3 and miR-NC group; d, p<0.05 comparing between miR-124 mimic + si-STAT3 and si-NC group.
activation of STAT3 by JAK kinase inhibitor, PCa cell had significantly weakened cell motility and migration potency. All these studies revealed the role of miR-124 up-regulation in weakening malignant biological features of PCa cells, as supported by our results. In this study, STAT3 down-regulation weakened proliferation or invasion of PCa cells, as consistent with Abdulghani et al\textsuperscript{17}. This work revealed the role of miR-124 down-regulation in inducing STAT3 up-expression and in facilitating PCa occurrence, whilst miR-124 up-regulation could weaken PCa proliferation, invasion or apoptosis resistance via targeted inhibition of STAT3 expression. All these results have not been reported before.

Conclusions

MiR-124 up-regulation significantly decreases STAT3, p-STAT3 and downstream Bcl-2 or Cyclin D1 expression in DU145 cells, whose invasion and malignant proliferation potency are weakened, along with induction of cell cycle arrest at G0/G1 phase to facilitate cell apoptosis.

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Conflict of interest

The authors declare no conflicts of interest.

References

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