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Original article

MicroRNA-4728 mediated regulation of MAPK oncogenic signaling in papillary thyroid carcinoma

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ABSTRACT

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer that accounts for 85% of thyroid cancers. MicroRNAs (miRNAs) have been reported to play important roles in the biological processes in cancer. In this study, we analyzed the biological role of miR-4728 in human PTC process in human PTC cell lines in vitro. MiRNA-4728 was observed to down-regulated in human PTC tissues and PTC cell lines. Additionally, miR-4728 inhibited PTC cell proliferation. Further study demonstrated SOS1 was repressed by miR-4728 and overexpression of miR-4728 down-regulated both the mRNA and protein levels of SOS1. Moreover, miR-4728 overexpression also decreased the MAPK signaling activity. These observations suggested that miR-4728 could inhibit the process of human PTC through regulating MAPK signaling pathway. And, appropriate regulation of miR-4728 might be vital to improve human PTC treatment.

1. Introduction

Aberrant gene regulation features significantly in Papillary thyroid carcinoma (PTC) like other cancers. MiRNAs were well known as small RNA molecules that regulate gene expression at the post-transcriptional level and drive cells towards transformation (Sotiropoulou et al., 2009). Recently, more than 1400 miRNAs have been verified and many of them are highly conserved in human (Griffiths-Jones et al., 2010). It has been showed that more than 60% of all mRNAs are predicted to be under miRNA control and contribute to many cancers (Wang et al., 2008; Bartel et al., 2009). Currently, aberrant miRNAs have also been reported to mediate gene expression in PTC (Graham et al., 2015; Ab Mutalib et al., 2016). Usually, these genes regulated by the abnormal expressed miRNAs were oncogenes or tumor suppressor genes (Hodge et al., 2011; Srivastava et al., 2013). So it might be a way to regulate tumor progress by appropriate miRNA regulation.

PTC is counted for 80% of the thyroid cancers and is one of the most quickly increasing cancers in cancer patients (Siegel et al., 2012). The surgical removal, radioactive iodine ablation, chemotherapy and radiotherapy are present clinical treatment of PTC. In order to find better and novel therapeutic strategies to treat PTC, it is becoming more and more important to understand the biological mechanism involved in PTC.

Although much is known about the profiling of miRNAs in many tumors, the mechanistic details of miRNA regulation are still not fully elucidated in PTC. It has been reported that overexpression of miR-375 suppressed PTC cell proliferation and induces cell apoptosis by targeting ERBB2 (Wang et al., 2016). In addition, miR-21 was shown to be over expressed in human PTC and promote the cell proliferation and invasion (Zhang et al., 2014). However, there was no report about the biological effects of miR-4728 on human PTC. In this study, we identified that miR-4728 was down-regulated in human PTC tumors and PTC cells. Additionally, MAPK signaling pathway was found to be repressed after miR-4728 overexpression through SOS1 by in vitro assays. Our studies investigated the precise role of miR-4728 in human PTC and may pave the way to a new therapeutic target for PTC.

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2. Material and methods

2.1. Cell culture

Human PTC cell TPC-1, K1 and normal thyroid cells Nthy-ori 3-1 cells were used for this study. The cells were maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) and 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich). The cells were cultured in a humidified incubator supplemented with 5% CO_2 at 37 °C.

2.2. MiR-4728 transfection

miR-4728 mimics (mimic-miR-4728) or miRNA control mimics (mimic-Ctl) were transfected PTC cells or Nthy-ori 3-1 cells as described previously (Schmitt et al., 2015). Briefly, human PTC cells were transfected with 10 nM mimic-miR-4728 (Invitrogen) or 10 nM mimi-Ctl (Invitrogen) after 24 h culture using lipofectamine 2000 (Invitrogen) in antibiotic-free Opti-MEM medium (Gibco). After 6 h transfection, DMEM complete medium replace the culture medium.

2.3. RNA isolation and quantitative real-time PCR

Total RNA of tissues or cells was isolated with TRIzol reagent (Invitrogen) according to the manufacturer. RNAs were reverse transcribed into cDNA with 300 units of M-MLV reverse transcriptase (BRL, Gaithersburg, MD) after removing contaminating DNA by DNA-free DNase (Ambion, Austin, TX). For gene expression, the quantitative real-time PCR (qRT - PCR) assays were performed with gene specific probes (Applied Biosystems, Foster City, CA). GAPDH was used as an internal control.

To quantify miRNA expression, the total RNA was reverse transcribed with a miRNA-specific looped RT primer (Applied Biosystems). MiR-4728 was tested with miRNA-specific Taqman minor groove binder probes (Applied Biosystems). U6 was used as an internal control. All Taqman qRT- PCR studies were performed in triplicate on an ABI 7500 system (Applied Biosystems) and the data were presented as mean ± SE.

2.4. Cell proliferation assay

CCK-8 assay was used to determine cell proliferation according to the description of the manufacturer. Briefly, cells with transfection of mimic-miR-4728 or mimic-Ctl were seeded into 96-well plates at a density of 5000 cells per well. Cells without transfection were considered as blank control (BC). 1.5 h before the designed time point, CCK8 reagent was added to cells at a ratio of 1:10. After then, these cells were incubated in a humidified incubator supplemented with 5% CO_2 at 37 °C for the indicated period. The absorbance at OD450 represented the proliferation of these cells. All the studies were performed in triplicate with independent cell preparations. Data were presented as mean ± SE.

2.5. Dual-luciferase assay

MAPK signaling pathway activity was detected by luciferase reporter assay as previous description (Zhang et al., 2015a, 2015b). Briefly, cells were seeded in 96-well plate at a density of 2×10^4 cells per well. MAPK pathway-luciferase-reporter construct (SABiosciences, Frederick, MD) was co-transfected with mimic-miR-4728 or mimic-Ctl into cells with lipofectatmine 2000. After culture 48 h, the cells were harvested for the Dual Luciferase Reporter Assay (Promega).

For SOS1 target gene verification, SOS1 luciferase assay was performed as previous description as well (Wang et al., 2016). Mutant SOS1 3′-UTR was used as a corresponding control. MiR-4728 inhibitor and mimic-Ctl were purchased from Invitrogen. The wild type (WT) or mutant SOS1 reporter vectors were co-transfected with mimic-miR-4728 or inhibitor into TPC-1 cells by lipofectamine 2000. The cells were harvested for luciferase assay after 48 h culture.

Results are represented by dividing firefly luciferase activity by Renilla luciferase activity. All the studies were performed in triplicate with independent cell preparations. Data were presented as mean ± SE.

2.6. Western blotting

Protein extract from PTC cells were separated in a 4–20% gradient SDS-polyacrylamide gel (Invitrogen) and electrophoretically transferred onto a PVDF membrane (GE). After block, membranes were incubated with primary antibodies (Ab) SOS1 and GAPDH (Abcam) overnight. Corresponding HRP-conjugated secondary Abs (Bio-Rad) were added the next day after washing with TBST for 3 times. The proteins were visualized by ECL chemiluminescence and exposed to X-ray film. All the studies were performed in triplicate.

2.6.1. Statistics

The significance of differences between groups was evaluated by student *t*-test with SPSS 13.0. A probability (P) value less than 0.05 was considered as statistical significance.

3. Results

3.1. MiR-4728 level in PTC tissues and cell lines

Previous studies reported that miR-4728 was abnormally expressed in many types of human cancers (Persson et al., 2011; Newie et al., 2014, 2015), and involved in many biological functions. In this study, we first determined miR-4728 expression in PTC and normal tissues. 18 pairs of PTC and non-cancerous normal tissues were used to determine miR-4728 expression by qRT- PCR assay. U6 snRNA was the internal control. Fig. 1A shows that miR-4726 level in human PTC tissues was significantly down-regulated compared to normal tissues.

To decipher the biological function of miR-4728 in PTC, we next determined miR-4728 level in human PTC cells (K1 and TPC-1) and normal thyroid cells Nthy-ori 3-1 by qRT- PCR. Fig. 1B indicates that miR-4728 level in TPC-1 and K1 cells were significantly decreased than that of Nthy-ori 3-1 cells. These results suggest that down-regulation of miR-4728 may involve in the development and progression of PTC.

3.2. Overexpression of miR-4728 suppresses human PTC cell proliferation

The effects of miR-4728 on the cell proliferation of human PTC cells were evaluated by transfecting TPC-1 and K1 cells with a miR-4728 mimic or mimic-Ctl. The cell proliferation was detected by CCK8 assay. Fig. 2 shows that miR-4728 overexpression remarkably suppressed the proliferation of TPC-1 and K1 cells than mock cells after D2. Our results indicated that miR-4728 overexpression may induce cell growth inhibition in TPC-1 and K1 cells.

3.3. SOS1 is repressed by miR-4728

To deciper the mechanism of miR-4728 in PTC, the bioinformatics tool, Targetscan, was used to predict the potential genes of