Abstract. Accumulating evidence suggests that some microRNAs (miRNAs) are involved in papillary thyroid carcinoma (PTC) progression. However, it remains necessary to elucidate the underlying molecular mechanisms involved. In the present study, we investigated the role of microRNA-101 (miR-101) in PTC via targeting of Ras-related C3 botulinum toxin substrate 1 (Rac1). The results showed that miR-101 was significantly downregulated in PTC tissues compared with adjacent normal tissues. Restoration of miR-101 expression significantly inhibited cell proliferation in the K1 PTC cell line. Moreover, algorithm-based and experimental strategies verified Rac1 as a direct target of miR-101 in the K1 cell line. Taken together, these findings suggest that miR-101 inhibited PTC growth via the downregulation of Rac1 expression, providing a better understanding of miRNA-modulated signaling networks for future cancer therapeutics.

Introduction

Papillary thyroid carcinoma (PTC) is the most common pathological type of thyroid malignant tumor, accounting for >80% of all thyroid cancers and typically occurs in young women or children (1). PTC usually presents as palpable thyroid mass or nodule and may be associated with hoarseness, dysphagia, stridor or pain (2). Although the majority of the PTCs have a good prognosis by surgical resection combined with radioiodine and levothyroxine treatment, metastasis and recurrence occur for certain PTCs (3). Certain clinical and pathological characteristics have been associated with a poor prognosis, such as advanced age at diagnosis, larger primary tumor (≥3 cm), extrathyroidal invasion, lymph node metastasis and advanced tumor-node-metastasis (TNM) stage (4,5). Therefore, a clear understanding of the pathogenesis of PTC is intrinsically valuable in the identification of novel diagnostic, prognostic and therapeutic targets.

MicroRNAs (miRNAs) are a class of non-protein-coding RNAs that regulate gene expression at the post-transcriptional and translational levels (6). Accumulating evidence suggests that miRNAs play essential roles in the regulation of tumor cell proliferation, differentiation, apoptosis and metastasis (7). It is well known that microRNA-101 (miR-101) is a tumor-suppressive miRNA that is downregulated in several cancer types. For example, miR-101 was significantly downregulated in gastric cancer in comparison with normal gastric mucosas (8). Enhanced miR-101 expression suppressed colony formation ability as well as tumor cell motility of colorectal cancer (9). miR-101 expression is also involved in other cancer types including breast, liver and prostate cancer (10). However, the expression and biological functional roles of miR-101 in PTC are largely unknown.

The role of miR-101 in PTC via targeting of Rac1 was therefore investigated. The results demonstrated that miR-101 was clearly downregulated in PTC tissue compared with that in adjacent normal thyroid tissue and restoration of miR-101 expression was able to reduce the proliferation of K1 PTC cells. Furthermore, we found that miR-101 is involved in PTC progression by directly targeting Rac1. To the best of our knowledge, this is the first study to support the hypothesis that miR-101 downregulation is involved in the development of PTC.

Materials and methods

Cell culture. The K1 PTC cell line was purchased from the European Collection of Animal Cell Cultures (ECACC,
The cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum.

**Patients and tissue specimens.** A total of 10 cases of PTC, which had been clinically and histologically diagnosed at the First Affiliated Hospital of Sun Yat-sen University (China), were obtained following patient consent and approval from the Institutional Research Ethics Committee of Sun Yat-sen University.

**Plasmids and constructs.** pCMV-miR-101 was purchased from OriGene (Rockville, MD, USA). pCMV-hygro-Rac1 was purchased from Sino Biological (Beijing, China). 3'-UTRs of Rac1 were amplified and then cloned into a modified pGL3 control vector where SacII and PstI sites were introduced into the original XbaI site downstream of the luciferase gene. To mutate the binding sites within the 3'-UTR, a QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) was used according to the manufacturer's instructions.

**Western blot analysis.** Cells were harvested, washed with 1X phosphate-buffered saline and lysed in mammalian protein extraction reagent RIPA (Beyotime, Jiangsu, China). A bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) was used for protein quantification. Proteins were separated in SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA) and immunoblotted with antibodies. The primary antibodies used were anti-Rac1 (Epitomics, Burlingame, CA, USA) and anti-α-tubulin (Sigma-Aldrich, St. Louis, MO, USA).

**RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The primers used for detection of mature miR-101 and U6 were designed by and purchased from Ribio Biotech Corporation (Guangzhou, China). The primers used for mRNAs were: Rac1, forward: 5'-CTGATGCAGGCTCATCA AGT-3' and reverse: 5'-TCTCCAGGAAATGTTTG-3'; GAPDH, forward: 5'-TGGTGGACCTCATGCTCTAC-3' and reverse: 5'-CAGCAACTGAGGCCCTCTCCT-3'.

**Statistical analysis.** Data were expressed as mean ± SD. Statistical significance was determined with t-test or analysis of variance by the SPSS 13.0 software. P<0.05 was considered statistically significant difference.

**Results**

**miR-101 is downregulated in PTC.** To detect a possible involvement of miR-101 in PTC, a quantitative RT-PCR assay was performed to examine the expression level of miR-101 in 5 pairs of PTC tissues (T) and their adjacent non-cancerous thyroid tissues (N). As shown in Fig. 1, miR-101 was significantly downregulated in PTC tissues as compared with that in the adjacent non-cancerous thyroid tissues.

**miR-101 inhibits K1 cells proliferation.** To investigate the possible function of miR-101 in PTC, we transfected miR-101 into the K1 PTC cell line and examined its effect on cell growth. Results of MTT assay showed that the restoration of miR-101 expression resulted in the inhibition of the growth of K1 cells.
as compared with the vector control (Fig. 2A). In addition, the colony formation assay exhibited that miR-101-overexpressing K1 cells generated a significantly lower number of colonies in comparison with vector control cells (Fig. 2B). The EdU incorporation assay revealed that the restored expression of miR-101 resulted in reduced proliferation of thyroid cancer cells (Fig. 2C). These data demonstrate miR-101 is involved in thyroid cancer cell proliferation.

miR-101 directly targets Rac1 expression in PTC. To explore the mechanism of miR-101-induced cell growth inhibition, we searched putative targets of miR-101 using TargetScan (http://www.targetscan.org/) and miRanda (www.microrna.org/miranda_new.html). The conserved target gene Rac1 was identified and used for subsequent investigation (Fig. 3A). The protein level of Rac1 was significantly downregulated in miR-101-overexpressing K1 cells (Fig. 3B), whereas no significant change in the mRNA levels of Rac1 was observed (data not shown). Luciferase reporter assays were performed to investigate the direct interaction between miR-101 and Rac1. As shown in Fig. 3C, ectopic expression of miR-101 significantly inhibited the luciferase activity of a reporter construct containing the 3'-UTR Rac1. Moreover, when a mutation was introduced into the predicted miR-101 site in 3'-UTR Rac1, the reporter failed to respond to miR-101, suggesting the silencing was associated with the predicted target site. Taken together, these data suggest that Rac1 is a direct target of miR-101.

Figure 2. MicroRNA-101 (miR-101) inhibits K1 cell proliferation. (A) MTT assays were performed to investigate the effect of miR-101 on the proliferation of thyroid cancer cells at the indicated time points. *P<0.05. (B) Colony formation assay of indicated cells. (C) Quantification of EdU incorporation assays.

Figure 3. Rac1 is directly targeted by microRNA-101 (miR-101). (A) Schematic miR-101 putative target site in 3'-UTR of Rac1. (B) Western blot analysis of the protein levels of Rac1 in response to miR-101 overexpression. α-tubulin was used as a loading control. (C) Relative luciferase activity in K1 cells transfected with wild-type (WT) or mutant (mut) Rac1 3'-UTR, as well as with miR-101 and a vector-control.

Figure 4. Rac1 is functionally related with the effect of microRNA-101 (miR-101). (A) Depletion of Rac1 and (B) inhibition of the proliferation of indicated cells. (C) Re-expression of Rac1 and (D) partially antagonized the miR-101-induced inhibition of cell proliferation.
Rac1 is functionally related with the effect of miR-101. In order to investigate the role of Rac1 in PTC cell proliferation, we knocked down the expression of Rac1 in K1 cells (Fig. 4A). It was shown that the suppression of miR-101 led to the inhibition of cell proliferation (Fig. 4B). Moreover, re-expression of Rac1 in miR-101-expressing K1 cells (Fig. 4C) partially antagonized the miR-101-induced inhibition of cell proliferation (Fig. 4D). These data suggest that Rac1 is a functionally relevant target of the effect of miR-101 on PTC cell proliferation.

Discussion

A vital biological feature of tumors is their potential for unrestrained growth. The activation of oncogenes and the aberrant expression of tumor suppressor genes are the main factors leading to tumors (15). miRNAs are small, single-stranded RNAs that regulate gene expression (16). In recent studies, miRNAs have been known to be involved in the pathogenesis of cancer and miRNA expression profiles are associated with prognosis in various types of cancer (17). miRNAs with increased expression levels in tumors may function as oncogenes and promote cancer development by negatively regulating tumor suppressor genes. By contrast, the miRNAs frequently downregulated in cancer may function as tumor suppressor and inhibit cancer development by down-regulating oncogenes (18). miR-101 is a well-known tumor suppressive miRNA that is downregulated in several cancer types. Nevertheless, the expression and biological function of miR-101 in PTC remains to be clarified.

Liu et al (19) have identified that 248 miRNAs were significantly deregulated (P<0.01) in PTC tissues when compared with their matching normal thyroid tissues. Subsequently, hsa-miR-101 was identified. Consistent with results of that study, we found that miR-101 was downregulated in PTC, suggesting that miR-101 is a tumor suppressor. Furthermore, we investigated the role of miR-101 in the growth of PTC cells by MTT assay, colony formation and EdU incorporation assay. As expected, the ectopic expression of miR-101 inhibited the growth of PTC cells. Our results therefore indicate that miR-101 is involved in the negative regulation of PTC cell proliferation. However, the mechanism underlying the effect of miR-101 on tumor growth remains to be elucidated.

Target genes of miR-101 were identified to examine the molecular mechanism underlying the miR-101-induced suppression of PTC cell growth. According to the in silico analyses, we predicted Rac1 as a direct target, which encodes a small G-protein that is an important member of the Rho family (20). Rac1 is overexpressed in various types of cancer including breast, colon and lung carcinomas (21-23) and participates in all stages of tumor formation (24). However, the effect of Rac1 on PTC remains preliminary and whether miR-101 affects the growth of PTC cells through Rac1 remains to be determined. We have clearly demonstrated that miR-101 induced the inhibition of PTC cell proliferation by directly targeting Rac1. Depletion of Rac1 mimics the anti-proliferative effect of miR-101, indicating that Rac1 has a functional contribution to PTC cell proliferation. The results also showed that suppression of Rac1 cannot fully recapitulate the miR-101 effect on PTC cell proliferation and together with the fact that many other target genes modulated by miR-101 are found in other types of cancer, additional studies should be conducted to investigate other target genes regulated by miR-101 that contribute to PTC tumorigenesis.

Accumulated evidence have identified miRNAs that aid in the diagnosis of disease and serve as potential targets for therapy in the future (25). Recent successful preclinical therapeutic trials in cancers include miR-380-5p replacement in neuroblastoma (26) and miR replacement/anti-miR combination therapy in hepatoblastoma (27). Notably, certain miR-based therapies have been applied in the clinic, including blockade of miR-122 for chronic viral hepatitis (28-29). Our findings provide insight into the therapeutic implications of miR-101 in PTC.

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