Downregulation of miR-146b-5p via iodine involvement repressed papillary thyroid carcinoma cell proliferation

Yujia Pan1,2,3,*, Weikang Yun4,*, Bingshuai Shi5, Rongjun Cui2, Chi Liu2, Zhong Ding6, Jialin Fan2, Wenqian Jiang2, Jiebing Tang7, Tianhu Zheng2, Xiaoguang Yu2 and Ying Liu1

1Key Lab of Etiology and Epidemiology, National Health and Family Planning Commission, Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University, Harbin, Heilongjiang, China
2Department of Biochemistry and Molecular Biology, Harbin Medical University, Harbin, Heilongjiang, China
3College of Medical Laboratory Science, Guilin Medical University, Guilin, Guangxi, China
4Department of Radiation Oncology, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang, China
5Department of Thyroid Surgery, First Affiliated Hospital of Henan University, Kaifeng, Henan, China
6Kaifeng Center for Disease Prevention and Control, Kaifeng, Henan, China
7Department of Gastrointestinal Medical Oncology, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang, China

Correspondence should be addressed to Y Liu or X Yu: ly-da@163.com or yxg301@163.com

*(Y Pan and W Yun contributed equally to this work)

Abstract

miR-146b-5p is overexpressed in papillary thyroid carcinoma (PTC) and is thought to be a related diagnostic marker. Previous studies have indicated the effects of iodine on oncogenic activation. However, the effect of iodine on the proliferation of PTC cells and the associated underlying mechanisms remain unclear. We found that miR-146b-5p was downregulated and smad4 was upregulated in patients exposed to high iodine concentration by in situ hybridisation (ISH) and immunohistochemical (IHC). NaI (10−3 M) treatment downregulated miR-146b-5p and upregulated Smad4 in PTC cell lines. Luciferase assay was used to confirm that Smad4 is a target of miR-146b-5p. Furthermore, MTT assay and cell cycle analysis indicated that 10−3 M NaI suppressed cell proliferation and caused G0/G1 phase arrest. Real-time PCR and Western blotting demonstrated that 10−3 M NaI increased p21, p27, and p57 levels and reduced cyclin D1 levels in PTC cells. Our findings suggest that 10−3 M NaI increases Smad4 levels through repression of miR-146b-5p expression, curbing the proliferation in PTC.

Keywords

- papillary thyroid cancer
- miR-146b-5p
- smad4
- iodine

Introduction

Thyroid cancer has been one of the most prevalent endocrine malignancies and one of the most rapidly increasing cancers in many countries over the last several decades, with 85–90% of those being PTC (Xing et al. 2013). It is known that 60% of PTCs are associated with alterations in RET, RAS, or BRAF genes (Kimura et al. 2003, Kondo et al. 2006). In particular, RET/PTC rearrangements and the BRAF V600E mutation are the two most common genetic alterations (Rossi et al. 2015). In addition to the large number of studies concerning PTC neoplasia, the molecular events involved in its progress have received attention, especially the relationship between PTC and miRNA (Fiore et al. 2009).

miRNAs are a class of small noncoding RNAs, which consist of 18–22 nucleotides. They can regulate the
Iodine is an important mineral for health and is required to produce the key thyroid hormones thyroxine (T4) and triiodothyronine (T3). These hormones are essential for cellular metabolism, growth, and physical development (McNulty et al. 2017). Iodine can only be obtained from external sources, and is mostly acquired from food, but can also be present in water (Morreale de Escobar & Escobar del Rey 2006). Thyroid follicular cells can concentrate iodide for the production of thyroid hormones. Excessive serum iodine levels have an influence on thyroid gland auto-regulatory, curbing cell proliferation and thyroid function (Eng et al. 1999, Leoni et al. 2008). In addition, iodine excess may play a protective role in the activation of oncogenic RET/PTC3 in thyroid follicular cells, obstructing the phosphorylation of ERK and delaying thyroid differentiation markers loss (Flore et al. 2009). Recently, there are some epidemiological studies shown that the PTC incidence of radiation exposure in iodine-deficient areas is more marked than in areas where iodine is excessive, suggesting that iodine plays an important role in the process of PTC (Shakhtarin et al. 2003, Cardis et al. 2005). A previous study showed that iodine reduced miR-17-92 expression, thus regulating the proliferation of PTC (Fuziwara & Kimura 2014).

In this study, we found that 10^{-3} M NaI could inhibit the proliferation of PTC cell lines, and smad4 was predicted to be a direct target of miR-146b-5p. Moreover, 10^{-3} M NaI could downregulate the level of miR-146b-5p and upregulate the level of smad4. Therefore, our findings offer a new insight into the function of iodine in thyroid cancer, miR-146b-5p may be a potential therapeutic target for thyroid cancer treatment.

Materials and methods

Materials

Sodium iodide (NaI; 409286) was obtained from Sigma–Aldrich. DMEM medium (C11995500BT) and FBS (10099141) were obtained from Gibco® (Thermo Fisher, Life-Technologies). Lipofectamine® 3000 (L3000015), Opti-MEM® reduced serum media (31985070), and SYBR® Select Master Mix (4472908) were obtained from Invitrogen® (Thermo Fisher, Life-Technologies). PrimeScript® RT reagent kit (RR047A) with gDNA Eraser was obtained from Takara Bio. The following antibodies were used for immunohistochemical analyses: rabbit anti-Smad4 (bs-0585R), rabbit anti-NIS (sodium iodide symporter) (Beijing Bios Bio-Technology Co., Ltd., Beijing, China), and Max Vision™ HRP-polymer anti-rabbit IHC Kit (KIT-5004) (Fuzhou Maixin Biotech. Co., Ltd., Fuzhou, China). The following antibodies were used for Western blot analyses: rabbit anti-Smad4 (9515P), rabbit monoclonal anti-cyclin D1 (2978S), rabbit anti-p21 (2947), rabbit anti-p57 (2557) (Cell Signaling Technology), rabbit anti-p27 (sc-1641) (Santa Cruz Biotechnology), and mouse anti-α-tubulin (66031) (Proteintech Group, Rosemont, IL, USA). HRP-conjugated goat anti-rabbit (31460) and goat anti-mouse (31430) secondary antibodies were obtained from Invitrogen® (Thermo Fisher).

Patients and ethical approval

All PTC tissues from patients exposed to adequate iodine levels and high iodine were obtained from the Department of Thyroid Surgery of the First Affiliated Hospital of Henan University. All human tissues were acquired in accordance with the protocol approved by the Ethics Committee of Harbin Medical University (No: CDCDCIRB20120001), and written informed consent was obtained from each participant prior to the study.

HE staining and immunohistochemical analysis

Whole tissue samples were paraffin embedded and cut into 4-μm thick tissue sections, baked at 65°C for 12 h, and then cooled at 4°C. One section was used for HE staining and another was used for immunohistochemistry. For HE staining, the section was de-waxed and stained with hematoxylin and eosin. The section was then observed under a light microscope. For immunohistochemistry, sections were de-waxed, washed with PBS, and repaired in sodium citrate (pH=6.0). Subsequently, sections were incubated in 0.3% hydrogen peroxide at room temperature for 15 min, and then blocked with 10% fetal calf serum for 30 min and treated with primary antibody diluted 1:100 at 4°C overnight. Sections were further incubated with secondary antibody at 37°C for 30 min and developed

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using DAB. After washing three times with PBS, the sections were stained using hematoxylin for 3 min and sealed for imaging. The stained slides were viewed and photographed using an Olympus BX60 microscope (Olympus Corp.), and all images acquired by using the same conditions. Positively area density values were quantified using Motic Med 6.0 software (Xiamen, China).

**In situ hybridisation**

The expression of miR-146b-5p in PTC tissues was detected by digoxin in situ hybridisation (ISH). The digoxin-labelled miR-146b-5p probe sequence was ACTCCTGACTTAAGGTAT, and the probe was purchased from BioSense (Guangzhou, China). The digoxin ISH procedure was performed in accordance with the BioSense instructions. The staining scores were determined by microscopy on the basis of both the intensity and proportion of mir-146b-5p-positive cells in five random fields under a 40× objective. The proportion of positively stained tumor cells was graded according to the following: 0, no positive cells; 1, <10% positive cells; 2, 10–50% positive cells; and 3, >50% positive cells. The staining intensity of the cells was graded according to the following: 0 (no staining), 1 (light blue), 2 (blue) and 3 (dark blue).

**Plasmid construction**

The 3′UTR of the Smad4 gene was amplified by PCR, and the product was cloned into the psi-CHECK-2 vector (Promega) using XhoI and NotI sites. The primer sequences were as follows: Smad4 3′UTR-F, 5′-TAGGCGATCGCCTGGGTCTTTTACCGTTGGGG-3′ and Smad4 3′UTR-R, 5′-TTGCAGGGACTGGGCCTCACATCAATCAAGTACAAAATATC-3′. The mutation psi-CHECK-2-Smad4 3′UTR plasmid was created using the QuikChange® Site-Directed Mutagenesis kit (Stratagene, Agilent Technologies). All constructs were generated using the In-Fusion® Clone Kit (Clontech).

**Cell culture**

Two papillary thyroid cancer cell lines, TPC-1 cells were obtained from Rebecca Schwerppe's Lab (University of Colorado Cancer Center, Aurora, CO, USA). BCPAP cells were kindly provided by Sareh Parangi (Department of Surgery, Massachusetts General Hospital, Boston, MA, USA) and William Cance (Department of Surgery, University of Buffalo, Buffalo, NY, USA). The human kidney cells HEK-293T cells were obtained from the American Type Culture Collection (ATCC). All cells were incubated in DMEM medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin (Gibco, Thermo Fisher Scientific), and cells were grown at 37°C in a humidified atmosphere (5% CO₂/95% air).

**Cell treatments**

**High iodine treatment**

High concentration iodine treatment was performed by diluting a 1 M stock solution of sodium iodide (NaI) in medium to a final concentration of 10⁻³ M. PTC cells were treated with 10⁻³ M, 10⁻⁵ M, and 10⁻⁷ M NaI for 24, 48, 72, and 96 h. The control group included cells without any treatment.

**Cell transfection**

Transfection was performed with Lipofectamine® 2000 according to the manufacturer's instructions. Cells were transiently transfected with miR-146b-5p mimics, inhibitors, or a negative control (RiboBio Co., Ltd, Guangzhou, China) at a final concentration of 10⁻³ M.

**RNA extraction and RT-PCR**

Total RNA was extracted from cells using TRIzol® reagent (Ambion®, Life-Technologies), and reverse transcribed using the PrimeScript® RT reagent Kit with gDNA Eraser according to the manufacturer's instructions (TaKaRa Bio). To evaluate gene expression, cDNA was amplified with the SYBR® Select Master Mix using the ABI Prism 7500 System. A total of 2 µg RNA was used for RT. The mixture was incubated for 10 min at 25°C, then 37°C for 120 min, at last 5 min at 85°C. The protocol of qPCR as follows: denaturation at 95°C for 10 min, then 40 amplification cycles of 95°C for 5 s and 60°C for 60 s. Specific primers for miRs and U6 were obtained from Ribo (RiboBio Co., Ltd.). Other primer sequences are described in Table 1. Individual amplification curves with a threshold cycle (CT) were verified by visual examination to ensure that there was an exponential phase; test wells with CTs of >40 were disregarded (Plain et al. 2014).

**Western blot analysis**

Whole-cell lysate preparation and Western blot analysis were performed as previously described (Li et al. 2016);
however, equal amounts of protein (40 μg/lane) were loaded. The following antibodies were used: anti-Smad4 (diluted 1:1000), anti-cyclin D1 (diluted 1:1000), anti-p21 (diluted 1:1000), anti-p27 (diluted 1:1000), anti-p57 (diluted 1:1000), and anti-α-tubulin (diluted 1:2000); goat anti-rabbit and goat anti-mouse secondary antibodies were also used (diluted 1:5000). Protein bands were identified and analysed using Quantity One software (Bio-Rad).

**Luciferase reporter assays**

Luciferase reporter assays were performed as previously described (Qin et al. 2014). Briefly, HEK293T cells were co-transfected with plasmids and miR-146b-5p mimics or miR-146b-5p inhibitors. Cells were harvested 24 h after transfection, and firefly and Renilla luciferase activities were assessed using a Dual Luciferase Reporter Gene Assay Kit (RG027; Beyotime, Nantong, China).

**Cell viability assays**

**MTT assay**

MTT assays were utilised as the qualitative index of cell viability. Cultured cells were subjected to different treatments and treated cells were seeded in 96-well plates at a density of 9 × 10^3 per well. 20 μl of MTT (C0009) (5 mg/mL; Beyotime) solution was added to each well for 4 h. Subsequently, cells were dissolved in dimethyl sulfoxide (150 μL/well; Sigma) for 15 min. When the formazan crystals were completely dissolved, the optical absorbance at 490 nm was measured using a microplate reader.

**Cell counting**

Cells (4 × 10^4 cells/well) were collected as described previously. Then performed according to the protocol of Trypan Blue Staining Cell Viability Assay Kit (C0011) (Beyotime).

**Results**

**Expression of miR-146b-5p and Smad4 based on adequate- and high-iodine regions**

miR-146b-5p and Smad4 play important roles in PTC progression. To investigate the relationship between miR-146b-5p, Smad4 and iodine, papillary thyroid cancer tissues were collected from 20 patients at the Department of Thyroid Surgery of the First Affiliated Hospital of Henan University. Half of the patients were from areas with adequate iodine in Henan province, whereas the others lived in areas with high water iodine levels, also in Henan. As shown in Table 2, iodine levels were significantly associated with tumor size and TNM stage. Histologic examination revealed characteristics of thyroid follicular cells (FC) and PTC (Supplementary Fig. 1A, see section on supplementary materials given at the end of this article). ISH staining and RT-PCR confirmed remarkably lower miR-146b-5p

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**Table 1** Primer sequences used for RT-PCR analysis.

<table>
<thead>
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<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product length (bp)</th>
</tr>
</thead>
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<tr>
<td>p21</td>
<td>TGTCCGTCGCAAACCACATGC</td>
<td>AAGTCGAGTTCATCGCTC</td>
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<tr>
<td>p27</td>
<td>AACGTGCGAGTGTCTAACGG</td>
<td>CCCTCTAGGGGTGGTACGTTT</td>
<td>209</td>
</tr>
<tr>
<td>p57</td>
<td>CAGATGGAGGCGCTCTCAAGT</td>
<td>CCTGCTGAAATGCTTAATCC</td>
<td>173</td>
</tr>
<tr>
<td>TSHR</td>
<td>ATCCAGGAGGAGACTTTCAGA</td>
<td>TTTGAGGCTACAGGGCTCTA</td>
<td>274</td>
</tr>
<tr>
<td>NIS</td>
<td>CCATCGCTATGCCTCAAGT</td>
<td>CGTGCTCAATGTCATGCTCA</td>
<td>185</td>
</tr>
<tr>
<td>TG</td>
<td>AGGGAGGTATTAGCTGTTCC</td>
<td>CAATACCCGATACCTCAGGAA</td>
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</tr>
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<td>TPO</td>
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<tr>
<td>GAPDH</td>
<td>GAGTCAGCAGGTAGTGGTGTG</td>
<td>GACAAGCTTCCGGTTTCAG</td>
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expression levels in samples from high-iodine areas than in those from adequate-iodine areas (Fig. 1A and Supplementary Fig. 1B), and there was a significant association between miR-146b-5p expression and iodine levels (Supplementary Table 1). Based on immunohistochemistry straining of PTC specimens, higher expression of Smad4 and NIS (sodium iodide symporter, a type of thyroid-specific differentiation gene) was detected in samples from high iodine areas compared to that in samples from adequate iodine areas (Fig. 1B and Supplementary Fig. 1C).

**Effect of iodine on the expression of miR-146b-5p and Smad4**

In this study, we analysed the effect of iodine on miR-146b-5p and Smad4 in PTC cells. TPC-1 (harbouring a RET/PTC rearrangement) (Ishizaka et al. 1990) and BCPAP (with a BRAFV600E mutation) (Fabien et al. 1994) cells were treated with different concentrations of NaI (10⁻³, 10⁻⁵, 10⁻⁷ M) for 24 h and 48 h (Fig. 2A). The expression of miR-146b-5p decreased compared to that in the control group. Decreased expression was detected in the 10⁻³ M NaI group. Subsequently, PTC cells were treated with 10⁻³ M NaI, and Smad4 expression was determined by Western blotting analysis. At 48 h, cells treated with 10⁻³ M NaI showed increased Smad4 levels compared to those in the control group, whereas no effect was detected after 72 h of treatment (Fig. 2B).

10⁻³ M NaI promotes Smad4 expression via miR-146b-5p repression in PTC cells

To confirm that the expression of Smad4 is regulated by miR-146b-5p, wild-type and mutated miR-146b-5p binding sites on the Smad4 3' UTR were cloned into a luciferase reporter plasmid, creating psi-CHECK-2-Smad4-3' UTR-wt and psi-CHECK-2-Smad4-3' UTR-mut. The effects of these plasmids on Smad4 expression were then measured in PTC cells treated with 10⁻³ M NaI. The results showed that Smad4 expression was significantly upregulated in cells transfected with the wild-type plasmid, whereas no effect was detected in cells transfected with the mutated plasmid (Fig. 3A). These findings suggest that miR-146b-5p can regulate Smad4 expression through its binding sites in the Smad4 3' UTR.

**Figure 1**

miR-146b-5p is downregulated and Smad4 is upregulated in high-iodine regions. (A) Expression of miR-146b-5p in adequate-iodine and high-iodine areas (in situ hybridization, 40×); (B) Expression of Smad4 in adequate-iodine and high-iodine areas (immunohistochemistry, 20×).

**Table 2** Correlation between iodine areas and clinicopathological characteristics of papillary thyroid carcinoma patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
<th>Iodine areas</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>Adequate</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>Male</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>11</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>&lt;45</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥45</td>
<td>7</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td>≤1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;1</td>
<td>9</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td>I/II</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III/IV</td>
<td>11</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td>Negative</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>6</td>
</tr>
<tr>
<td>Multifocality</td>
<td></td>
<td>Yes</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>13</td>
</tr>
<tr>
<td>BRAF mutation</td>
<td></td>
<td>Yes</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>12</td>
</tr>
</tbody>
</table>
UTR-mut, respectively. Co-transfection of psi-CHECK-2-Smad4-wt with miR-146b-5p mimics markedly reduced luciferase activity, and transfection of anti-miR-146b-5p could abolished this effect. However, miR-146b-5p mimics did not affect the luciferase activity upon mutation of the miR-146b-5p binding site (Fig. 3A). In addition, we detected the luciferase activity was dramatically increased in $10^{-3}$ M NaI-treated cells, but this effect was reversed by transfection of miR-146b-5p mimic (Supplementary Fig. 2). To investigate the role of miR-146b-5p, $10^{-3}$ M NaI-induced Smad4 was detected by Western blotting in PTC cells, whereas overexpression of miR-146b-5p abolished this effect (Fig. 3B); meanwhile, inhibition of miR-146b-5p enhanced this effect (Fig. 3C). These data suggested that $10^{-3}$ M NaI might activate Smad4 expression by regulating miR-146b-5p.

10$^{-3}$ M NaI reduces viability and proliferation in PTC cells

To determine the iodine concentration with the greatest effect on the proliferation of PTC cells, TPC-1 and BCPAP cells were treated with different concentrations of NaI (as above) at different time points. MTT assays showed that cell proliferation was lower in the NaI group compared to that in the control group. A marked reduction was detected in the $10^{-3}$ M NaI group (Supplementary Fig. 3A). A similar result was observed with cell counting assays (Supplementary Fig. 3B).

To verify that high iodine contributes to the cell cycle distribution in PTC cells, DNA content was measured (Burgess et al. 2000) by flow cytometry. We observed that treatment of PTC cells with $10^{-3}$ M NaI enhanced the proportion of cells in the G0/G1 phase compared to that in control cells (Fig. 4A). Subsequently, mRNA levels of cyclin-dependent kinase inhibitors ($p21$, $p27$, $p57$) were examined by RT-PCR. All genes were upregulated after high-iodine treatment compared to the levels in the control group in PTC cells (Fig. 4B). We then examined levels of the cell cycle regulators cyclin D1, $p21$, $p27$ and $p57$ by Western blotting. Compared to the levels in the control group, cells treated with $10^{-3}$ M NaI exhibited significant downregulation of cyclin D1 and upregulation of $p21$, $p27$, and $p57$ (Fig. 4C and Supplementary Fig. 4).
Moreover, we detected the level of *cyclin D1* was decreased, and the levels of *p21*, *p27*, and *p57* were increased in high iodine area tissues (Supplementary Fig. 5).

Thyroid follicular cells are characterised by the expression of thyroid-specific differentiation genes including *NIS* (sodium iodide symporter), *TG* (thyroglobulin), *TPO* (thyroid peroxidase), and *TSHR* (thyroid-stimulating hormone receptor). We tested whether 10^{-3} M NaI could affect the expression of these genes in PTC cells. *TG* expression was increased in TPC-1 cells (Supplementary Fig. 6A and C), whereas *TG* and *TSHR* expression increased in BCPAP cells after treatment with 10^{-3} M NaI (Supplementary Fig. 6B and D).

**Discussion**

This study shows that treatment with high concentrations of iodine can adjust the proliferation of PTC cells and describes the underlying mechanisms responsible for this; specifically, high iodine might modulate levels of miR-146b-5p and downstream targets such as Smad4.

Iodine is an important mineral for health (McNulty et al. 2017), but its role in PTC is controversial. Previous studies have shown that high iodine promotes carcinogenesis. However, during thyroid cancer activation, excess iodine has an anti-oncogenic role in thyroid follicular cells. In addition, it has been reported that the incidence of PTC in Tasmania recently demonstrated a paradoxical fourfold rise, despite a contemporaneous decrease in iodine intake (Burgess et al. 2000). The current study showed that 10^{-3} M NaI represses proliferation and regulates expression of cell-cycle-related genes in TPC-1 and BCPAP cells, but the molecular mechanism is unclear.

Recent studies have reported that miRNA is involved in the development of PTC. Han et al. (2017) reported that miR-148a suppresses proliferation and invasiveness of PTC by directly targeting CDK8. miR-144 was found to target E2F8, leading to PTC progression (Sun et al. 2017). Geraldo et al. discovered that miR-146b-5p is overexpressed in PTC and promotes PTC proliferation via smad4, the regulation of iodine to miR-146b-5p in PTC is still mysterious (Geraldo et al. 2012). Previous studies indicated that ionizing radiation (Iglesias et al. 2017) and diet (Vini & Harmer 2002) were associated with PTC. In our study, we found that miR-146b-5p was upregulated in patients exposed to high iodine compared to those from areas with adequate iodine. Additionally, 10^{-3} M NaI regulated miR-146b-5p, thyroid-associated gene expression, cycle-related-gene expression, and repressed PTC proliferation. Furthermore, MTT assays elucidated that the inhibitory impact of 10^{-3} M NaI on the proliferation of PTC cells could be abolished by miR-146b-5p overexpression (Supplementary Fig. 7). The results revealed that 10^{-3} M NaI represses PTC proliferation by inhibiting miR-146b-5p expression.

Recent studies have reported that miR-146b-5p regulates the proliferation of PTC cells (Geraldo et al. 2012, Czajka et al. 2016). In addition, NfκB (Pacifico & Leonardi 2010), MAPK (Shih et al. 2002), and TGF-β (Geraldo et al. 2012) signalling is related to the
proliferation of thyroid cancer cells. It has also been reported that the NF-κB downstream effector signal transducer and activator of transcription 3 (STAT3) increases miR-146b expression (Xiang et al. 2014), and TGFβ and PI3K signalling hyperactivated leading to higher levels of miR-146b (Riesco-Eizaguirre et al. 2015). Interestingly, the TGF-β pathway has contrasting influences in a multitude of cancers, serving as a tumor suppressor in epithelial-derived tumors and as a tumor promoter in mesenchyme-derived tumors and epithelial-derived tumors undergoing epithelial-to-mesenchymal transition (Massague 1998). In thyroid cancer cells, cell growth is notably inhibited by TGF-β signalling (Geraldo et al. 2012). Smad4, a predominant protein in canonical TGF-β signaling, was previously recognized as a target of miR-146b-5p in PTC and PCCl3 cells (D’Inzeo et al. 2010). However, whether Smad4 can be regulated by miR-146b-5p during conditions of high iodine, and consequently inhibit proliferation, was previously unknown. In this literature, we detected that Smad4 is markedly elevated in response to high iodine concentrations and that transfection of miR-146b-5p can inhibit Smad4 expression, and then we found that the high iodine inhibited PTC cell lines proliferation.
suggesting that Smad4 may be a downstream effector of miR-146b-5p during high iodine-mediated inhibition of proliferation. In fact, the mechanism in which high iodine inhibits thyroid cancer occurrence is complex, not only a single factor could explain. PTEN and IRAK1 were the downstream targets of miR146b-5p. Previous studies showed that miR-146b inhibits PTEN expression and hyperactivating the PI3K signalling pathway, promoting the proliferation and invasion of PTC (Ramírez-Moya et al. 2018). Chou et al. illuminated that miR-146b promotes aggressive tumor characteristics in PTC by suppressing the expression of IRAK1 (Chou et al. 2016). But its specific mechanistic contributions to PTC progression remain to be further characterized.

The relationship between cancer cell proliferation and cell cycle changes is interrelated. Therefore, we used flow cytometry to detect changes in cell cycle stages and compared these data to qRT-PCR and western blotting results. We observed that cells treated with 10⁻³ M iodine were induced to undergo G₁ cell cycle arrest and that a subset of CDK inhibitors including p21, p27, and p57 (at the mRNA and protein level) was upregulated in these cells, whereas cyclin D₁ was suppressed.

Thyroid follicular cells express specific genes (i.e., NIS and TSHR), they are involved in iodine metabolism and thyroid physiology. Thyroid follicular cells transport iodine through NIS, and it is a crucial thyroid-specific differentiation marker (Dohan & Carrasco 2003). In this finding, the expression of NIS in samples from patients in adequate iodine areas was lower than that in samples from patients in areas of excess iodine. Moreover, high iodine upregulated the mRNA and protein levels of thyroid-specific genes (TG and TSHR) in PTC cells. These results indicated that high iodine may restore TSHR and TG expression in PTC cells.

In conclusion, we indicated that 10⁻³ M NaI inhibits proliferation in PTC cells and that the inhibition of proliferation may be occurred via miR-146b-5p by targeting of Smad4. However, these results were obtained in tissues and cell lines, and thus, the effect of 10⁻³ M NaI in vivo has not been verified. Therefore, clinical testing is required to further verify the role of iodine in PTC.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JME-19-0198.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References
Eng PH, Cardona GR, Fang SL, Previti M, Alex S, Carrasco N, Chin WW & Braverman LE 1999 Escape from the acute Wolff-Chaikoff effect is associated with a decrease in thyroid sodium/iodide symporter messenger ribonucleic acid and protein. Endocrinology 140 3404–3410. (https://doi.org/10.1210/endo.140.8.6893)
Fiore AP, Fuziwara CS & Kimura ET 2009 High iodine concentration attenuates RET/PTC3 oncogene activation in thyroid follicular cells. Thyroid 19 1249–1256. (https://doi.org/10.1089/thy.2008.0408)
Fuziwara CS & Kimura ET 2014 High iodine blocks a Notch/miR-19 loop activated by the BRAF(V600E) oncoprotein and restores the response to TGbeta in thyroid follicular cells. Thyroid 24 453–462. (https://doi.org/10.1089/thy.2013.0398)
Han C, Zheng W, Ge M, Wang K, Xiang Y & Wang P 2017 Downregulation of cyclin-dependent kinase 8 by microRNA-148a...
Ishizaka Y, Ushijima T, Sugimura T & Nagao M 1990 cDNA cloning and characterization of ret activated in a human papillary thyroid carcinoma cell line. *Biochemical and Biophysical Research Communications* **168** 402–408. ([https://doi.org/10.1016/0006-291x(90)92335-w](https://doi.org/10.1016/0006-291x(90)92335-w))
Ramírez-Moya J, Wert-Lamas I & Santisteban P 2018 MicroRNA-146b promotes PI3K/AKT pathway hyperactivation and thyroid cancer progression by targeting PTEN. *Oncogene* **37** 3369–3383. ([https://doi.org/10.1038/s41388-017-0088-9](https://doi.org/10.1038/s41388-017-0088-9))
Shakhtarin VV, Tsyb AF, Degli Uberti E, et al. 2017 BRAF(V600E) and PTEN status are independent markers of malignancy and progression in thyroid cancer. *Oncogene* **36** 7534–7540. ([https://doi.org/10.1038/s41388-017-0088-9](https://doi.org/10.1038/s41388-017-0088-9))
Shih A, Davis FB, Lin HY & Davis PJ 2002 Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. *Journal of Clinical Endocrinology and Metabolism* **87** 221–232. ([https://doi.org/10.1210/jcem.87.3.8345](https://doi.org/10.1210/jcem.87.3.8345))

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