Roles of SHARP1 in thyroid cancer

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Abstract. SHARP1 is a basic helix-loop-helix transcription factor involved in various cellular processes, including proliferation and differentiation. The present study assessed the role of SHARP1 in the progression and invasion of thyroid cancer. PCR and western blot analysis demonstrated that in thyroid cancer tissues, SHARP1 was significantly downregulated at the mRNA and protein level compared with that in normal tissues. Furthermore, SHARP1 was downregulated in the TT and TPC-1 thyroid cancer cell lines compared with a normal thyroid cell line, while it was upregulated in other thyroid cancer cell lines. Overexpression of SHARP1 in TT and TPC-1 cells significantly inhibited the cell viability, migration and invasion *in vitro*. Furthermore, the protein and mRNA levels of HIF-1 α were found to be decreased in TT and TPC-1 cells following forced overexpression of SHARP1. In addition, silencing of HIF-1 α reduced the viability, migration and invasion of TT and TPC-1 cells. In conclusion, the present study indicated that SHARP1 acts as a tumor suppressor in thyroid cancer and that its downregulation may contribute to the proliferation, migration and invasion of thyroid cancer cells through mechanisms possibly involving HIF-1 α , suggesting that SHARP1 may be an important therapeutic target for the treatment of thyroid cancer.

Introduction

Thyroid cancer is the most common endocrine malignancy and its incidence is increasing (1). The molecular mechanisms underlying the development and progression of thyroid have remained to be fully elucidated. Genetic and epigenetic alterations in the major signaling pathways are central to these mechanisms and genes affected represent novel molecular markers and therapeutic targets, providing a basis for further research and clinical development of treatment strategies for thyroid cancer (2,3).

SHARP1, also known as basic helix-loop-helix family member e41 (BHLHE41), BHLHB3 or Dec2, is a basic helix-loop-helix transcription factor that has complex roles in cellular differentiation, apoptosis and tumor progression (4-6). It is expressed at high levels in the brain and skeletal muscle, at moderate levels in the pancreas and heart, and at low levels in the lung and placenta, while it is barely detectable in the liver and kidneys (7,8). SHARP1 has also been reported to be involved in mutant p53-mediated metastasis (9,10). Montagner *et al* (11) identified a significant association between HIF activity and SHARP1 expression in a cohort of triple-negative breast cancer (TNBC) patients. Furthermore, SHARP1 suppresses breast cancer metastasis and negatively regulates vascular endothelial growth factor (VEGF) expression (11,12). However, whether and how SHARP1 contributes to the development and progression of thyroid cancer has remained elusive.

Hypoxia in tissues is responsible for cell metabolism reprogramming, thus increasing cell proliferation, transformation and cancer progression (13). Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator and helps cells adapt to hypoxia. The transcriptional activity of HIF-1 is regulated by HIF-1 α , which activates multiple target genes involved in cancer biology, inducing cell proliferation, survival, apoptosis and angiogenesis (14-16). A number of studies showed that a reduction in HIF-1 α protein was a consequence of proteasome-dependent degradation by SHARP1 (17,18). This led to the hypothesis that SHARP1 acts as a global inhibitor of HIF-1 α activity. However, the influences of SHARP1 on HIF-1 α expression in thyroid cancer cells have not been assessed to date, to the best of our knowledge.

The present study revealed that SHARP1 was downregulated in certain thyroid cancer cell lines as well as in thyroid cancer tissues. Overexpression of SHARP1 inhibited the viability, migration and invasion of thyroid cancer cells, while reducing the protein levels of HIF-1 α in parallel. These results indicated that SHARP1 functions as a tumor suppressor in thyroid cancer, possibly via reduction of HIF-1 α levels, and may therefore represent a potential therapeutic target for the treatment of thyroid cancer.

Materials and methods

Cell culture. All culture media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G and

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100 mg/ml streptomycin (all from Thermo Fisher Scientific, Inc., Waltham, MA, USA). The thyroid cancer cell lines TT, TPC-1, FTC-133 and ARO as well as the Nthy-ori 3-1 normal thyroid cell line were obtained from the Cell Bank of Academia Sinica (Shanghai, China). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) in a humidified incubator containing 5% CO₂ in air at 37°C.

Patient samples. A total of 30 pairs of thyroid cancer tissues and matched normal tissues were obtained from patients (n=12 women and n=18 men; age, 14-91 years; median age, 53 years) who underwent surgery between October 2012 to February 2015, at Yangpu Hospital (Shanghai Tongji University School of Medicine, Shanghai, China). The normal tissues were resected within ≥ 5 cm of the tumor margin during surgery. No patients had received radiotherapy or chemotherapy. The study was approved by the Ethics Review Committee of the institutional review board of Yangpu Hospital (Shanghai Tongji University School of Medicine, Shanghai, China) and written informed consent was obtained from every patient. Tissues were centrifuged at 400 x g at 25°C for 20 sec for homogenization and immediately placed on ice, and then centrifuged at 400 x g at 4°C for 10 min and stored at 80°C prior to experiments.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from thyroid cancer cells with TRIzol reagent (Thermo Fisher Scientific, Inc.) as previously described (19) and stored at -80°C. Complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific, Inc.). The DyNamo Flash SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland) was used for PCR amplification according to the manufacturer's instructions to determine the mRNA levels of SHARP1 and HIF-1 α genes. The primer sequences (sense/antisense) used were as follows: SHARP1, 5'-GACCAACTGCTTCACACT TTC-3' and 5'-GCTGTTCGTTTCCTCTGTTTC-3'; HIF-1 α , 5'-TCGGCGAAGTAAAGAATC-3' and 5'-TTCCTCACA CGCAAATAG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-CACCCACTCCTCCACCTTTG-3' and 5'-CCACCACCCTGTTGCTGTAG-3' (all obtained from Sangon Biotech Co., Ltd., Shanghai, China). The PCR reaction mixture contained 12.5 μ l DyNamo Flash SYBR Green qPCR mix (Thermo Fisher Scientific, Inc.), 0.5 μ l forward/reverse primers, 9.5 μ l ddH₂O and 2 μ l cDNA. The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec, and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. Data collection was performed using an ABI 7500 (Thermo Fisher Scientific, Inc.) and relative quantification of gene expression was performed using the 2^{- $\Delta\Delta$ C_q} method (20). Relative quantification was performed by normalizing the signals of different genes to the GAPDH signal.

Western blot analysis. Total protein was isolated from thyroid cancer cell lines with radioimmunoprecipitation buffer (Wuhan Amyjet Scientific Co., Ltd., Wuhan, China), and the protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Total

protein (30 μ g) was separated using 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Wuhan Amyjet Scientific Co., Ltd.) and transferred to polyvinylidene fluoride membranes (Sigma-Aldrich, St. Louis, MO, USA), followed by blocking in non-fat milk overnight at 4°C. The membrane was first incubated with antibodies against SHARP1 (mouse polyclonal; 1:1,000; cat. no., ab57739; Abcam, Cambridge, MA, USA), HIF-1 α (mouse polyclonal; 1:1,000; cat. no., ab113642; Abcam) and GAPDH (rabbit monoclonal; 1:1,000; cat. no. #5174; Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at 25°C. The membranes were then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (cat. no. A0208; 1:1,000; Beyotime Institute of Biotechnology, Haimen, China) and goat anti mouse IgG (cat. no. A0216; 1:1,000; Beyotime Institute of Biotechnology) secondary antibodies for 1 h at 37°C, and washed three times with Tris-buffered saline with Tween-20 (Amresco, Solon, OH, USA). SHARP1 and HIF-1 α , and then with anti-GAPDH antibody as a loading control. The membranes were then probed with secondary antibodies labeled with horseradish peroxidase and signal intensity was determined using Image J software 1.46 (National Institutes of Health, Bethesda, MD, USA).

Antibodies of SHARP1 and HIF-1 α were purchased from Abcam (Cambridge, MA, USA) and GAPDH (Cell Signaling Technology, Inc., Danvers, MA, USA). All primary antibodies were used at a 1:1,000 dilution.

Overexpression vector construction and transfection. To construct the SHARP1 overexpression vector, a sequence was designed by Sangon Biotech Co., Ltd. and inserted into the pLenti6/V5-DEST vector (Thermo Fisher Scientific, Inc.). The overexpression vectors were transfected into TT and TPC-1 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. An empty vector was used as a negative control (NC) and the thyroid cancer cells were analyzed 48 h after transfection.

Transfection of HIF-1 α small interfering (si)RNA. siRNA specific to HIF-1 α (5'-TCGGCGAAGTAAAGAATC-3') was obtained from Genesil Biotechnology (Wuhan, China). The TT and TPC-1 cells were plated onto 96-well plates at a density of 2x10³ cells/well and were transfected with siRNA (40 nM) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A non-specific scramble siRNA sequence (Genesil Biotechnology) was used as a negative control. Cells were analyzed 48 h after transfection.

Cell viability assay. A Cell Counting Kit (CCK)-8 assay (Beyotime Institute of Biotechnology, Inc., Haimen, China) was used to determine the cell viability according to the manufacturer's instructions. Following transfection with siHIF-1 α for 48 h, TT and TPC-1 cells were plated onto 96-well plates at a density of 2x10³ cells/well. After incubation for 0, 12, 24, 48 or 72 h, 10 μ l CCK-8 reagent was added to each well, followed by incubation at 37°C for 1 h. The absorbance was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm and analyzed using Microplate manager 6 software (Bio-Rad Laboratories, Inc.).

Migration and invasion assays. The migratory and invasive capacity of the cells following transfection with SHARP1 overexpression vector or HIF-1 α silencing were examined using a Transwell culture system comprised of Transwell inserts (5 μ m; Corning, Corning, NY, USA), with membranes coated with or without Matrigel (2.5 mg/ml; BD Biosciences, Franklin Lakes, NJ, USA). Following transfection with siHIF-1 α for 48 h, suspensions of 10⁵ TT and TPC-1 cells/ml in DMEM with 1% FBS were prepared and cells were seeded into the upper wells of optionally pre-coated Transwells at 5x10⁴ cells per well. The lower wells of the Transwells contained DMEM with 10% FBS. After 48 h of incubation, cells that had transgressed through the membrane were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde and stained by 0.5% crystal violet. Images of the lower sides of the membranes were captured and cells counted under a microscope (CX41RF; Olympus Corporation, Tokyo, Japan).

Statistical analysis. Values are expressed as the mean $\pm$ standard deviation. The paired, two-tailed Student's t-test was used to analyze the significance of differences between groups. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Expression of SHARP1 in thyroid cancer cell lines and tissues. In order to elucidate the role of SHARP1 in thyroid cancer, its expression levels were initially examined in the thyroid cancer cell lines TT, TPC-1, FTC-133 and ARO, and compared with those in the Nthy-ori 3-1 normal thyroid cell line at the mRNA and protein level. The results indicated that SHARP1 expression in TT and TPC-1 cells was significantly decreased in comparison to that in Nthy-ori 3-1 cells ($P < 0.01$) (Fig. 1A and B). Therefore, subsequent experiments were performed on TT and TPC-1 cells. Furthermore, the expression of SHARP1 was significantly downregulated in thyroid cancer tissues compared to their matched normal tissues ($P < 0.01$) (Fig. 1C). These results suggested that SHARP1 may act as a tumor suppressor in thyroid cancer.

SHARP1 suppresses the viability of TT and TPC-1 cells. In order to explore the biological significance of SHARP1 in the genesis of thyroid cancer, TT and TPC-1 cells were stably transfected with SHARP1 overexpression vector. The efficacy of transfection was examined by RT-qPCR and western blotting (Fig. 2A and B). The mRNA expression of SHARP1 was increased by 3.75 and 2.35 fold in TT and TPC-1 cells, respectively. The protein expression of SHARP1 was increased by 1.34- and 1.14-fold in TT and TPC-1 cells, respectively ($P < 0.01$ vs. NC) (Fig. 2B). Next, the effects of SHARP1 on the proliferation/viability of TT and TPC-1 cells were assessed. At 72 h after transfection with SHARP1 overexpression vector, the number of viable TT and TPC-1 cells was reduced by ~43 and ~42%, respectively ($P < 0.01$ vs. NC) (Fig. 2C and D). These results suggested that SHARP1 may function as a tumor suppressor in TT and TPC-1 cells through inhibition of cell proliferation/viability.

SHARP1 suppresses the migration and invasion of TT and TPC-1 cells. The migratory capacities of TT and TPC-1 cells with stably overexpressing SHARP1 were examined using Transwell assays over 48 h. The number of migratory TT and TPC-1 cells with forced SHARP1 overexpression decreased by 61.4 and 53.6% compared with the NC groups, respectively ($P < 0.01$) (Fig. 3A). Furthermore, Transwell invasion assays with Matrigel-coated membranes showed that SHARP1 overexpression decreased the invasive capacity of TT and TPC-1 cells by 73.4 and 70.1% compared with the NC groups ($P < 0.01$) (Fig. 3B). These results suggested that SHARP1 may function as a tumor suppressor in TT and TPC-1 cells through inhibition of cell migration and invasion.

SHARP1 reduces HIF-1 α levels in TT and TPC-1 cells. To elucidate the mechanisms by which SHARP1 exerts its effects, the influence of SHARP1 expression on HIF-1 α levels was assessed. At the mRNA and protein level, HIF-1 α was significantly decreased in SHARP1-overexpressing TT and TPC-1 cells ($P < 0.01$ vs. NC) (Fig. 4A). The association between SHARP1 and HIF-1 α was further investigated by analyzing the relative protein levels of HIF-1 α in the 30 paired tumor and normal tissues from thyroid cancer patients, showing that HIF-1 α was significantly increased in tumor tissues compared with normal tissues ($P < 0.01$) (Fig. 4B). These results indicated that SHARP1 may directly or indirectly regulate the levels of HIF-1 α in thyroid cancer.

HIF-1 α knockdown reduces the viability, migration and invasion of TT and TPC-1 cells. As the above results and previous studies indicated that SHARP1 affects the expression of HIF-1 α in thyroid cancer cells (17,18), the present study examined the effects of HIF-1 α knockdown on the viability, migration and invasion of TT and TPC-1 cells. The knockdown efficiency of a lentiviral vector containing shRNA targeting HIF-1 α was confirmed by western blot analysis ($P < 0.01$ vs. NC) (Fig. 5A). HIF-1 α silencing significantly reduced the proliferation/viability and repressed the migratory and invasive capacities of TT and TPC-1 cells ($P < 0.01$ vs. NC) (Fig. 5B-D). These findings suggested that HIF-1 α , whose levels were shown to be affected by SHARP1, is important for the viability, migration and invasion of TT and TPC-1 cells.

Discussion

Although thyroid cancer is the most common endocrine tumor, the underlying molecular mechanisms have remained to be fully elucidated. SHARP1 has been previously reported to be a clock gene (21,22), a transcriptional repressor (23) and importantly, a tumor suppressor in lung cancer (24). The present study reported a potential role for SHARP1 as a tumor suppressor in thyroid cancer, which it may exert by decreasing the levels of HIF-1 α . SHARP1 was found to be decreased in tumor tissues compared with that in normal tissues; furthermore, SHARP1 was decreased at the mRNA and protein level in TT and TPC-1 cells compared with that in other thyroid cancer cell lines and the Nthy-ori 3-1 normal thyroid cell line. By contrast, a previous study showed that in human breast cancer cells SHARP1 expression was increased compared with that in normal human breast cells (25). The expression of

Figure 1. Expression of SHARP1 in thyroid cancer cell lines and tissues. (A) mRNA and (B) protein expression of SHARP1 in various thyroid cancer cell lines. (C) Expression of SHARP1 in 30 paired thyroid cancer tissues and their corresponding normal tissues. All samples were assessed in triplicate for each group and the experiment was repeated at least twice. Values are expressed as the mean $\pm$ standard deviation. ** $P < 0.01$ vs. Nthy-ori 3-1 cells or normal tissue. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 2. Effects of SHARP1 on cell viability. Vector-mediated overexpression of SHARP1 in TT and TPC-1 cells was confirmed (A) at the mRNA level by reverse-transcription quantitative polymerase chain reaction analysis and (B) at the protein level by western blot analysis. (C and D) SHARP1 overexpression significantly inhibited the proliferation of TT and TPC-1 cells as determined by a Cell Count Kit-8 assay. Values are expressed as the mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. NC. All samples were assessed in triplicate for each group and the experiment was repeated at least twice. OD, optical density; NC, cells transfected with empty vector (negative control); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SHARP1, cells transfected with SHARP1 transfection vector.

SHARP1 was diffuse in different tumors, suggesting regulation of the protein via a combination of the tumor genotype and the microenvironment (11). High expression of SHARP1 in primary breast cancer tumors has been found to be an indicator of favorable prognosis (26).

In the present study, stable vector-mediated overexpression of SHARP1 significantly inhibited the proliferation/viability of TT and TPC-1 cells, which was consistent with the results of previous studies on endometrial cancer cells (17) and disseminated tumor cells in a model of dormant head and

Figure 3. Effects of SHARP1 on cell migration and invasion. (A) A Transwell assay was performed to determine the migration of TT and TPC-1 cells. (B) A Transwell assay with Matrigel-coated membranes was performed to determine the invasion of TT and TPC-1 cells. Representative images of crystal violet-stained cells on the lower sides of the membranes are shown (magnification, x200). All samples were assessed in triplicate for each group and the experiment was repeated at least twice. Values are expressed as the mean $\pm$ standard deviation. ** $P < 0.01$ vs. NC. NC, cells transfected with empty vector (negative control); SHARP1, cells transfected with SHARP1 transfection vector.

neck squamous cell carcinoma (27). In addition, SHARP1 is known to suppress invasion, migration and metastasis by inhibiting HIFs. Loss of SHARP1 led to an increase in MII-cell (TNBC non-metastatic MCF10Atk1 cells) migration, which was attenuated by concomitant silencing of HIF-1 α (11). To gain insight into the mechanisms by which SHARP1 inhibits malignant progression, the present study further assessed the migration and invasion of TT and TPC-1 cells following SHARP1 overexpression. The results demonstrated that overexpression of SHARP1 resulted in a significant inhibition of migration and invasion of TT and TPC-1 cells. Due to its anti-proliferative and anti-metastatic roles in human thyroid cancer, SHARP1 may be a potential therapeutic target worth pursuing.

The results of the present study raise the question of whether and how SHARP1 affects the levels and expression of HIF-1 α . SHARP1 binds to the HIF-1 α sub-unit and directly shuttles it to the proteasome for degradation under normoxic

as well as hypoxic condition, followed by downregulation of HIF-1-responsive genes (28). In TNBC cell lines, overexpression of SHARP1 led to the suppression of the protein levels and transcriptional activity of HIF-1 α under hypoxia (29,30). However, in the present study, the mRNA and protein levels of HIF-1 α were significantly decreased in TT and TPC-1 cells with stable expression of SHARP1 under normoxic conditions, compared with those in the NC group. These results suggested that SHARP1 is a hypoxia-independent regulator of HIF-1 α levels. Indeed, low SHARP1 expression has been shown to contribute to high HIF activity and low metastatic capacity. In a previous study, overexpressed HIF-1 α and SHARP1 co-immunoprecipitated in Cos7 cells, and SHARP1 overexpression repressed HIF-1 α -dependent control of the VEGF-A promoter (31).

In the present study, HIF-1 α was identified as a target of SHARP1 and SHARP1 overexpression was shown to lead to the downregulation of HIF-1 α as well as inhibition of the

Figure 4. SHARP1 downregulates HIF-1 α expression. (A) mRNA (upper panel) and protein (lower panel) expression of HIF-1 α in TT and TPC-1 cells with stable overexpression of SHARP1. (B) Relative protein expression of HIF-1 α in 30 paired thyroid cancer tissues and normal tissues. Values are expressed as the mean $\pm$ standard deviation. All samples were assessed in triplicate for each group and the experiment was repeated at least twice. ** $P < 0.01$ vs. NC or normal tissue. NC, cells transfected with empty vector (negative control); SHARP1, cells transfected with SHARP1 transfection vector; HIF, hypoxia-inducible factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

proliferation/viability, migration and invasion of TT and TPC-1 cells. Furthermore, knockdown of HIF-1 α had similar effects on the proliferation/viability, migration and invasion of TT and TPC-1 cells. The findings of the present study may therefore indicate that the tumor suppressor role of SHARP1 may, at least in part, be mediated via the regulation of HIF-1 α expression. It is important to note that HIF-1 α is activated not only by hypoxia within the tumor but also by oncogenic stimulation (32). Knockdown of HIF-1 α has been shown to reduce the migration and invasion of glioma cells (33,34). This regulatory role of HIF-1 α on migration and invasion have also been found in colon (35), lung (36) and gastric cancer (37). These studies supported the notion that the inhibition of the proliferation/viability, migration and invasion of thyroid cancer cells may, at least in part, be due to downregulation of HIF-1 α expression, which was demonstrated to be an effect of SHARP1 overexpression.

In conclusion, the present study showed that SHARP1 was downregulated in certain thyroid cancer cell lines as well as in thyroid cancer tissues, and that its ectopic expression inhibited the proliferation/viability, migration and invasion of the TT and TPC-1 thyroid cell lines. HIF-1 α was found to be overexpressed in thyroid cancer tissues, and SHARP1 overexpression decreased the levels of HIF-1 α in TT and TPC-1 cells under normoxic conditions. Furthermore, knockdown of HIF-1 α inhibited the proliferation/viability, migration and invasion of TT and TPC-1 cells. These results indicated that SHARP1 may have important roles in the proliferation of thyroid cancer cells and the development of metastasis, possibly via decreasing HIF-1 α , and that SHARP1 may represent a potential therapeutic target for the treatment of thyroid cancer.

Figure 5. Silencing of HIF-1 α inhibits cell viability, migration and invasion of thyroid cancer cells. (A) Knockdown of HIF-1 α in TT cells by siRNA was confirmed by western blot analysis. Effects of siHIF-1 α on (B) viability and (C) migration and invasion of TT cells. Values are expressed as the mean $\pm$ standard deviation. All samples were assessed in triplicate for each group and the experiment was repeated at least twice. ** $P < 0.01$ vs. siControl. OD, optical density; siHIF-1 α , small interfering RNA targeting hypoxia-inducible factor-1 α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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