Original Article

Downregulation of Notch-regulated Ankyrin Repeat Protein Exerts Antitumor Activities against Growth of Thyroid Cancer

Bing-Feng Chu[1,2], Yi-Yu Qin[3], Sheng-Lai Zhang[2], Zhi-Wei Quan[2], Ming-Di Zhang[2], Jian-Wei Bi[4]

[1]Graduate School, Shanghai Second Military Medical University, Shanghai 200433, China
[2]Department of General Surgery, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China
[3]Clinical College, Yancheng Institute of Health Sciences, Yancheng, Jiangsu 224000, China
[4]Department of First General Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

Bing-Feng Chu and Yi-Yu Qin contributed equally to this work.

Abstract

Background: The Notch-regulated ankyrin repeat protein (NRARP) is recently found to promote proliferation of breast cancer cells. The role of NRARP in carcinogenesis deserves extensive investigations. This study attempted to investigate the expression of NRARP in thyroid cancer tissues and assess the influence of NRARP on cell proliferation, apoptosis, cell cycle, and invasion in thyroid cancer.
Methods: Thirty-four cases with thyroid cancer were collected from the Department of General Surgery, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine between 2011 and 2012. Immunohistochemistry was used to detect the level of NRARP in cancer tissues. Lentivirus carrying NRARP-shRNA (Lenti-NRARP-shRNA) was applied to down-regulate NRARP expression. Cell viability was tested after treatment with Lenti-NRARP-shRNA using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis and cell cycle distribution were determined by flow cytometry. Cell invasion was tested using Transwell invasion assay. In addition, expressions of several cell cycle-associated and apoptosis-associated proteins were examined using Western blotting after transfection. Student's *t*-test, one-way analysis of variance (ANOVA), or Kaplan–Meier were used to analyze the differences between two group or three groups.
Results: NRARP was highly expressed in thyroid cancer tissues. Lenti-NRARP-shRNA showed significantly inhibitory activities against cell growth at a multiplicity of infection of 10 or higher ($P < 0.05$). Lenti-NRARP-shRNA-induced G1 arrest (BHT101: 72.57% ± 5.32%; 8305C: 75.45% ± 5.26%) by promoting p21 expression, induced apoptosis by promoting bax expression and suppressing bcl-2 expression, and inhibited cell invasion by suppressing matrix metalloproteinase-9 expression.
Conclusion: Downregulation of NRARP expression exerts significant antitumor activities against cell growth and invasion of thyroid cancer, that suggests a potential role of NRARP in thyroid cancer targeted therapy.

Key words: Apoptosis; Cell Cycle Checkpoints; Notch-regulated Ankyrin Repeat Protein; Thyroid Neoplasms

Introduction

Thyroid cancer which is the most common malignancy of the endocrine system has shown an increasing incidence worldwide in recent years. According to pathology, thyroid cancer is classed into papillary adenocarcinoma, follicular carcinoma, medullary carcinoma, and anaplastic thyroid carcinoma (ATC). ATC accounting for 1%–2% of all thyroid carcinomas has a high degree of malignancy and displays highly invasive behavior usually presenting with lymph node metastasis or distant metastasis. In contrast to well-differentiated cancers (papillary and follicular carcinoma), patients with ATC have a poorer prognosis with a median survival of <1 year.[1,2] To date, no effective and standard treatments for ATC were established. A number of studies have shown that no survival benefit from traditional treatments including resection, radiotherapy, and chemotherapy alone or combined were observed in patients

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Address for correspondence: Dr. Jian-Wei Bi, Department of General Surgery, Changhai Hospital, Second Military Medical University, No. 168, Changhai Road, Shanghai 200433, China
E-Mail: bjw3721@126.com

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with ATC. Therefore, it is imperative to explore a novel effective therapeutic strategy for ATC.

Recent advancement in molecular biology has enlightened us on the potential mechanisms of thyroid cancer tumorigenesis and development. It has been documented that thyroid cancer is a complex disease which involves a series of gene-environment interactions in a progressive process associated with dysfunction in multiple genes, including abnormal activation or inactivation of oncogenes or tumor suppressor genes, such as *RET* gene rearrangement, *C-myc* gene amplification, *BRAF*, and *p53* mutation inactivation.[3-7]

Notch-regulated ankyrin repeat protein (NRARP) is a negative feedback regulator in Notch signaling pathway and regulated by Notch protein.[8] On one hand, overexpression of Notch protein can promote NRARP expression; on the other hand, NRARP has feedback inhibition on the activity of Notch by inducing degradation of Notch receptor intracellular domain (NICD).[9] In the present study, we found the overexpression of NRARP in thyroid cancer tissues and thyroid cancer cell lines (BHT101 and 8305C) and evaluated the effects of downregulation of NRARP expression on cell proliferation, cell cycle, invasion, and apoptosis.

Methods

Patient samples

A total of 34 cases (11 males and 23 females; age range: 39–72 years) with thyroid carcinoma were collected from the Department of General Surgery, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine between 2011 and 2012. Tumor tissues and corresponding normal tissues adjacent to tumors were obtained from patients undergoing radical resection with or without neck lymph node dissection. The samples were stored at −70°C immediately after resection. Twenty of 34 cases had cervical lymph node metastases. All patients were followed up for 3 years after the operation, and no patient was lost to follow-up. The study was approved by the Ethical Committee of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine and all patients consented to participate in the study.

Immunohistochemistry

Frozen sections were cut at 5 μm thickness and fixed in cold acetone for 15 min at 4°C and then rinsed with phosphate buffered saline (PBS) for 5 min. Then, the slides were treated with 3% hydrogen peroxide for 20 min for blocking peroxidase in the tissue and subsequently rinsed well with PBS. After blocked with goat serum, the slides were incubated with monoclonal antibody against NRARP (Santa Cruz, USA) overnight at 4°C. After being washed in PBS for 5 min, the slides were incubated for 30 min with the secondary antibody. After being washed three times in PBS for 5 min, the slides were stained with 3,3-diaminobenzidine (Merck, Germany).

Cell culture and treatment

Human ATC cell lines BHT-101[10] and 8305C[11] were purchased from China Center for Type Culture Collection (Shanghai, China). Normal human thyroid follicular cell line Nthy-ori 3-1 was purchased from the European Collection of Cell Cultures (Salisbury, UK). Nthy-ori 3-1 was grown at 37°C in complete Roswell Park Memorial Institute 1640 medium (GIBCO, USA), while BHT-101 and 8305C were maintained at 37°C in Dulbecco's modified Eagle medium (GIBCO, USA). All media were supplemented with 10% fetal bovine serum (Sigma, USA) and 1% penicillin/streptomycin (GIBCO, USA) in 5% CO_2 incubator. Subcultures were maintained at 80% confluence and passaged by 0.25% trypsin (GIBCO, USA).

To produce cells deficient in NRARP, Lentivirus carrying NRARP-shRNA (Lenti-NRARP-shRNA) (Santa Cruz) and Lenti-CON (Santa Cruz) were transfected into cells. In brief, BHT-101 and 8305C were cultured in 12-well plates for 24 h, then 5 μg/ml Lenti-NRARP-shRNA or Lenti-CON was added into the wells. After 24 h with infection, cells were cultured in fresh medium overnight. To obtain stable-transfected cells lines, cells were subcultured with 5 ug/ml puromycin for 2 weeks.

Water soluble tetrazolium-1 assay

The viability of cells was analyzed using water soluble tetrazolium-1 (WST-1), (Beyotime, China) assay. Briefly, 5×10^3 of BHT-101 and 8305C cells were seeded in 96-well microplates overnight. Cells were divided into three groups: cells with Lenti-NRARP-shRNA (multiplicity of infection [MOI] = 0.01, 0.1, 1, 10, 100, 1000), with Lenti-CON (MOI = 0.01, 0.1, 1, 10, 100, 1000), or with PBS. After incubation for 48 h at 37°C, the culture medium was removed and the cells were rinsed twice with PBS. Then, 10 μl of WST-1 reagent was added to each well. The absorbance of WST-1-derived formazan was measured using a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA) at 450 nm. Cell survival rate = (optical density [A] of experiment group − A of background)/(A of control group − A of background) ×100%.

Mouse xenografts

To evaluate the effects of *NRARP* on the proliferation of BHT-101 and 8305C cells *in vivo*, mouse models with BHT-101 and 8305C xenografts were used. Nude mice were raised in the specific pathogen-free environment. All animal procedures were approved by the Ethical Committee of Xinhua Hospital, Shanghai Jiaotong University School of Medicine. Thirty-six of BALB/c nude male mice (4-week-old, weight: 25.0 ± 1.5 g) were divided into three groups: the group inoculated with Lenti-NRARP-shRNA transfected cells (n = 12), the group inoculated with Lenti-CON transfected cells (n = 12), and the group inoculated with culture medium (n = 12). Briefly, BHT-101 and 8305C cells (1×10^7 cells per mouse) were injected subcutaneously into the right flanks of the mice. Tumors were formed after 2 or 3 weeks. Tumor formation was monitored every 5 days, and tumor volume based on caliper measurements was calculated by the following formula: tumor volume = 1/2 (length × width^2). On day 20th after injection, mice were sacrificed and tumors were weighed.

Cell cycle analysis

The effects of NRARP on cell cycle distribution were determined using flow cytometry (FCM) analysis. BHT-101 and 8305C cells were seeded in a 6-well plate overnight. Lenti-NRARP-shRNA was added to the wells of the plates for 48 h. After washed twice by PBS, the cells were fixed in 70% precooling ethanol overnight. The cells were then stained with 100 μg/ml RNaseA and 50 μg/ml propidium iodide (PI) (Sigma, USA) in PBS. Samples were run on an fluorescence activated cell sorting (FACS) Calibur flow cytometer (Becton-Dickinson Bioscience, Franklin Lakes, NJ, USA) and DNA content was analyzed using FlowJo Software 9.1 (Tree Star Inc., Ashland, Oreg., USA).

Invasion assays

Transwell assay (Corning, USA) was used to detect the invasion of BHT101 and 8305C cells after transfection with Lenti-NRARP-shRNA. Briefly, 5×10^4 cells in 200 μl of medium were added to the (Corning, Corning, NY, USA). After 24 h of incubation in complete medium, cells that invaded the (Corning, Corning, NY, USA) and passed through the filters were stained with 0.1% crystal violet for at least 15 min. The noninvading cells were then removed from inside with a cotton swab. Stained cells were counted under a microscope (BX50; Olympus, Tokyo, Japan).

Apoptosis analysis

Annexin V/PI double staining assay was used to analyze apoptosis of BHT101 and 8305C cells after transfection with Lenti-NRARP-shRNA. Briefly, cells transfected Lenti-NRARP-shRNA for 24 h or 48 h were trypsinized, then rinsed with precooled PBS, and resuspended in annexin V-FITC binding buffer. Annexin V-FITC and PI were added to the cell suspension. The cell suspension was gently vortexing and then incubated for 5 min at 4°C protected from light. After that samples were analyzed by the FACS Calibur (Becton-Dickinson) within 1 h.

Western blotting

Western blotting was used to analysis protein expression levels. Cells were collected after transfection with Lenti-NRARP-shRNA for 48 h. Total protein was extracted according to the manufacturer's instructions, and the concentration was determined by the Bradford assay. About 25 μg of protein samples were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a 0.2 μm polyvinylidene difluoride membrane. The membrane was blocked in 5% nonfat milk. Membranes were probed with primary antibody (1:1000) (Santa Cruz, USA) overnight at 4°C, washed three times in Tris-Buffered Saline Tween-20 (TBST), incubated with anti-mouse or anti-rabbit horseradish peroxidase antibody (1:5000) (Santa Cruz, USA) for 2 h at room temperature and then washed three times in TBST. The signal was visualized by an enhanced chemiluminescence solution (ECL Plus; Pierce, USA) and was exposed to Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA). β-actin was used as loading control.

Statistical analysis

All statistical analyses were performed by using IBM SPSS Statistics for Windows, Version 20.0, (IBM Corp, Armonk, NY, USA) and data expressed as a mean ± standard deviation (SD). Student's *t*-test, one-way analysis of variance (ANOVA), or Kaplan–Meier were used to analyze the differences between two group or three groups. All experiments in this study were performed independently at least three times. A $P < 0.05$ was considered statistically significant.

Results

Notch-regulated ankyrin repeat protein and Notch are highly expressed in thyroid cancer tissues

The immunohistochemistry (IHC) score of NRARP protein was calculated by multiplying the percentage of positive cells and the intensity of NRARP protein. The positive rate was scored as 0 (positive cells: <5%), 1 (5%–25%), 2 (25%–50%), or 3 (>50%). The intensity of staining was also scored as 0 (negative), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). The IHC score were determined as: − (score 0–1), + (score 2–3), ++ (score 4–6), and +++ (score > 6). According to IHC score, the patients were divided into low expression group (− or +) and high expression group (++ or +++).

As shown in Figure 1a-1c, the higher intensity and more positive cells of NRARP staining were present in thyroid cancer tissues and involved lymph node tissues than noncancer tissues. IHC score of NRARP further revealed that a higher level of the protein was expressed in cancer tissues than in noncancer tissues ($t = 7.86$, $P = 0.006$), and in metastatic lymph node than in thyroid cancer tissues ($t = 10.22$, $P = 0.013$) [Figure 1d]. The Kaplan–Meier analysis showed that NRARP protein level negatively correlated with survival of patients with ATC. Patients with higher level of NRARP protein had shorter survival than those with lower level ($\chi^2 = 6.96$, $P = 0.031$) [Figure 1e].

In vitro study showed that NRARP and Notch proteins are highly expressed in BHT101 and 8305C and lowly expressed in Nthy-ori 3-1 cells, while Lenti-NRARP-shRNA significantly down-regulated the level of NRARP protein in BHT101 and 8305C cells [Figure 1f and 1g].

Downregulation of Notch-regulated ankyrin repeat protein expression significantly inhibits the proliferation of BHT101 and 8305C cells *in vitro* and *in vivo*

WST-1 assay [Figure 2a and 2b] showed that after transfection with Lenti-NRARP-shRNA, the cell viability of BHT101 and 8305C was significantly decreased (BHT101: $F = 79.36$, $P = 0.025$; 8305C: $F = 64.59$, $P = 0.014$). Due to the concentration-dependent manner of inhibitory effects of Lenti-NRARP-shRNA, cell proliferation was not significantly affected at an MOI of 0.01–1 but was significantly suppressed at an MOI of 10 or higher.

Mouse xenograft models showed that the overall rate of tumor formation of BHT101 and 8305C cells transfected with Lenti-NRARP-shRNA was 50% (6/12), while the tumor formation rate in control groups (including Lenti-CON and Control group) was 100% (12/12). In addition, the size and weight of tumor xenografts in Lenti-NRARP-shRNA group were much smaller than those of control groups

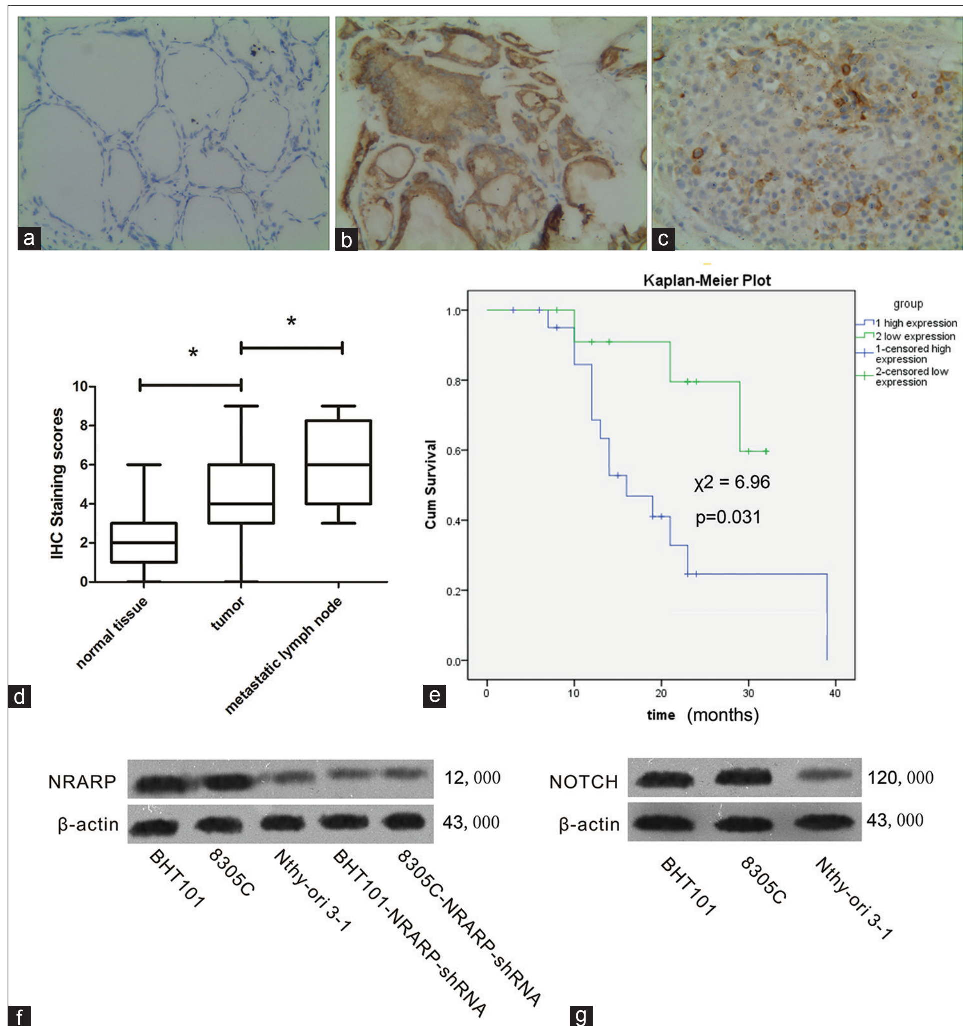

Figure 1: Immunohistochemical staining of NRARP in normal thyroid tissues, thyroid cancer tissues, and metastatic lymph nodes. Immunohistochemical staining, original magnification ×200 of NRARP in normal thyroid tissues (a), in thyroid cancer tissues (b), and in metastatic lymph nodes (c). (d) Immunohistochemistry score of *NRARP*. (e) The Kaplan–Meier analysis of overall survival of thyroid cancer patients according to the NRARP level in thyroid cancer. (f) Western blotting analysis of NRARP expression in BHT101, 8305C, and Nthy-ori 3-1 cells. (g) Western blotting analysis of Notch expression in BHT101, 8305C, and Nthy-ori 3-1 cell. *$P < 0.05$. NRARP: Notch-regulated ankyrin repeat protein.

(size: BHT101, $F = 31.55$, $P = 0.000$; 8305C: $F = 42.67$, $P = 0.000$. Weight: BHT101: $F = 26.13$, $P = 0.000$; 8305C: $F = 30.57$, $P = 0.000$) [Figure 2c and 2d].

Downregulation of Notch-regulated ankyrin repeat protein expression induces G1 phase arrest in BHT101 and 8305C cells

As shown in Figure 3a, G1 population of BHT101 (72.575±5.32%) and 8305C (75.455 ± 5.26%) cells was significantly increased after transfection with Lenti-NRARP-shRNA compared to the two control groups (BHT101: $F = 109.26$, $P = 0.000$; 8305C: $F = 209.31$, $P = 0.000$). In addition, Lenti-NRARP-shRNA-induced p21 protein expression, while it inhibited the expression of cyclin D1 protein [Figure 3b].

Downregulation of Notch-regulated ankyrin repeat protein expression induces apoptosis in BHT101 and 8305C cells

As shown in Figure 4a-4c, the population of apoptotic cells was significantly increased after transfection with Lenti-NRARP-shRNA for 24 h or 48 h in BHT-101 cell line (24 h: $F = 11.42$, $P = 0.009$; 48 h: $F = 25.51$, $P = 0.000$), and in 8305C cell line (24 h: $F = 8.97$, $P = 0.016$; 48 h: $F = 17.44$, $P = 0.000$). In addition, apoptosis-associated proteins including bax and bcl-2 were also detected in cell lysates by immunoblotting. Lenti-NRARP-shRNA promoted the expression of bax and the activation of caspase-3, while it inhibited the expression of bcl-2 protein [Figure 4d].

Downregulation of Notch-regulated ankyrin repeat protein expression attenuates BHT101 and 8305C cell invasion

Invasion assays showed that the number of migratory cells was significantly decreased after treatment with Lenti-NRARP-shRNA (BHT-101: $F = 23.12$, $P = 0.000$; 8305C: $F = 29.75$, $P = 0.000$) [Figure 5a and 5b]. Western blotting analysis showed that Lenti-NRARP-shRNA significantly decreased expression of matrix metalloproteinase-9 (MMP-9) protein [Figure 5c].

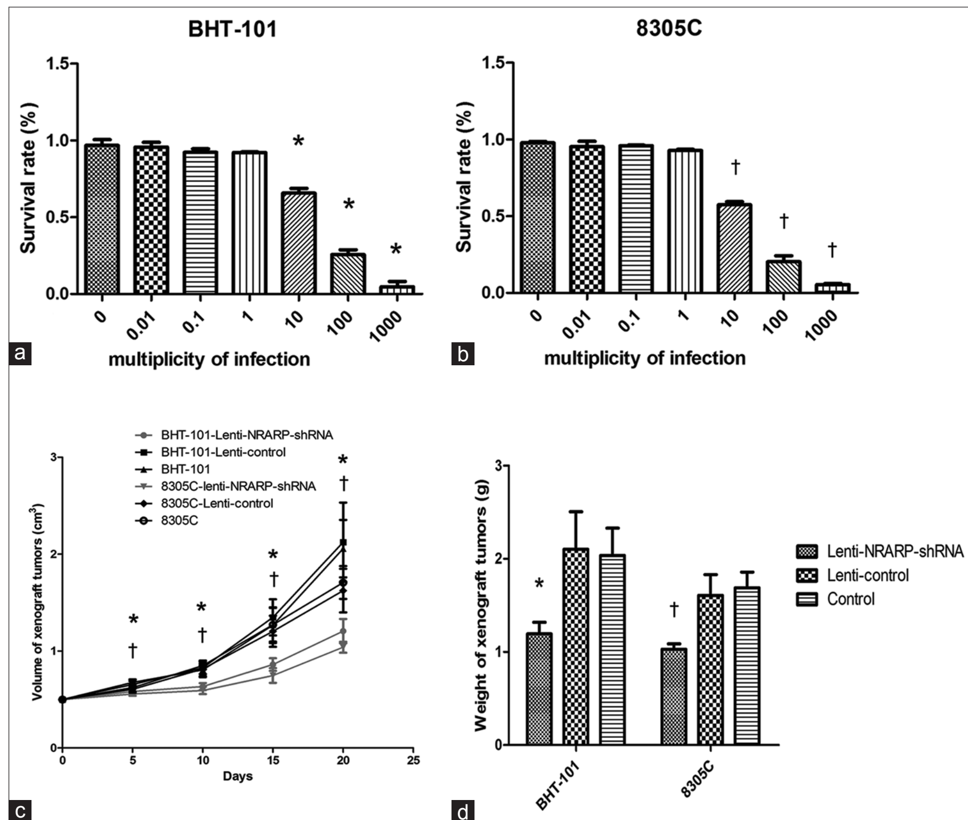

Figure 2: Knockdown of *NRARP* significantly inhibits the proliferation of BHT101 and 8305C cells *in vitro* and *in vivo*. (a) Knockdown of *NRARP* significantly inhibits the proliferation of BHT101 cells *in vitro*. (b) Knockdown of *NRARP* significantly inhibits the proliferation of 8305C cells *in vitro*. (c) The volume of tumor treated with or without knockdown of *NRARP*. (d) The weight of tumor treated with or without knockdown of *NRARP*, *, †indicates the difference was statistically significant compared to Lenti-control and control, respectively. *NRARP:* Notch-regulated ankyrin repeat protein.

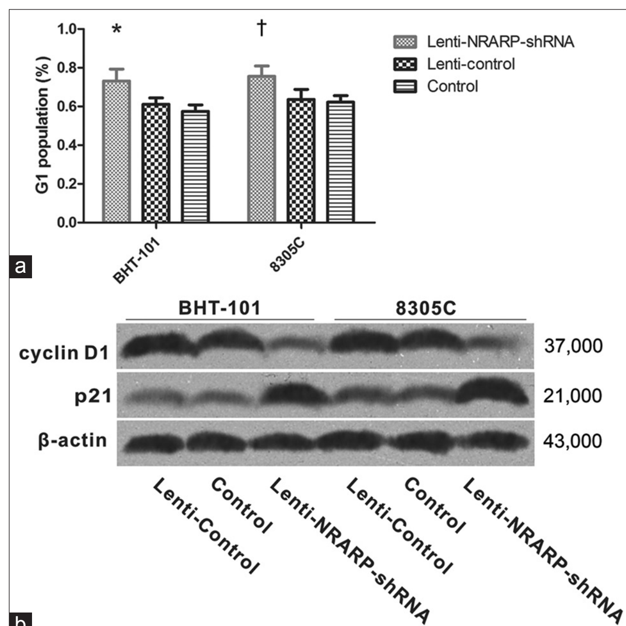

Figure 3: Knockdown of *NRARP* induces G1 phase arrest in BHT101 and 8305C cells. (a) The effects of knockdown of *NRARP* on cell cycle of BHT101 and 8305C cells. (b) Western blotting analysis of p21 and cyclin D1 expression in *NRARP* knockdown BHT101 and 8305C cells. *, † indicate the difference was statistically significant compared to Lenti-control and control, respectively. NRARP: Notch-regulated ankyrin repeat protein.

Discussion

Thyroid carcinoma is one of the most aggressive malignancies of head and neck tumors and is responsible for the majority of death from all endocrine malignancies.[12] The annual incidence of thyroid cancer for male is approximately 3/100,000, while it is about 9/100,000 for female, which ranks first in the increasing incidence of cancers for female cancers.[13] ATC, an undifferentiated thyroid cancer with aggressive phenotype and poor prognosis, is responsible for more than half deaths of all thyroid cancer. Clinical presentation is frequently characterized as a rapidly growing neck mass with associated compressive symptoms and lymph node metastasis.[14] Until now, few published papers have shown a significant survival benefit from the current therapies. In such a circumstance, new and effective therapeutic strategies based on molecular underpinnings of carcinogenesis and growth of ATC received our attention.

Notch signaling is known to play important roles in the regulation of diverse developmental processes including cell proliferation, differentiation and apoptosis, and deregulation and this signaling cascade has been linked to many cancers. A number of studies have revealed that *Notch* gene may have dual effects on tumorigenesis varying with cancer types. In breast cancer or prostate cancer, Notch seems to act as an oncogene,[15]

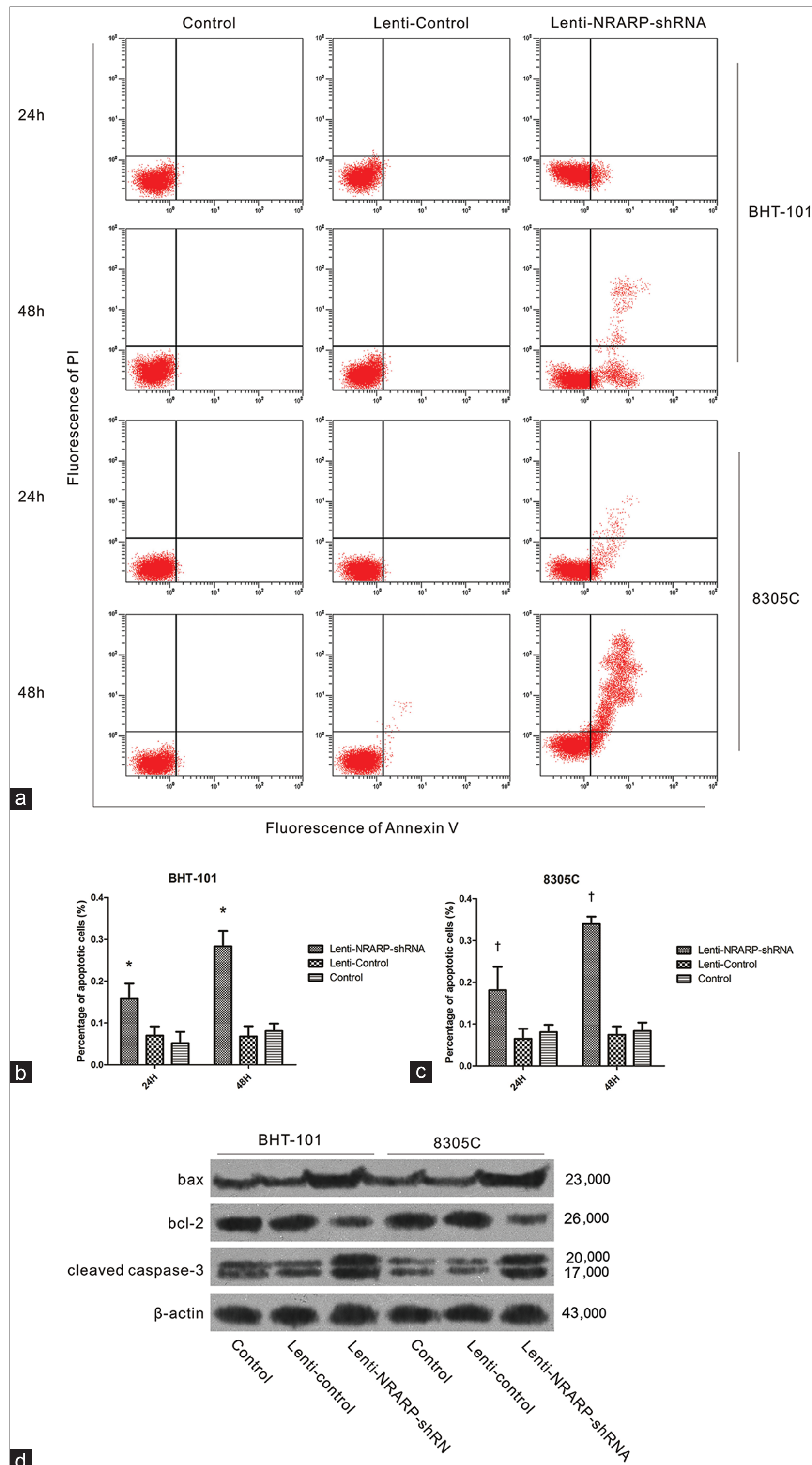

Figure 4: Knockdown of *NRARP* induces apoptosis in BHT101 and 8305C cells. (a) The apoptotic cell death was assessed by measuring the fluorescence intensity of cells staining with annexin V/FITC and PI. Legend for cytogram: the lower left quadrant includes the viable cells, which are negative for annexin V/FITC binding (annexin V−) and exclude PI (PI−); the lower right quadrant include early apoptosis cells, which are positive for annexin V/FITC binding (annexin V+) but PI−; the upper right quadrant represent the late apoptotic cells, which are annexin V+ and show PI uptake (PI+); the upper left quadrant represents necrotic cells, which are annexin V−/PI+. (b) FACS analysis confirmed that the early and late apoptotic cell death in *NRARP* knockdown BHT101 cells was significantly higher than that in control group. (c) FACS analysis confirmed that the early and late apoptotic cell death in *NRARP* knockdown 8305C cells was significantly higher than that in control group. (d) Western blotting analysis the expression of bax, bcl-2, and caspase-3. *, †indicates the difference was statistically significant compared to Lenti-control and control, respectively. PI: Propidium iodide; NRARP: Notch-regulated ankyrin repeat protein; FACS: Fluorescence activated cell sorting.

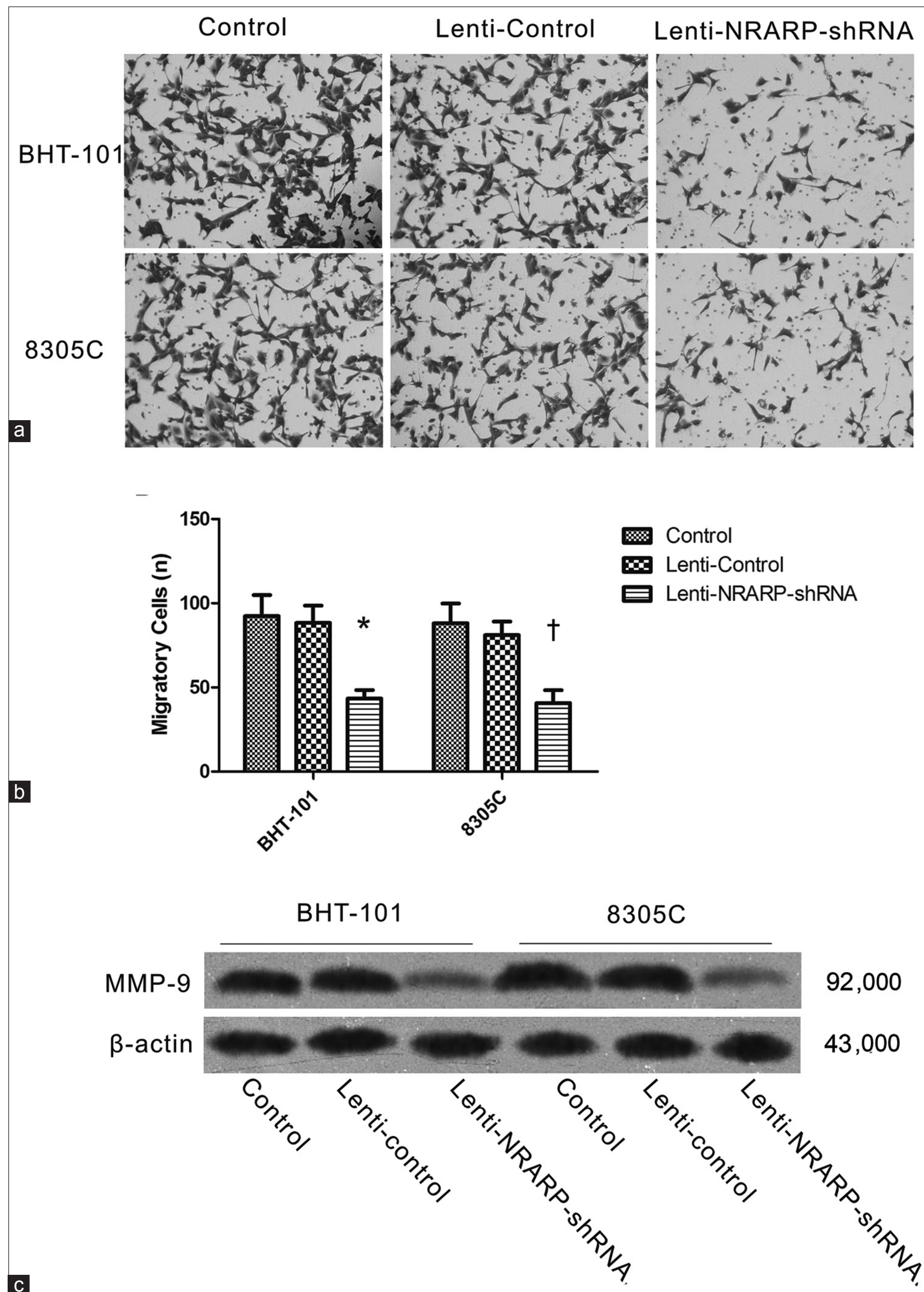

Figure 5: Knockdown of *NRARP* attenuates BHT101 and 8305C cell invasion. (a) Migratory cells were stained with crystal violet (original magnification ×100). (b) Less Lenti-NRARP-shRNA transfected cells invading to the bottom chamber, suggested decreased invasiveness. (c) Western blotting showed the reduced expression of MMP-9 in *NRARP* knockdown BHT101 and 8305C cells. *, †indicates the difference was statistically significant compared to Lenti-control and control, respectively. NRARP: NOTCH-regulated ankyrin repeat protein; Lenti-NRARP-shRNA: Lentivirus carrying NRARP-shRNA; MMP-9: Matrix metalloproteinase-9.

while in bladder cancer it possesses characteristics as a tumor suppressor gene.[16] Moreover, it may act as both oncogene and tumor suppressor gene in prostate cancer simultaneously.[17] An important role for Notch signaling in thyroid cancer has been well documented and the members of Notch signaling are abnormally expressed in thyroid cancer.[18,19] *NRARP* encodes a small evolutionarily conserved protein containing two ankyrin repeats which is a component of a negative feedback system to attenuate Notch pathway-mediated signaling. Notch may promote the expression of NRARP, and NRARP suppresses the activation of Notch signaling by promoting degradation of Notch intracellular domain (NICD). To our knowledge, the present study originally reported the expression and regulation of NRARP and its association with cancer cell functions *in vitro* and *in vivo* in thyroid cancer.

In our study, NRARP protein was highly expressed in ATC tissues and ATC cell lines compared to normal thyroid tissue and follicular cells. The expression of Notch increased in

parallel with NRARP in ATC tissues and cell lines, which indicated that the overexpression of NRARP may result from the abnormally high expression of Notch in ATC cells. Overexpression of Notch was also found in other types of thyroid cancer. The feedback of NRARP on Notch signaling seemed through inhibiting the activation of downstream pathway of Notch but it had little effect on the expression of Notch.

The paradoxical effects of overexpressed Notch on cancer cells may depend on the cellular context. In the case of ATC, it is more likely that high concentration of Notch protein would play an antitumor role.[20] We found that downregulation of NRARP expression could inhibit the growth of ATC cells both *in vitro* and *in vivo*. Moreover, downregulation of NRARP-induced G1 arrest, inhibited invasion, and promoted apoptosis. How *NRARP* gene participates in the regulation of growth, apoptosis, and invasion of ATC cells is not clear, but we assumed that overexpression of NRARP could block Notch signaling and inhibited the activation of *Notch* target genes by degrading NICD. When NRARP was down-regulated by Lenti-NRARP-shRNA, the inhibitory feedback on the downstream pathway of Notch was reduced, Notch signaling was over-activated and the antitumor action of Notch was restored. Our study suggested that the expression of bax and the activation of caspase-3 were reinforced and the expression of bcl-2 protein was attenuated in Lenti-NRARP-shRNA-treated ATC cells indicating a possible pathway involved in NRARP-regulated apoptosis of ATC cells. In addition, it has been demonstrated that activation of Notch suppressed the expression of *WNT* target genes in human colorectal cancer cells through epigenetic modification.[21] We found the expression of MMP-9, a downstream target of *WNT* signaling, was significantly suppressed and invasion was also inhibited after treatment with Lenti-NRARP-shRNA. It was very likely that downregulation of NRARP promoted activation of Notch, which subsequently inhibits *WNT* signaling and cell invasion. Apparently, *NRARP/Notch*-regulated proliferation, apoptosis, differentiation, and invasion of cancer cells remain to be explored in more details.

It was interesting to find a significantly higher expression of NRARP in metastatic lymph node tissue than in ATC tissue. The highly aggressive nature of ATC was closely related to extremely high expressed NRARP. On the other hand, increased NRARP may also correlate to less differentiated cancer, and therefore, the expression of NRARP in poorly differentiated ATC would be compared with other types of thyroid cancer in the future study. Nevertheless, our research data along with the clinical finding showing that NRARP protein level could be negatively correlated with patient prognosis which was similar to the breast cancer data[22] suggesting that *NRARP* may serve as a prognostic marker and as a potential target for thyroid cancer targeted therapy.

Our investigation suggested that Notch could be up-regulated in thyroid cancer to over-activate Notch signaling pathway which may inhibit ATC cell proliferation, induce G1 arrest, inhibit cell invasion, and promote apoptosis. However, NRARP as a feedback regulator of Notch pathway would be activated by overexpressed Notch, exerting negative effect on the activation of the target genes of Notch, and attenuating antitumor effects accordingly. Downregulation of NRARP expression could reverse the inhibitory feedback of NRARP on Notch signaling. Therefore, *NRARP* gene may act as an oncogene in the tumorigenesis and development of thyroid cancer. *NRARP* gene may serve as a potential target for thyroid cancer therapy. Though a series of functional studies were carried out to demonstrated the role of NRARP in proliferation, apoptosis, and invasion of thyroid cancer, the exact molecular mechanisms by which NRARP regulated proliferation, apoptosis, and invasion have not been completely elucidated in this study. In addition, the optimal way to down-regulate the expression of NRARP needs to be investigated in future studies.

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Conflicts of interest

There are no conflicts of interest.

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