GATA4 is upregulated in nasopharyngeal cancer and facilitates epithelial-mesenchymal transition and metastasis through regulation of SLUG

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Abstract. GATA4, a member of the GATA family, serves a key function in several types of cancer, including hepatoblastoma, gastric cancer and breast cancer. However, the function of GATA4 in nasopharyngeal cancer (NPC) is largely unknown. The present study revealed that GATA4 was upregulated in NPC tissue samples and the NPC cell line, 5-8F. Furthermore, the expression of GATA4 was associated with tumor size, metastasis and poor prognosis. Transwell invasion and wound healing analyses demonstrated that GATA4 promoted cell invasion and migration, respectively. Western blotting and reverse transcription-quantitative polymerase chain reaction revealed that GATA4 overexpression decreased the expression of epithelial markers and increased the expression of mesenchymal markers. By contrast, GATA4 inhibition increased the expression of epithelial markers and decreased the mesenchymal markers. Additionally, chromatin immunoprecipitation and dual-luciferase reporter assays revealed that GATA4 promoted epithelial-mesenchymal transition through transcriptionally activating SLUG. Cell counting kit-8 and colony formation assays were performed to analyze the effect of GATA4 on cell proliferation. The results indicated that GATA4 facilitated cell proliferation in NPC. In conclusion, GATA4 acts as an oncogene and serves crucial roles in NPC and GATA4 may find a potential application as therapeutic option in NPC.

Introduction

Nasopharyngeal cancer (NPC) derives from the epithelial lining of the nasopharynx (1). Although the incidence rate of NPC is relatively low in the majority of the world, representing about 0.7% of the global cancer burden, it is comparatively higher in southern China and Southeast Asia with ~7.3% (2,3). Previous studies have revealed that inheritance, environmental influences and the Epstein-Barr virus serve essential functions in NPC development (4-9).

The GATA family contains GATA1, GATA2, GATA3, GATA4, GATA5 and GATA6 (10). They all contain a zinc finger domain, which can bind to the specific consensus DNA sequence, 5'-A/TGATAA/G-3' (11). Multiple reports demonstrated that the GATA family serves essential functions in cell proliferation and differentiation (12,13). The GATA family is separated into two subfamilies; the GATA1/2/3 subfamily and the GATA4/5/6 subfamily. Several previous studies have indicated that GATA4 regulates gene transcription by binding to GATA elements (14,15). Additionally, GATA4 was demonstrated to interact with other transcription factors, including Tbx5 and Nkx2-5 (16). GATA4 acts as a transcriptional regulator, regulating hypertrophic growth of the heart (17). Defects or mutations of GATA4 contribute to a series of cardiac problems, including abnormal ventral folding, congenital heart disease as well as hypoplasia of the ventricular myocardium (18). Additionally, GATA4 is crucial to the gastrointestinal, respiratory and reproductive systems and cancer (19). In addition, GATA4 contributes to cancer development and serves as a potent prognostic predictor (20-23). However, the molecular mechanism of GATA4 in NPC remains poorly understood.

Tumor metastasis occurs due to epithelial-mesenchymal transition (EMT) in cancer cells (24,25). EMT is a crucial phenotypic event; it regulates embryonic development, wound healing and tissue remodeling (26). The main characteristics of EMT are decreased epithelial markers and increased mesenchymal markers (26). Several studies have indicated that GATA4 regulates EMT in many types of cancer (27,28). However, the function of GATA4 in NPC remains unknown.

The current study demonstrated that GATA4 was upregulated in NPC and the expression of GATA4 was associated with tumor size, metastasis and pathological grade. Additionally, the upregulation of GATA4 was associated with a poor prognosis in NPC. In addition, GATA4 enhanced EMT through the regulation of SLUG expression in NPC and facilitated...
cell invasion. Furthermore, cell proliferation was also demonstrated to be regulated by GATA4. In conclusion, the current study indicated that GATA4 acts as an oncogene in NPC.

**Materials and methods**

**Cell culture and tissue samples.** The human NPC cell line, 5-8F, was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin-streptomycin solution at 37°C with 5% CO₂. The human immortalized normal nasopharyngeal epithelial cell line, NP69 obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), was cultured in Keratinocyte-SFM medium supplemented with epidermal growth factor (EGF; both Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO₂.

The adjacent normal tissue (ANT; 0.25 cm from tumor tissue) and tumor tissue samples were obtained from patients who were diagnosed with NPC. A total of 42 pairs of ANT and NPC specimens were collected from Department of Otorhinolaryngology, The Second Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) between April 2015 and October 2016. A total of 19 males and 23 females participated in the study with a mean age of 55.3±4.3 years (range, 35-82 years). The current study was approved by The Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine. All patients provided written informed consent.

**Transfection.** Cells (3x10⁵) were transfected with 2.5 µg pcDNA3.1 empty vector (Youbio, Hunan, China), 2.5 µg FLAG-GATA4 (pcDNA3.1-GATA4; Youbio) or 30 nM small interfering (si)RNA scrambled (SCR) or GATA4 siRNA (siGATA4; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according the manufacturer's protocol. Following 48 h transfection at 37°C, cells on the undersurface were fixed with 4% paraformaldehyde (PFA; VWR; Newtown, PA, USA) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich; Epsom, UK). The cells were blocked by 5% non-fat milk at 37°C for 1 h, and then incubated with primary antibodies at 4°C overnight. The membranes were washed three times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Finally, blots were visualized using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). The antibodies used were as follows: Rabbit anti-GATA4 (1:2,000; cat, 195503-1-AP; Proteintech Group, Inc., Chicago, IL, USA), rabbit anti-TWIST (1:1,000; cat, 25465-1-AP; Proteintech Group, Inc.), rabbit anti-a-catenin (1:3,000; cat, 12831-1-AP; Proteintech Group, Inc.), rabbit antibodies from the EMT kit (1:2,000; cat, 9783; Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse anti-β-actin (1:3,000; cat, 6276; Abcam, Cambridge, UK), goat anti-rabbit (HRP-conjugated; 1:5,000; cat, ab205718; Abcam) and goat anti-mouse (HRP-conjugated; 1:3,000; cat, ab205719; Abcam).

All the experiments were repeated at least three times.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from tissue samples and cells using a Qiagen RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Then, cDNA was reverse transcribed from mRNA at 42°C for 30 min using a PrimeScript RT-PCR kit (Takara Bio, Inc., Otsu, Japan). Subsequently, Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Inc.) was used to perform qPCR analyses in an Applied Biosystems 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: 5 min at 98°C, followed by 34 cycles of denaturation at 98°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 35 sec. Primers were as follows: GATA4 forward, 5’-CCCCATCTCTAGATATGTGTGAC-3’ and reverse, 5’-CCGCTTACCTTTGTTGAG-3’; E-cadherin forward, 5’-AACATCATGTAGCAGAC-3’ and reverse, 5’-GATACTCTCGAGTCGTT-3’; Twist forward, 5’-TTAGACAAGAGATTTACCC-3’ and reverse, 5’-GGAGCGGTCTTCTCCTTCTC-3’; Snail forward, 5’-GTGGCTTCTGCTTCTCCT-3’ and reverse, 5’-GAGCCCTCATCAATCCAC-3’; N-cadherin forward, 5’-CAGGCGGTATGATGATG-3’ and reverse, 5’-GTGGCTGCTTCTCCT-3’; vimentin forward, 5’-CTCCAAGAGGAGGAGAACTAC-3’ and reverse, 5’-GATTTTGAGCAGTCATCTGAC-3’; Slug forward, 5’-ACACAATCGTGATATTTCCC-3’ and reverse, 5’-AGTGTAGGAGAGAAGAG-3’; Western blotting. Whole protein was extracted from cells and tissues using radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 1% NP-40] with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The concentration of protein was measured using a bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, equal amount of proteins (40 µg) were separated by 8% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked by 5% non-fat milk at room temperature for 1 h, and then incubated with primary antibodies at 4°C overnight. The membranes were washed three times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Finally, blots were visualized using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). The antibodies used were as follows: Rabbit anti-GATA4 (1:2,000; cat, 195503-1-AP; Proteintech Group, Inc., Chicago, IL, USA), rabbit anti-TWIST (1:1,000; cat, 25465-1-AP; Proteintech Group, Inc.), rabbit anti-a-catenin (1:3,000; cat, 12831-1-AP; Proteintech Group, Inc.), rabbit antibodies from the EMT kit (1:2,000; cat, 9783; Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse anti-β-actin (1:3,000; cat, 6276; Abcam, Cambridge, UK), goat anti-rabbit (HRP-conjugated; 1:5,000; cat, ab205718; Abcam) and goat anti-mouse (HRP-conjugated; 1:3,000; cat, ab205719; Abcam).

All the experiments were repeated at least three times.

**Transwell invasion assay.** A Transwell invasion assay was performed using Transwell chambers (8 µm pore size; Costar; Corning Incorporated, Corning, NY, USA) according to the manufacturer's protocol. In brief, GATA4 was overexpressed or knocked down in 5-8F cells. Following a 48-h transfection, 20,000 cells were placed into the upper chamber, which was coated with Matrigel (BD Biosciences, San Jose, CA, USA). The cells in the upper chamber were maintained with serum-free RPMI medium 1640, the lower plate was filled with RPMI medium 1640 which contained 10% FBS. Following a 16-h incubation at 37°C, cells on the underside were fixed with
were cloned into a pGL3-basic plasmid (Beyotime Institute of Biotechnology) at 4°C overnight. Protein G agarose beads (60 µl) were used to purify antibody-bound DNA fragments at 4°C over 1 h. Beads were then washed with each 1 ml low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer and TE buffer (all Merck KGaA) at 4°C. DNA was isolated from the immunoprecipitates using a DNA kit (Tiangen Biotech Co., Ltd.) and quantified using RT-qPCR analysis with the specific primer pairs: Slug, forward 5'-TTTGCCTGTGGAT-3' and reverse 5'-TGGATTTTTTGCTGTTGATG-3'; twist, forward 5'-AAAGGATGGACC TGAAACGG-3' and reverse 5'-GGCAAATCGGAAGC GCAA-3'. The reaction mixture contained 2 µl sample, 1 µl primer (10 nM), 10 µl 2X MIX buffer (TransGen Biotech Co., Ltd., Beijing, China) and 7 µl RNase-free. RT-qPCR conditions were as follows: 5 min at 98°C, followed by 35 cycles of denaturation at 98°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 20 sec. Input DNA and DNA immunoprecipitated by anti-IgG antibody served as a positive and negative control, respectively. The results were calculated using the 2-ΔΔCt method (29).

**Statistical analysis.** All data were presented as mean ± standard deviation. SPSS (version 18.0; SPSS, Inc., Chicago, IL, USA) was used to analyze data. The association between GATA4 expression and clinical information was analyzed by Pearson's χ² test. Kaplan-Meier plots and log-rank tests were used to analyze the rates of survival. Comparisons between two groups were analyzed by Student's t-test. Multiple groups were analyzed by one-way analysis of variance followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**GATA4 is upregulated in NPC tissues and the cell line, 5-8F.** In order to explore the function of GATA4 in NPC, 42 paired NPC tissue and ANT samples were collected from patients who were diagnosed with NPC. The expression of GATA4 was detected by RT-qPCR. As presented in Fig. 1A, the expression of GATA4 in tumor tissue was significantly higher compared with its expression in ANT. Western blotting was performed to detect the protein level of GATA4. Consistent with the RT-qPCR analysis, the expression of GATA4 was markedly higher in tumor tissue compared with ANT (Fig. 1B). Additionally, the association between GATA4 expression and clinicopathology of NPC was analyzed. High GATA4 expression was significantly associated with large tumor size, metastasis and high pathological grade (Table I). In addition, according to survival curves analysis, the patients with high GATA4 expression had lower survival rates compared with the patients who had low GATA4 expression (Fig. 1C). Next, the expression of GATA4 was further investigated in the NPC cell line, 5-8F; and the human immortalized normal nasopharyngeal epithelial cell line, NP69, was used as control. The results demonstrated that the protein and mRNA levels of GATA4
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Table I. Clinicopathological variables in 42 nasopharyngeal cancer patients.

<table>
<thead>
<tr>
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<th>High (n=29)</th>
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<td>≥55</td>
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<td>6</td>
<td>16</td>
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<td>7</td>
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Figure 1. GATA4 is upregulated in nasopharyngeal cancer tissues and the cell line, 5-8F. (A) The relative mRNA expression levels of GATA4 in ANT and tumor tissue samples were detected and quantified using RT-qPCR. (B) GATA4 protein levels in ANT and tumor tissue samples were detected using western blotting (n=42). (C) The association between the expression of GATA4 and the survival rate of patients was analyzed by the Kaplan-Meier method. (D) GATA4 protein levels in NP69 and 5-8F cells were detected using western blotting. (E) The relative mRNA expression levels of GATA4 in NP69 and 5-8F cells were detected and quantified using RT-qPCR. *P<0.05. ANT, adjacent normal tissue; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
were higher in 5-8F cells compared with that of NP69 cells (Fig. 1D and E). The results indicated that GATA4 serves an essential function in NPC.

**Overexpression of GATA4 promotes NPC cell migration and invasion.** Previous studies by our group indicated that GATA4 was positively correlated with metastasis (22,30). Therefore, the role of GATA4 in the regulation of NPC cell migration and invasion was explored. The human highly metastatic NPC cell line, 5-8F, was used for further experiments. 5-8F cells were transfected with the empty vector, FLAG-GATA4, SCR or siGATA4. Western blotting and RT-qPCR were performed to determine GATA4 expression in 5-8F cells following transfection. The results revealed that the protein and mRNA levels of GATA4 were markedly (Fig. 2A) and significantly (Fig. 2B) increased, respectively, in 5-8F cells transfected with FLAG-GATA4 compared with the vector group. The protein and mRNA levels of GATA4 were markedly and significantly decreased, respectively, in 5-8F cells transfected with siGATA4 compared with the SCR group. Transwell and wound healing assays were performed to determine the effect of GATA4 expression on cell invasion and migration, respectively (magnification, x20). *P<0.05. FLAG-GATA4, vector overexpressing GATA4; si, small interfering RNA; SCR, scramble siRNA; siGATA4, siRNA against GATA4.

**GATA4 facilitates EMT in NPC cells.** EMT is a complex process, which is associated with metastasis. Therefore, the effect of GATA4 on EMT was explored. The 5-8F cells were transfected with the empty vector, FLAG-GATA4, SCR or siGATA4. Western blotting and RT-qPCR were then used to determine the GATA4 expression in the cells following
transfection. As expected, the protein levels of the epithelial markers (E-cadherin and α-catenin) were markedly decreased and mesenchymal markers (N-cadherin and vimentin) were markedly increased when GATA4 was overexpressed compared with the vector group (Fig. 3A). Additionally, knocking down GATA4 expression markedly increased the epithelial markers and decreased the mesenchymal markers compared with the SCR group. The results were similar and significant when the mRNA levels were analyzed (Fig. 3B). These results revealed that GATA4 facilitates EMT in NPC cells.

**SLUG is transcriptionally activated by GATA4.** To further decipher the molecular mechanisms of GATA4 in EMT, the expression of genes that serve roles in EMT (SLUG, SNAIL, TWIST and ZEB1) were analyzed to identify whether they are regulated by GATA4. Western blotting demonstrated that the protein expression of SLUG and TWIST was increased when GATA4 was overexpressed; in contrast, the protein expression of SLUG and TWIST was decreased when GATA4 was depleted (Fig. 4A). The results were similar and significant when the mRNA levels were analyzed (Fig. 4B). However, the expression of SNAIL and ZEB1 did not significantly change at the protein and mRNA level. Thus, it was assumed that GATA4 transcriptionally regulated SLUG and TWIST. To validate this hypothesis, a chromatin immunoprecipitation (ChIP) assay was performed to detect the interaction between GATA4, and promoter regions of **SLUG** and **TWIST**. The results of the ChIP assay demonstrated that GATA4 directly bound the promoter region of **SLUG**, but not the promoter region of **TWIST** (Fig. 4C). Subsequently, a dual-luciferase reporter assay was performed in 5-8F cells, which were co-transfected with pGL3-SLUG or pGL3-TWIST and Renilla, and incubated with increasing concentrations of pcDNA3.1-GATA4. GATA4 significantly increased the luciferase activity of the pGL3-SLUG group, but not in the pGL3-TWIST group (Fig. 4D). These results suggested that GATA4 transcriptionally activates **SLUG**. Although GATA4 did not transcriptionally regulate **TWIST**, GATA4 may regulate **TWIST** through the regulation of other proteins.

**GATA4 promotes cell proliferation in NPC cells.** Due to the association between GATA4 expression and tumor size, the role of GATA4 in the regulation of NPC cell proliferation was explored using CCK-8 and colony formation assays. The colony formation assay revealed that overexpression of GATA4 in 5-8F cells significantly increased the number of colonies compared with the vector group, but GATA4 inhibition in 5-8F cells significantly decreased the number of colonies compared with the SCR group (Fig. 5A). The CCK-8 assay demonstrated that overexpression of GATA4 significantly promoted cell proliferation compared with the vector group (Fig. 5B). However, GATA4 inhibition significantly suppressed 5-8F cell proliferation compared with the SCR group. These results suggested that GATA4 promotes cell proliferation in NPC cells.

**Discussion**

The results of the current study revealed that GATA4 was upregulated in patients’ tissue samples and the 5-8F NPC cell line. In addition, the increased expression of GATA4 in these samples and cells was associated with larger tumor size, high pathological grade, metastasis and poor prognosis. The survival curve analysis indicated that patients with high GATA4 expression survived for ~145 months and patients with low GATA4 expression survived for ~175 months, suggesting that GATA4 may be a novel predictor of NPC. Metastasis is an important event in cancer development, previous reports demonstrated that nasopharyngeal carcinoma frequently led to lymph node metastasis (31,32). As stated above, the current study demonstrated that high GATA4 expression was associated with metastasis. Several functional experiments, including Transwell invasion and wound healing assays, were performed to investigate the mechanism of GATA4 on cell migration and invasion. The results indicated that GATA4 significantly promoted migration and invasion in 5-8F cells.

EMT is a crucial phenotypic event; it regulates embryonic development, wound healing and tissue remodeling (33,34). A number of studies have indicated that GATA4 regulates EMT in several cancers (27,28). However, the role of GATA4 in EMT remains unclear. In order to explore whether GATA4 regulates EMT, GATA4 was overexpressed or knocked down in 5-8F cells. It was demonstrated that the overexpression of GATA4 increased the expression of N-cadherin and vimentin, whereas...

Figure 3. GATA4 facilitates epithelial-mesenchymal transition in nasopharyngeal cancer cells. 5-8F cells were transfected with an empty vector, FLAG-GATA4, SCR or siGATA4. (A) Western blotting was used to detect the protein levels and (B) reverse transcription-quantitative polymerase chain reaction analysis was used to detect and quantify the mRNA expression levels of E-cadherin, α-catenin, N-cadherin and vimentin. *P<0.05. FLAG-GATA4, vector overexpressing GATA4; si, small interfering RNA; SCR, scramble siRNA; siGATA4, siRNA against GATA4.
Figure 4. *SLUG* is transcriptionally activated by GATA4. The (A) protein expression levels and (B) mRNA expression levels of epithelial-mesenchymal transition-associated transcription factors in 5-8F cells transfected with an empty vector, FLAG-GATA4, SCR or siGATA4. Protein expression was detected using western blotting, and mRNA expression was detected and quantified using reverse transcription-quantitative polymerase chain reaction analysis. (C) Chromatin immunoprecipitation analysis was used to detect and quantify the interaction between GATA4 and the promoter regions of *SLUG* and *TWIST* in 5-8F cells. (D) A dual-luciferase reporter assay was performed to determine the effect of GATA4 on the transcriptional regulation of *SLUG* and *TWIST*.* P<0.05. FLAG-GATA4, vector overexpressing GATA4; si, small interfering RNA; SCR, scramble siRNA; siGATA4, siRNA against GATA4; Ig, immunoglobulin.

Figure 5. GATA4 promotes cell proliferation in NPC cells. (A) Colony formation and (B) Cell counting kit-8 assays quantified cell proliferation of 5-8F cells transfected with an empty vector, a vector overexpressing GATA4, SCR or an siRNA against GATA4 (magnification, x20). *P<0.05. **P<0.05 vs. the vector group. #P<0.05 vs. the SCR group. si, small interfering RNA; SCR, scramble siRNA; siGATA4, siRNA against GATA4; OD, optical density.
the expression of E-cadherin and α-catenin was decreased. Conversely, GATA4 inhibition increased the expression of E-cadherin and α-catenin, but the expression of N-cadherin and vimentin was decreased. Therefore, GATA4 promoted EMT in 5-8F cells.

EMT is regulated by several transcription factors, including SLUG, SNAIl, TWIST and ZEB1 (35-38). Subsequently, ChIP and luciferase reporter assays demonstrated that GATA4 transcriptionally activated SLUG. Although previous studies have demonstrated that TWIST is also regulated by GATA4 (39), the current study did not identify GATA4 as a transcriptional regulator of TWIST. Thus, it is assumed that GATA4 may regulate TWIST through other proteins. In summary, GATA4 promotes EMT through transcriptionally activating SLUG in NPC.

Cell proliferation is also an important event in cancer development (40). Due to the association between the expression of GATA4 and tumor size, it was hypothesized that GATA4 may regulate cancer cell proliferation. As expected, the current study revealed that GATA4 facilitated cell proliferation in 5-8F cells.

To the best of our knowledge, the current study is the first study to demonstrate that GATA4 is upregulated in NPC and serves as an oncogene. The high expression of GATA4 predicted a poor prognosis for patients with NPC. Additionally, GATA4 promoted metastasis and proliferation in NPC cells. Therefore, GATA4 may be a novel biomarker for NPC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

YZ and BY conceived and designed the present study. YZ, HC and BY performed the experiments. YZ and BY wrote the paper. All authors reviewed and edited the manuscript. The final version of the manuscript was read and approved by all authors, and each author believes that the manuscript represents honest work.

Ethics approval and consent to participate

The current study has been approved by The Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine. All patients were informed that their tissues would be used in the current study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


