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*Original Article*

# GATA2 promotes metastasis and infers poor outcomes for nasopharyngeal carcinoma patients

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**Abstract:** GATA family act as pioneer factors, GATA2 gene encodes a zinc-finger transcription factors that play a critical role in numerous cell development process. Our results showed that high expression of GATA2 in NPC associated with pathological grade, tumor size and poor prognosis. We also found GATA2 can promote EMT through transcriptional activity TWIST, a key transcription factor which promote EMT, thereby increase cancer cell metastasis and invasion capacity. Our findings reveal a novel mechanism of EMT in Nasopharyngeal carcinoma and support the pursuit of GATA2 as a potential biomarker for nasopharyngeal carcinoma aggressiveness and a potential target for nasopharyngeal carcinoma intervention.

**Keywords:** GATA2, EMT, TWIST

## Introduction

Nasopharyngeal carcinoma, short for NPC,is an epithelial tumor which located in the nasopharynx. NPC is predominantly occurred in the Southeast Asia and southern part of China [1, 2]. It is highly chemo sensitive and radiosensitive, hence the survival rate has been improved obviously depend on the advances in chemo radiotherapy [3, 4]. Nevertheless, NPC has a character different from other cancers, that is tendency to metastasize, therefore the NPC patients normal occurred distant metastasis and has a poor prognosis [5, 6]. Therefore, in order to more effective diagnosis and cure NPC, more in depth investigate the molecular mechanism of NPC is concernment. There are numerous studies revealed that GATA family act as pioneer factors [7, 8]. Recently, GATA2 was shown to have a high expression in several carcinomas, for instance prostate cancer [9, 10]. In addition, high GATA2 expression also strongly linked to poor prognosis in prostate cancer patients [9]. But the role of GATA2 in NPC is still unknown.

To determine the role of GATA2, we first performed western blot and immunohistochemistry, result demonstrated that GATA2 was remarkably high expression in NPC cell lines and tissues. Moreover, its expression level also positive relate to tumor size, lymph node metastasis and pathological grade. High expression of GATA2 simultaneously predict poor prognosis. To further confirm above results, we carried out several research in vitro. By GATA2 siRNA, we down-regulated GATA2 and found that GATA2 could inhibit NPC cells proliferation and migration. The oppose results can be seen in cells which over express GATA2. Besides, we also observed that GATA2 made a great differences to NPC cells during EMT. Above data suggested that GATA2 might promote NPC progression and provide a new biomolecule for NPC diagnosis and prognosis.

## Materials and methods

### *Patients and tissue samples*

Retrieved 186 NPC specimens from the Guangxi Medical University as well as chosen at random during 2010 and 2015. Every patients never undergone the anti-tumor therapies. The chronic inflammatory nasopharyngeal epithelium tissue was as non-tumor tissue, it was from patients who were suspected to have nasopharyngeal carcinoma, however excluded by patho-

logical diagnosis. We analyzed the expression of GATA2 in the tumor and non-tumor samples of NPC.

*Immunohistochemistry*

Immunohistochemistry (IHC) was used to analyze the expression of GATA2 in NPC. First fixed the specimens with formalin, and embedded in paraffin, nest sectioned at 4 mm thickness, then transferred to microscope slides. All sections were dewaxed in xylene, subsequently rehydration in graded ethanol. Followed by incubated them in pH 6.0 citrate buffer and retrieve the antigen in an autoclave. Immersed into 0.3% hydrogen peroxide about 30 min to inactivated endogenous peroxidase. To exclude nonspecific binding, incubated specimens in 10% goat serum 4°C overnight or 1 hr at room temperature. Followed by incubated sections with anti-GATA2 antibody at 4°C overnight. According to the manufacture instructions to stain by peroxidase-anti-peroxidase method (DAKO, Hamburg, Germany), DAB solution was used to demonstrate the reaction. Rinsing in water for 40 min, and counterstained with hematoxylin, next dehydrated. Finally, mounted them in mounting medium for interpretation. Determined the specificity of the antibody by using IgG (1:150, Santa Cruz Biotechnology) at the same concentration.

*Cell culture*

Cultured NPC cell lines 5-8F, CNE2 in RPMI medium 1640 (Gibco) supplemented with 10% FBS (fetal bovine serum) at 37°C with 5% $CO_2$. And NP69, an immortalized normal nasopharyngeal epithelial cell line, was cultured in Keratinocyte-SFM medium supplemented with EGF (epidermal growth factor) (Invitrogen) at 37°C with 5% $CO_2$.

*RNA isolation and quantitative real-time PCR*

After using the Trizol reagent (Sigma) to extracted total RNA, the cDNA samples were produced using a Trans Script First-Strand cDNA Synthesis SuperMix (TransGen). Gene transcripts was analyzed using Power SYBR Green PCR Master Mix.

The primers used for qRT-PCR were as follows: GAPDH as an internal control, and the primers were forward: 5'-CATTTCCTGGTATGACAACG-3'; reverse: 5'-CACAGGGTACTTTATTGATGGT-3'; GATA2 forward: 5'-TGTGCAAATTGTCAGACGAC-3'; reverse: 5'-CCTGTTAACATTGTGCAGCT-3'; E-cadherin forward: 5'-CTACAGACAATGGTTCTCCA-3'; reverse: 5'-GAAGAATATAGTTCGAGGTTCTGG-3'; N-cadherin forward: 5'-TCAAAGCCTGGAACATATGTG-3'; reverse: 5'-TTCTCGATCAAGTCCAGCTG-3'; Fibronectin forward: 5'-CATCACAGGTTTACAACCAGG-3'; reverse: 5'-GTTGGATGGTGCATCAATGG-3'. They were purchased from Thermo Fisher Scientific Inc. Reversetranscription at 42°C for 30 min, next 2 min at 93°C for predenature followed by 36 cycles of 93°C for 20 s, 57°C for 20 s as well as at 72°C for 30 s. ΔΔCt method was used to calculate the Ct-value, all results were expressed as $2^{\Delta\Delta Ct}$. All experiments were performed at least three times.

*Transient transfection with siRNAs*

We purchased GATA2 siRNA and control siRNA from GenePharma. After CNE2 cells were grown to 30%-40% confluence, transfected siRNA into CNE2 cells using RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's method. The efficiency of siRNA was Determined the efficiency of GATA2 siRNA by the mRNA and protein levels in the 48-hr post transfected cells.

*Western blot*

Lysis cells with RIPA Lysis Buffer containing cocktail on ice. BCA Protein Assay Kit was used to measured protein concentration. Equal amount of total cellular protein (20 μg) was Separated equal amount of total protein (40 μg) with 10% SDS-PAGE and transferred protein onto the PVDF membranes (Millipore, MA). Blocked the membrane with 5% nonfat milk in TBST buffer for 1 hr at room temperature, next incubated overnight at 4°C with primary antibodies, follow by incubated with HRP-linked secondary antibodies (Santa Cruz Biotechnology, 1:3000) for 1 hr at room temperature. Immunoreactive band was visualized by ECL reagent (Millipore). The antibodies used were as follows: GATA2 antibody (Santa, USA, 1:500), anti-E-cadherin (Abcam, USA, 1:10000), anti-N-cadherin (Abcam, USA, 1:10000), anti-Vimentin (Abcam, USA, 1:5000).

*Cell proliferation assays*

To explore the function of GATA2 on cell proliferation, utilize control siRNA or GATA2-siRNA to

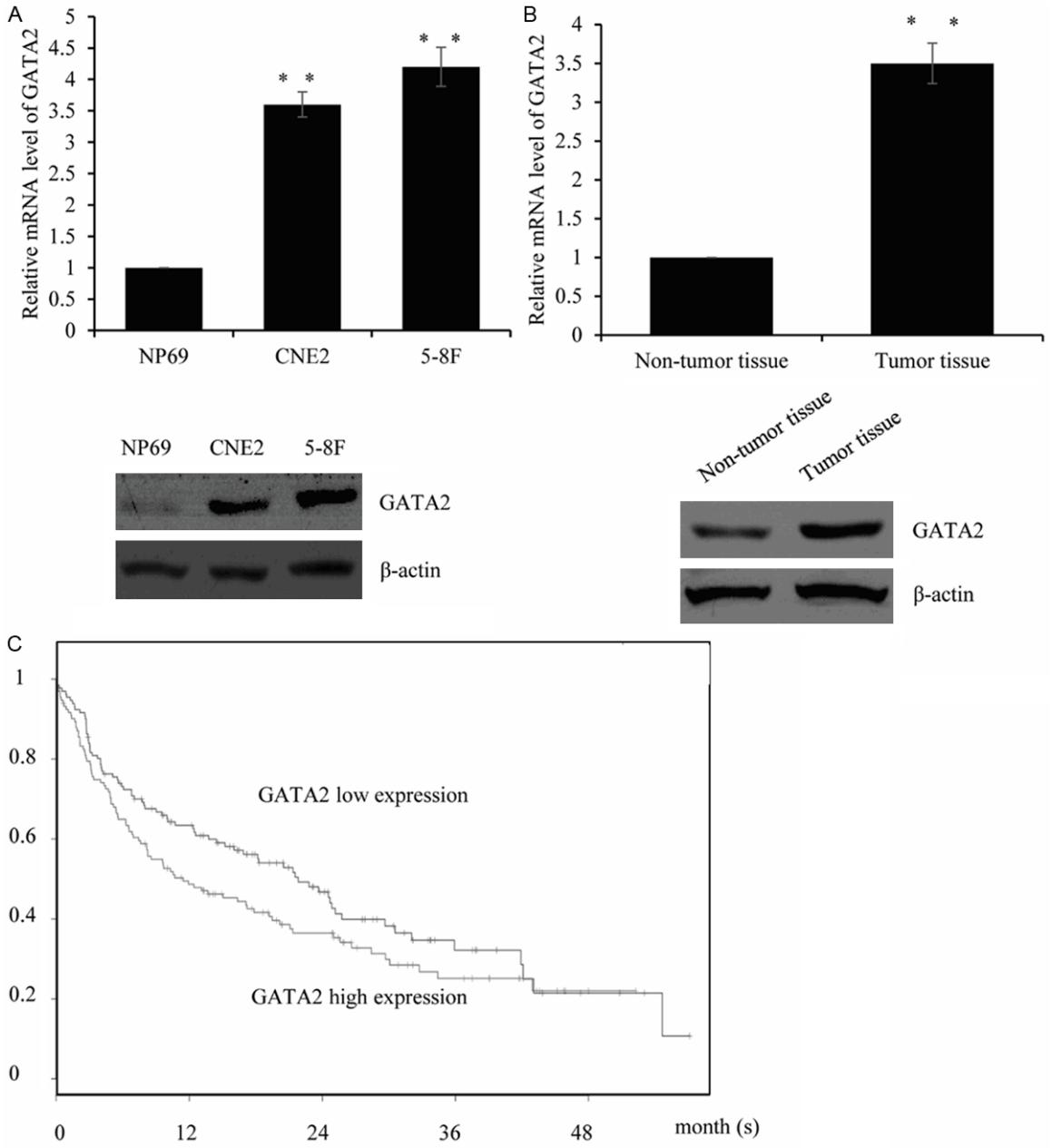



**Figure 1.** GATA2 is high expressed in NPC and predicted poor prognosis of NPC patients. A. The mRNA (up) and protein levels (down) of GATA2 between Human immortalized normal nasopharyngeal epithelial cell line NP69 and poorly differentiated nasopharyngeal carcinoma cell lines CNE2 and highly metastaticnasopharyngeal carcinoma cell lines 5-8F were detected by qRT-PCR and Western blotting. B. The mRNA (up) and protein levels (down) of GATA2 between non-tumor tissue and tumor tissue from NPC patients were detected by qRT-PCR and Western blotting. C. The Kaplan-Meier survival curves were analyzed by the online tool.

knockdown GATA2 in CNE2 cells, and then placed 5 × $10^3$ cells onto 96-well plate. Then at different time points add 10 μl CCK-8 solution per 100 μl media and incubated for 1 hr and measure the absorbance at 450 nm. Repeat all the experiments at least three times.

*Wound healing assay*

Analyze cell motility by wound healing assay, seeded 4 × $10^5$ cells which transfected with either control-siRNA or GATA2-siRNA on 6-well plates. While the cells were grown to almost

**Table 1.** Clinic pathologic variables in 186 NPC patients

| Variables | No. (n=186) | GATA2 protein expression | | *P* value |
|---|---|---|---|---|
| | | Low (n=80) | High (n=106) | |
| Gender | | | | |
| Male | 110 | 45 | 65 | 0.486 |
| Female | 76 | 35 | 41 | |
| Age | | | | |
| ≥60 | 91 | 43 | 48 | 0.523 |
| <60 | 95 | 37 | 58 | |
| Tumor size | | | | |
| Small (≤3 cm) | 76 | 46 | 30 | <0.001 |
| Large (≥3 cm) | 110 | 34 | 76 | |
| Pathological grade | | | | |
| I-II | 100 | 54 | 46 | 0.001 |
| III-IV | 186 | 26 | 60 | |
| Lymph node metastasis | | | | |
| Yes | 112 | 30 | 82 | <0.001 |
| No | 74 | 50 | 24 | |
| Drinking | | | | |
| Yes | 69 | 35 | 34 | 0.260 |
| No | 107 | 45 | 62 | |

80% confluence, scraping the cells to created wound with a 100 μl pipette tip. Cells were incubated at 37°C, follow by observe the migrated distance of cells in the microscope every 12 hr. Measured the relative migrating distance by the wound width/the original distance measured at 0 hr.

*Transwell migration assay*

Migration assays were performed by a Millipore chamber with a polycarbonate filter of 8 mm pore size (Millipore). High invasion NPC cell 5-8F were transfected with either control-siRNA or GATA2-siRNA, and placed to the upper chamber which contain media without serum, and the bottom chambers with complete DMEM media. Incubate cell for 16 hr in 37°C with 5% $CO_2$, used a cotton to remove the cells in the upper chamber and stain the lower membrane surface with 0.5% crystal violet. Counted the cells and repeated all the experiments for three times.

*Statistical analysis*

All the experiments were performed three independent times. Statistical analysis was performed using SPSS18.0 software (IBM Company). Analyze the correlation between the clinic pathological features of NPC and GATA2 expression by $\chi^2$ test. Using Student's t-test to analyzed the differences between groups.

## Results

*GATA2 is high expressed in NPC and predicted poor prognosis of NPC patients*

In order to determine the function of GATA2 in NPC, we first detected its expression at mRNA level and protein levels in NPC cell lines. The qRT-PCR was used to demonstrate that mRNA level of GATA2, results indicated GATA2 up regulate in poorly differentiated nasopharyngeal carcinoma cell lines CNE2 and highly metastaticnasopharyngeal carcinoma cell lines 5-8F compare with human immortalized normal nasopharyngeal epithelial cell line NP69 (**Figure 1A**). Western blot also confirmed GATA2 high expression in CNE2 and 5-8F compared with NP69 (**Figure 1A**). Subsequent, we collect NPC patients' tissue samples and inflammatory nasopharyngeal epithelium tissues. The similar result can be seen in qRT-PCR and Western blot, which represent mRNA and protein level of GATA2 (**Figure 1B**). Above results demonstrate that GATA2 was up regulate in NPC tissues and cell lines.

To further confirm our result, we collected 186 NPC samples from NPC patients, then performed immunohistochemically analysis. Interesting, GATA2 also exhibited abnormal immunoreactivity in NPC samples, however it had low expression in inflammatory nasopharyngeal epithelium tissues. Furthermore, we found GATA2 high expression was positive correlation with several clinic pathologic in NPC, such as tumor size, lymph node metastasis and pathological grade. But there are no significant correlation with gender, age and drinking (**Table 1**). Besides, we analyze survival curve in NPC by bioinformatics website. The data revealed that high expression of GATA2 achieved a lower overall survival rate than that with low expression of GATA2 (Hazard Ratio =1.25, P=0.01617,

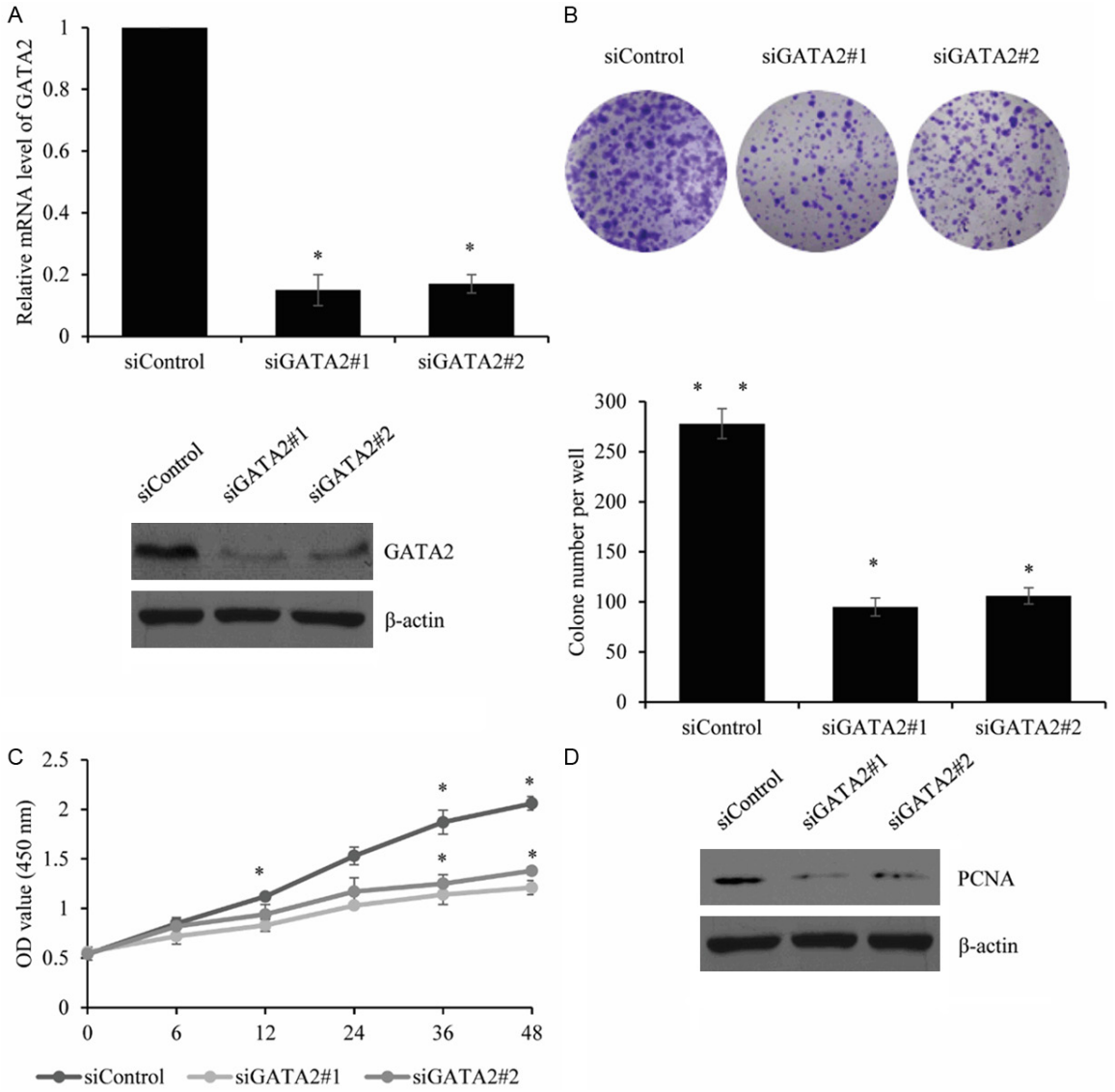
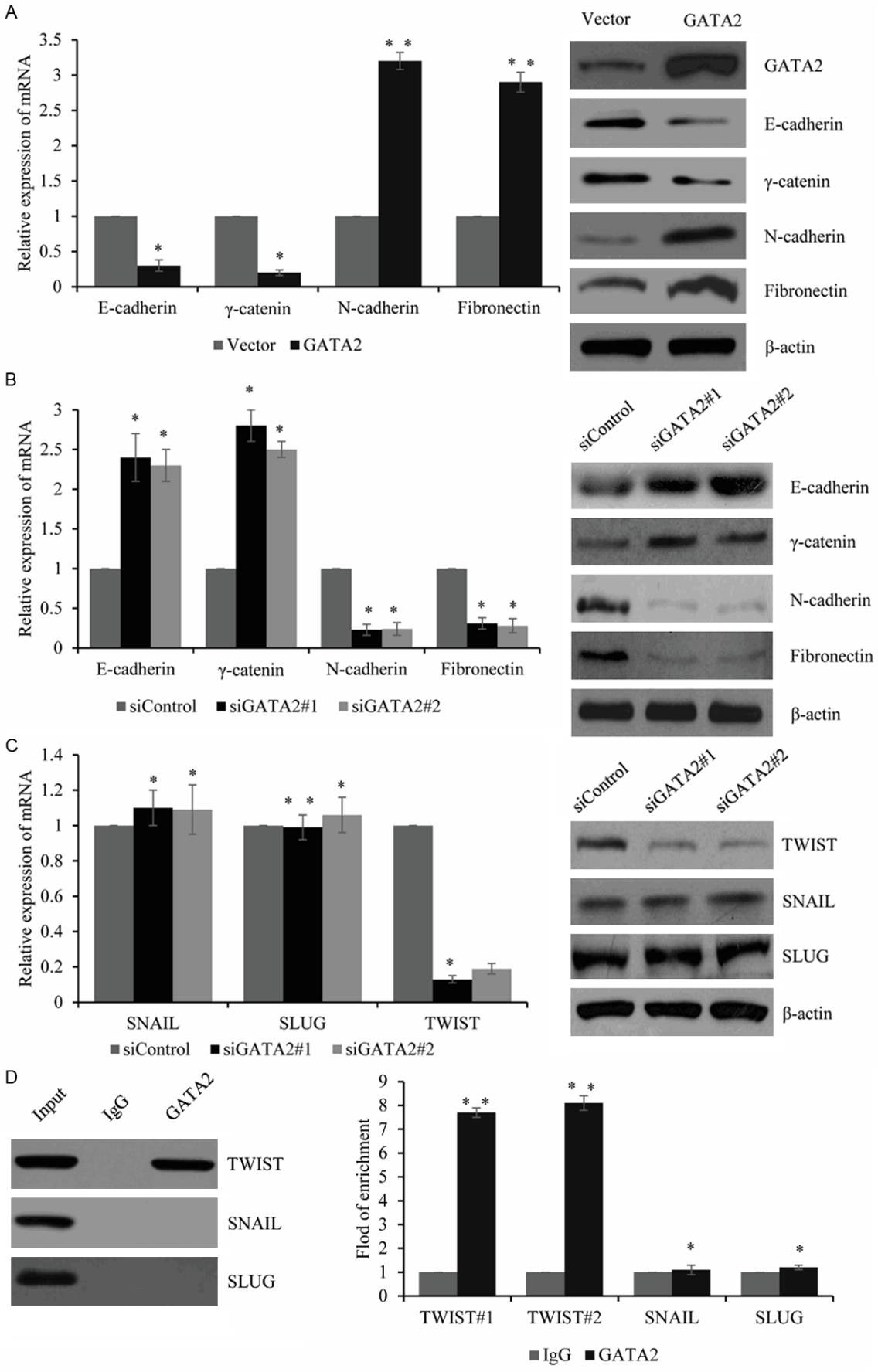



**Figure 2.** GATA2 promotes CNE2 cells growth. A. The qRT-PCR (up) and Western blot (down) were used to detect the efficiency of GATA2 siRNA in CNE2 cells. B. Knockdown GATA2 by GATA2 siRNA in CNE2 cells, and performedmonolayer colony formation assay, graph indicated the number of cells per well. were transfected with shSIRT6 or SCR, and performed CCK-8 assay to draw the growth curves. C. CCK-8 assay was used to draw the growth curves in CNE2 cell which transfected either control siRNA or GATA2 siRNA. D. Knockdown GATA2 by GATA2 siRNA in CNE2 cells, and detected PCNA protein expression.

**Figure 1C**). In conclusion, we hypothesized that GATA2 play a crucial role in NPC.

*GATA2 promotes CNE2 cells growth*

Our above results show that GATA2 was relationship with tumor size, so we assumed that GATA2 maybe promote tumorigenesis. In order to verify our hypothesis, we first knock down GATA2 by two different siRNA in CNE2 cells. As shown in **Figure 2A**, qRT-PCR revealed that mRNA levels of GATA2 were reduce more than 85% in CNE2 cells which transfected GATA2 siRNA, compared with control group which CNE2 cells transfected control siRNA. Western bolt revealed that protein level of GATA2. Next, monolayer colony formation assay was performed. The data revealed that cells with an interference of GATA2 effectively suppress cell tumorigenesis, including foci formation frequency assay and cell growth rate assay in vitro assays (**Figure 2B** and **2C**). However, while we over expressed GATA2, an opposite results can be seen (data not shown). Meanwhile, PCNA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Relative expression of mRNA
E-cadherin
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Fibronectin
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Relative expression of mRNA
siControl
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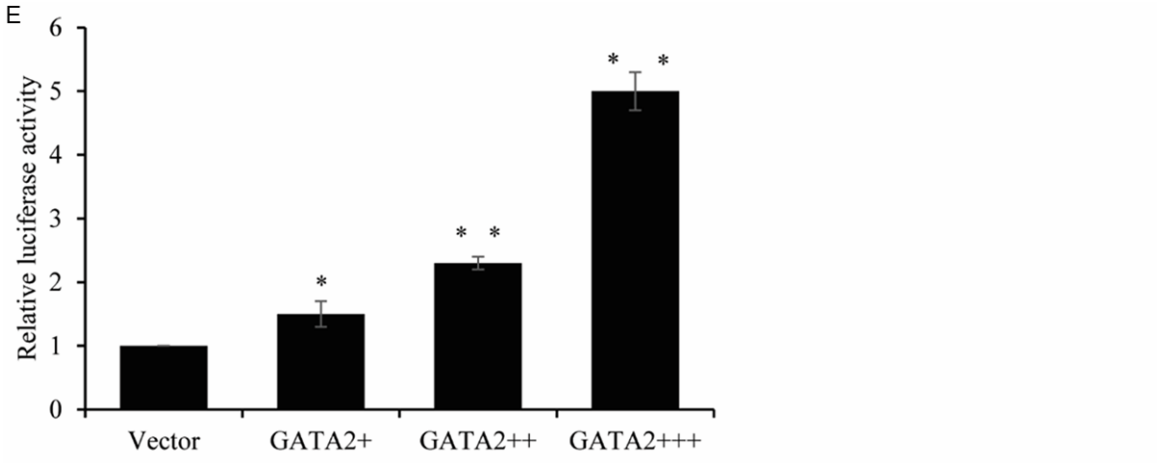


**Figure 3.** GATA2 promotes EMT in CNE2 cells by inducing Twist. A. The qRT-PCR (left) and Western blot (right) analysis all demonstrate a criticalreduce epithelial markers, whereas increased mesenchymal marker in CNE2 cells which over express GATA2. B. The qRT-PCR (left) and Western blot (right) analysis demonstrate a significantupregulate of epithelial markers, whereas downregulate of mesenchymal marker in CNE2 cells which GATA2 was depleted. C. The qRT-PCR (left) and Western blot (right) were performed to detected TWIST, SNAI1 or SLUG mRNA and protein level in CNE2 cells which GATA2 was depleted. D. ChIP analyses was performed with GATA2 antibody, subsequent qRT-PCR was performed with specific primers for *TWIST*, *SNAIL* and *SLUG* mRNA. E. Luciferase reporter assay was used to explore whether TWIST was transcriptional activated by GATA2.

was decrease when GATA2 was depletion. In brief, the high expression of GATA2 strongly influenced NPC tumorigenesis.

*GATA2 promotes EMT in CNE2 cells by inducing Twist*

Epithelial-mesenchymal transition (EMT) is a complex process, it not only influences cancer development, but also regulates cancer cell migration and invasive capacity. We next examined whether GATA2 play role on NPC cells was associated with EMT. We over expressed GATA2 in CNE2 cells, the qRT-PCR and western blot all showed that the epithelial marker, E-cadherin and γ-catenin, were dramatic decrease. Simultaneously, the mesenchymal marker, N-cadherin and Fibronectin, were significant increase when over express GATA2 in CNE2 cells (**Figure 3A**). While we used siRNA to knockdown GATA2 in CNE2 cells, the oppose result can be seen, the epithelial markers were dramatic increase, and the mesenchymal markers were significant reduce (**Figure 3B**). Collectively, GATA2 take participate in EMT.

To further investigate the molecular mechanisms that GATA2 regulates EMT, we decided to identify transcription factors which expression was influenced by GATA2. Since several transcription factors play crucial function in EMT process, including SLUG, SNAI1 and TWIST, then we testified whether GATA2 regulated their expression in CNE2 cells. As shown in **Figure 3C**, qRT-PCR demonstrate that TWIST was significantly decrease in CNE2/siGATA2 cells, suggesting that TWIST was regulate by GATA2. However SNAI1 and SLUG wereslightly depend on GATA2 in CNE2 cells (**Figure 3C**). Similar results can be seen in protein level by Western blot, it confirmed that GATA2 depleted reduced the expression of TWIST in CNE2 cells (**Figure 3C**). ChIP and qChIP assay all confirmed the recruitment of GATA2 in the region of the *TWIST promoter region* in CNE2 cells (**Figure 3D**). Furthermore, luciferase reporter assay also confirmed that GATA2 transcription stimulation TWIST (**Figure 3E**). Together, GATA2 promote EMT through transcriptional stimulation TWIST.

*Interference of GATA2 expression inhibited the migration of CNE2 cells*

Cancer cell metastasis was a critical reason that result in NPC patients have a poor prognosis. Our study also demonstrated that GATA2 high expression correlation with lymph node metastasis and poor prognosis. So we subsequently investigated the effect of GATA2 on cancer cell migration with transwell migration assays and wound-healing assay. We knockdown GATA2 in highly metastatic nasopharyn-

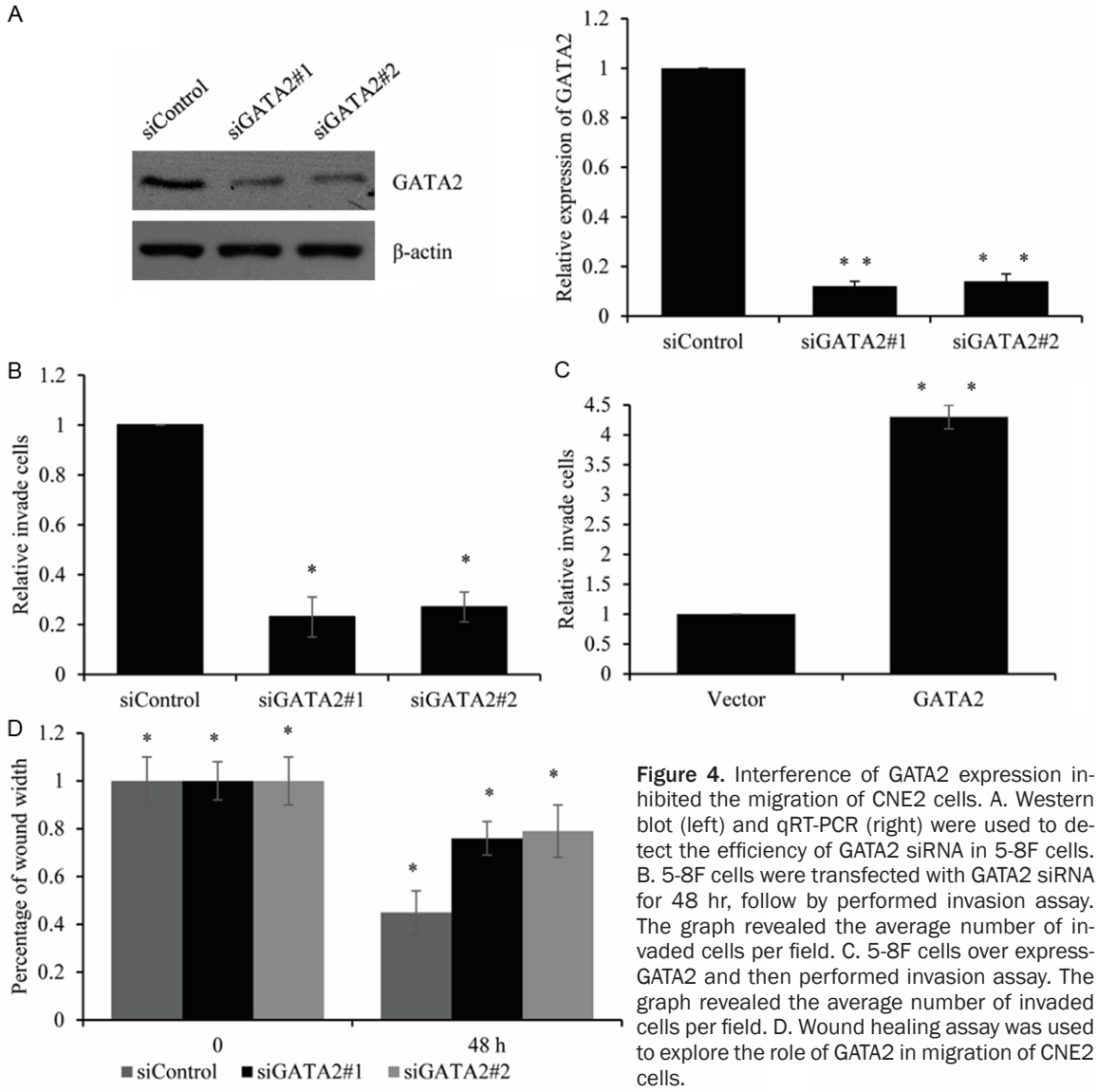


**Figure 4.** Interference of GATA2 expression inhibited the migration of CNE2 cells. A. Western blot (left) and qRT-PCR (right) were used to detect the efficiency of GATA2 siRNA in 5-8F cells. B. 5-8F cells were transfected with GATA2 siRNA for 48 hr, follow by performed invasion assay. The graph revealed the average number of invaded cells per field. C. 5-8F cells over express-GATA2 and then performed invasion assay. The graph revealed the average number of invaded cells per field. D. Wound healing assay was used to explore the role of GATA2 in migration of CNE2 cells.

geal carcinoma cell lines 5-8F and detected the efficiency of GATA2 siRNA in 5-8F cells (**Figure 4A**). Then performed transwell assay, first serum starvation for 24 hr and planted cells in transwell chamber. After stain with 0.5% crystal violet, we found that the number of invade cell was significantly decrease in cell which GATA2 was depletion, compared with control group (**Figure 4B**). Reciprocally, the number of invaded cell was as twice as control group when 5-8F cells over expressed GATA2 (**Figure 4C**). Together, it indicated that GATA2 would significant enhance the invasion ability of 5-8F cells. We next performed wound-healing assay, the similar results can be seen. Within 48 hr, the control group had longer migrating distance than GATA2 depleted cells (**Figure 4D**). All the results supported that GATA2 obviously stimulate cell migration.

## Discussion

Nasopharyngeal carcinoma is one of the most popular carcinoma in Southeast Asia and China [11]. The distant metastasis is one reason of poor prognosis of NPC [12]. Although there are some studies about NPC [13-16], the detail molecular mechanisms of NPC were still unknown. Nowadays, there are more and more studies reported that gene change play an important role in cancer progression, so finding a novel molecular is a key to explore the moleculesmechanism of NPC. It may help us to discover a new prognostic markers and potential therapeutic targets.

In the current experiments, we detected that GATA2 expression was higher in NPC cell lines and patient tissues samples. Then we performed IHC to verify whether the expression of GATA2 in NPC samples is high, and we found GATA2 is high expression in NPC patient tissue sample than inflammatory nasopharyngeal epithelium tissues. Meanwhile, we found that that high GATA2 expression was associated with pathological grade, tumor size and poor prognosis Survival curve analysis indicated that low expression of GATA2 have longer survival rate than high expression of GATA2.

Since high expression of GATA2 was associated with tumor size and lymph node, we assume that GATA2 maybe regulate tumor cell growth and metastasis. In order confirmed our hypothesis, we first used siRNA to knockdown GATA2 and then monolayer colony formation assay was showed that cells which depleted GATA2 critical suppress tumor cell proliferation. The similar result can be seen in CCK-8 assay. Meanwhile, when GATA2 was depleted, PCNA also reduce. In brief, the high expression of GATA2 strongly influenced NPC tumorigenesis.

An important event which influence nasopharyngeal carcinoma patients' prognosis is metastasis, nasopharyngeal carcinoma always has a tendency to metastasize to lymph nodes. Next, we used transwell migration assays and wound-healing assays to investigate the function of GATA2 in NPC migration. The results revealed that slicing GATA2 could dramatically suppress cells migrating ability.

Epithelial-mesenchymal transition (EMT) is a complex process that regulated by numerous mechanisms, such as transcript factors, signal pathway and so on. One obvious characters of EMT is cells phenotype changed from epithelial to the mesenchymal and enhance cells migratory and invasive capacity [17]. E-cadherin and γ-catenin as epithelial marker are currently reduced, however mesenchymal marker, N-cadherin and fibronectinare normal increase. EMT always closely associated with tumor malignant. As GATA family play a critical roles in carcinogenesis, several studies had been found the correlation of GATA with EMT. But the role of GATA2 in EMT is still unknown. In order to investigate the mechanisms of GATA2 in EMT, we utilized GATA2 siRNA to knocked down GATA2 in CNE2 cells and found the epithelial marker E-cadherin and γ-catenin were increased, but the mesenchymal marker vimentin and N-cadherin were decreased. Moreover, a series of experiments indicated that EMT associated transcript factor, TWIST, is regulated by GATA2. In summary, GATA2 promote EMT through transcript activity TWIST.

In brief, we have demonstrated that GATA2 is a potent oncogenic in NPC. It not only could predict prognostic information of NPC patients, but also was influence growth and metastasis of nasopharyngeal carcinoma cells. All results represented that GATA2 a potential biomarker for nasopharyngeal carcinoma aggressiveness and a potential target for nasopharyngeal carcinoma intervention.

## Disclosure of conflict of interest

None.

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