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Long non-coding RNA HOTAIR promotes HLA-G expression via inhibiting miR-152 in gastric cancer cells

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ABSTRACT

Recent studies have shown that the long non-coding RNA HOTAIR plays critical roles in tumor biology, including cancer progression and metastasis. However, the potential biological role HOTAIR in tumor escape remains undefined. Here, HOTAIR expression was measured in sixty paired gastric cancer (GC) tissue samples by real-time PCR, and then subjected to correlation analysis with human leukocyte antigen (HLA)-G levels which show close links with tumor escape mechanisms. Significant HOTAIR overexpression was observed in GC tissues, as well as strong positive correlations with HLA-G levels in both tissue and peripheral blood samples, detected by real-time PCR and ELISA assays respectively. Further gain- and loss-of-function studies indicated that HLA-G could be upregulated HOTAIR at both mRNA and secretion levels in vitro. On the other hand, bioinformatics analysis indicated the interaction between HOTAIR and miR-152, which shows potential regulation on HLA-G. And, altered miR-152 expression in GC tissues was also identified, and showed negative correlation with HOTAIR expression. Moreover, the negative regulation of miR-152 on HLA-G was verified in GC cells, while miR-152 induced decrease of HLA-G 3′UTR activity could be attenuated by HOTAIR co-overexpression with the assistant of mutation studies. Therefore, it was concluded that HOTAIR overexpression might also get involved in tumor escape mechanisms, involving HLA-G upregulation via inhibiting miR-152. Furthermore, this study recommended the potential application of HOTAIR in GC immunotherapy for better prognosis and improved survival.

1. Introduction

Gastric cancer (GC) is not only one of the most frequently diagnosed cancers and but also one of the leading causes of cancer death worldwide [1]. Furthermore, GC is among the most frequent malignant tumors in East Asian countries [2]. Although early diagnosis and improved outcome have been observed after curative resection, most GC patients are diagnosed at an advanced stage missing the most appropriate times for diagnosis and treatments. Since conventional chemotherapy and irradiation have little effect on the treatment for advanced GC, more attentions have been paid to novel effective therapeutic modalities such as immunotherapy [3].

Cancer immune surveillance is considered to be an important host protection process to inhibit carcinogenesis and to maintain cellular homeostasis [4]. Some tumor rejection antigens have been used as potential targets for specific immunotherapy in GC patients, which results in tumor rejection [5,6]. However, many neoplasms can escape such host immune surveillance [7]. The human leukocyte antigen-G (HLA-G), a newly identified member of the non-classical MHC family, shows important role in the escape of tumor cells from host immune surveillance by suppressing functions of various immune cells [8]. HLA-G associated immune escape in GC has also been characterized in recent studies [9].

Long non-coding RNAs (lncRNAs), a new class of non-coding RNAs (ncRNAs) with lengths ranging from 200 bp to 100 kbp, have been receiving increased attentions, especially in cancer

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research [10]. Amounting functional lncRNAs have been well characterized in the regulation on gene expression at various levels, including chromatin modification, transcription and post-transcriptional processing [11]. HOX transcript antisense intergenic RNA (HOTAIR), one of the well-studied lncRNAs, is initially identified to regulate gene expression by mediating the modulation of chromatin structure [12], while subsequent studies provide abundance evidence for the important role of HOTAIR in tumor biology [13], including tumorigenesis, metastasis, and drug resistance. Recent studies also reveal HOTAIR upregulation in GC, as well as underlying molecular mechanisms [14]. However, the biological role of HOTAIR in tumor immune escape remains undefined, let alone underlying molecular mechanism.

In the present study, the correlation between HOTAIR and HLA-G expression in tissues from GC patients was analyzed wherein a strong positive relationship was observed, indicating the biological potential role of HOTAIR in tumor immune escape. Further studies were performed to investigate the regulation of HOTAIR on HLA-G expression in GC cells. MiR-152, as one candidate from microRNAs that showing abnormal expression in GC tissues or cells and potential targeting on HLA-G expression, was picked up here to undergo further bioinformatics and correlation analysis with HOTAIR. Finally, the regulation of miR-152 on HLA-G was introduced to underlying molecular mechanism within the regulation of HOTAIR on HLA-G expression.

2. Materials and methods

2.1. Patients and samples

Sixty GC tissues and matched adjacent tissue samples were collected from 60 patients who underwent surgical resection for GC at Qilu Hospital of Shandong University from January 2013 to August 2013. Paraffin-embedded, formalin-fixed tumor sections were prepared. And, corresponding peripheral blood samples were also collected one day before surgery for EDTA-plasma preparation [15], and frozen at −80 °C until use. None of the patients had received immunosuppressive drugs or chemotherapy before surgical resection. Written informed consent was obtained from each patient and the work was approved by local Ethics Committee.

2.2. Cell culture

Two human GC cell lines, SGC7901 and MGC-803, were obtained from the Chinese Academy of Sciences (Shanghai, China), and cultured in RMPI-1640 medium, supplemented with 10% FBS (Invitrogen, NY, USA) and antibiotics (1% streptomycin/penicillin, Sigma–Aldrich) at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Real-time PCR

Total RNA was isolated from cells and tissues using Trizol (Invitrogen). For the detection of HLA-G mRNA and HOTAIR, RNA was reverse transcribed into cDNA primed by oligo-dT primers using SuperScript III Reverse Transcriptase (Invitrogen), following the manufacturer's instructions, with GAPDH used as an endogenous control. For the detection of mature miR-152, the enrichment of small RNA was carried out with the mirVana miRNA Isolation Kit (Ambion, Austin, TX), with Small nuclear RNA U6 as control. Quantitative real-time PCR (qRT-PCR) was performed on ABI 7300 SYBR Premix Ex Taq from Takara (Tokyo, Japan). Primers used for real-time PCR were listed in Table 1. Data analysis was done by the ΔCT method for relative quantification.

Table 1
Primers used for real-time PCR.

Primers	Sequences (5′-3′)
HLA-G-F	GAGGAGACACGGAACACCAAG
HLA-G-R	GTCGCAGCCAATCATCCACT
HOTAIR-F	TTTGGACTGTAAAATATGGC
HOTAIR-R	TTCTGACACTGAACGGACT
GAPDH-F	GTGAAGCAGGCGTCGGA
GAPDH-R	AGCCCCAGCGTCAAAGG
miR-152-F	ACACTCCAGCTGGGACAAGTCGGAG
miR-152-R	CTCAACTGGTGTCGTGGAGTCGGCAATTCAAGGTTCTGTGATACT
U6–F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT

2.4. ELISA assays

EDTA-plasma samples or cell culture supernatants after 48 h incubation in serum-free medium were prepared for ELISA assays. Soluble HLA-G concentration was measured using sHLA-G kit (Biovendor&Exbio, Praha, CZ) according to the manufacturer's instructions. Three independent experiments were repeated for each point.

2.5. Bioinformatics

In silico prediction of the interaction between HOTAIR transcript and miR-152 was performed using DIANA TOOLS (http://diana.imis.athena-innovation.gr/DianaTools) as described before [16]. In additional, Mut-HOTAIR transcript was emerged according to the binding sites of miR-152 within HOTAIR transcript.

2.6. Plasmids and small interfering RNAs (siRNAs)

HOTAIR overexpression plasmid was constructed using pCDNA3.1 (Invitrogen, Shanghai, China) vector with inserted human full-length HOTAIR cDNA, that synthesized with primers (sense: 5′-CCAGTTCTCAGGCGAGAGCC-3′; antisense: 5′-TTTATATTCAGGACATGTAA-3′). Mutation in the HOTAIR binding-sites module of miR-152 was introduced by site-directed mutagenesis, resulting in Mut-HOTAIR overexpression plasmid. All plasmid vectors for transfection were extracted by DNA Midiprep kit (Qiagen, Hilden, Germany). Small interfering RNAs (siRNAs) and scrambled negative control siRNA (si-NC) that purchased from Invitrogen (Invitrogen, CA, USA) were used for HOTAIR inhibition. The sequences of three individual HOTAIR siRNAs are as follows: siHOTAIR-1: GAACGGGAGUACAGAGAGAUU; siHOTAIR-2: CCACAUGAACGCCCAGAGAUU; siHOTAIR-3: UAACAAGACCAGAGAGCUGUU.

2.7. Cell transfection

MiR-152 mimics and inhibitor with their relative negative control RNA were obtained from GenePharma (Shanghai, China). SGC7901 and MGC-803 cells (2.5×10^5) were plated in 6-well culture plates and transfected after incubation for 24 h. Plasmid, siHOTAIR, miR-152 mimics or inhibitor was introduced into GC cells respectively using Lipofectamine2000 (Invitrogen) in Opti-MEM medium (Invitrogen) according to the manufacturer's instructions.

2.8. Luciferase assay

A Firefly luciferase reporter comprising 3′UTR of HLA-G was generated using pGL3 vector (Promega, Madison, WI) and amplified cDNA of HLA-G 3′UTR with the following primers: HLA-G-3′UTR-forward: 5′-GGGGTACCGATGGGTGAGTTCAACGAGA-3′; HLA-G-

Table 2
Characteristics of gastric cancer patients.

Variable	Patients
Number of cases	60
Sex	
Male/Female	35/25
Age at diagnosis (mean)	55.8
Clinical pathology features	
TNM stage	
I	10
II	19
III	31
Lymph node metastasis	
Negative	11
Positive	49
Histologic grade	
Well	7
Poor	53

3′UTR-reverse: 5′-CCCTCGAGAGGGTGGGTGTCATCAGG-3′. Then, pGL3-HLA-G was co-transfected with plasmid, siHOTAIR, miR-152 mimics or inhibitor for luciferase reporter assay. After 24 h incubation, luciferase activity was measured using the Dual Luciferase Reporter 1000 Assay System (Promega).

2.9. Statistical analysis

The results are presented as mean ± SD. Correlations were evaluated by Pearson's correlation. Differences between groups were analyzed using a one-way ANOVA or χ^2 test. Statistical analyses were performed using SPSS 17.0 computer software (SPSS Inc., Chicago, IL). And, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical and demographic characteristics

Retrospective examination was performed of sixty GC patients who underwent surgery for gastric cancer at the Qilu Hospital of Shandong University. Patient characteristics are shown in Table 2. The mean age of patients at diagnosis was 55.8 years and the male/female ratio was 35/25.

3.2. HOTAIR expression showed positive correlation with HLA-G expression in GC tissues and concentration in peripheral blood

As a first step to investigate the relationship between HOTAIR and HLA-G, the level of HOTAIR and HLA-G expression were determined in tissues samples from GC patients by real time PCR, while HLA-G concentration was analyzed in peripheral blood samples using ELISA assays. HOTAIR expression showed significant upregulation in GC tissues compared with normal controls ($P < 0.01$; Fig. 1A). Furthermore, Pearson's correlation analysis showed a strong positive relationship between HOTAIR and HLA-G expression ($R^2 = 0.4805$, $p < 0.001$; Fig. 1B), while similar results was also observed for HOTAIR expression and HLA-G concentration ($R^2 = 0.4865$, $p < 0.001$; Fig. 1C). These results implied that HOTAIR might regulate HLA-G expression in GC carcinogenesis.

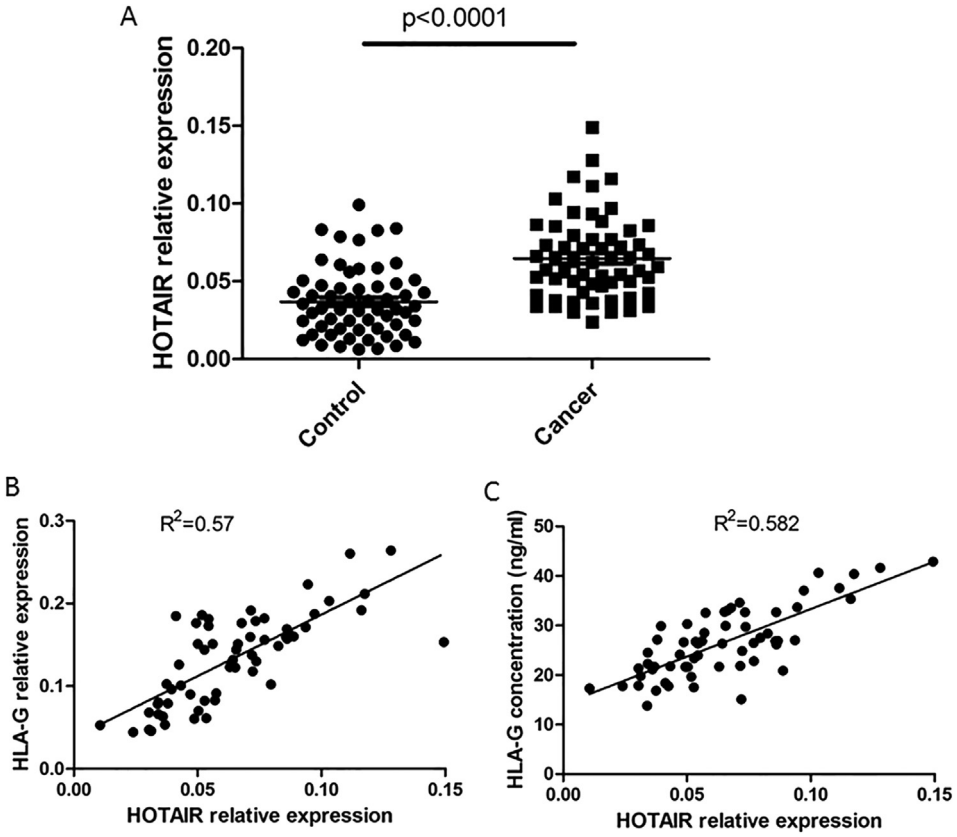

Fig. 1. Correlation analysis between HOTAIR and HLA-G expression in GC tissues, as well as HLA-G level in peripheral blood. HOTAIR relative expression was analyzed by real time PCR in GC tissues from sixty GC patients (Cancer) as compared to normal ones (Control) (A, $p < 0.0001$). And, Pearson's correlation was performed to analyze the correlations between HOTAIR and HLA-G expression in GC tissues (B, $R^2 = 0.57$; $p < 0.001$) or HLA-G level in corresponding peripheral blood (C, $R^2 = 0.582$; $p < 0.001$).

3.3. HOTAIR promoted HLA-G expression in GC cells

For further investigation about the potential regulation of HOTAIR on HLA-G expression, HOTAIR expression was dysregulated by HOTAIR overexpression plasmid or siHOTAIR transfection in both SGC7901 and MGC-803 cells. After dysregulated HOTAIR expression was confirmed, HLA-G expression and secretion were both detected in vitro. As shown in Fig. 2 A, 16.1-fold increase or 3.7-fold decrease of HOTAIR expression was verified in HOTAIR overexpressing or inhibiting SGC7901 cells respectively, while 16.7-fold increase or 3.7-fold decrease was observed in corresponding MGC-803 cells. Moreover, approximate 2-fold increase or 0.5-fold decrease of HLA-G expression was detected in HOTAIR over-expressing or inhibiting cells respectively (Fig. 2B), while HLA-G secretion showed similar variation trend in corresponding cell culture supernatants (Fig. 2C). These results declared that HOTAIR could promote HLA-G expression in GC cells.

3.4. HOTAIR showed direct interaction with miR-152 and negative regulation on miR-152 expression

Previous studies have shown the regulation of miR-152 on HLA-G expression, so the interaction between HOTAIR and miR-152 was speculated to be involved in regulation of HOTAIR on HLA-G. Firstly, bioinformatics analysis for the predicted interaction revealed three potential binding domains within HOTAIR transcripts and miR-152 (Fig. 3A), while “GCACUG” as common sequences module within these binding domains was replaced by “AAGAGA” to emerge a new Mut-HOTAIR transcript for following mutation studies. Moreover, decreased expression of miR-152 was detected in GC tissues ($p = 0.0003$; Fig. 3B), in line with previous studies. Additionally, correlation analysis showed a strong negative relationship between HOTAIR and miR-152 expression ($R^2 = 0.4134$, $p < 0.001$; Fig. 3C), indicating that abnormal HOTAIR expression might also lead to miR-152 dysregulation due to their interactions. Therefore, miR-152 expression was also detected in HOTAIR overexpressing or inhibiting cells. Results showed that HOTAIR could induce significant downregulation of miR-152 in vitro, while Mut-HOTAIR showed no significant effect on miR-152 downregulation, with miR-152 mimics or inhibitor transfection as positive control ($P < 0.01$; Fig. 3D). Furthermore, HOTAIR inhibition led to obviously upregulated miR-152 ($P < 0.01$; Fig. 3E). Thus, HOTAIR could negatively regulated miR-152 expression probably through their direct interaction.

3.5. HOTAIR induced HLA-G up-regulation via inhibiting miR-152 expression

Since miR-152 dysregulation was illustrated under abnormal HOTAIR expression, HOTAIR induced HLA-G up-regulation might owe to inhibited miR-152 expression in HOTAIR overexpressing cells. In order to demonstrate this speculation, the regulation of miR-152 or HOTAIR on HLA-G expression was compared through gain- and loss-of-function studies. As shown in Fig. 4, relative expression (Fig. 4A), concentration (Fig. 4B) or 3′UTR activity (Fig. 4C) of HLA-G was consistently downregulated or upregulated

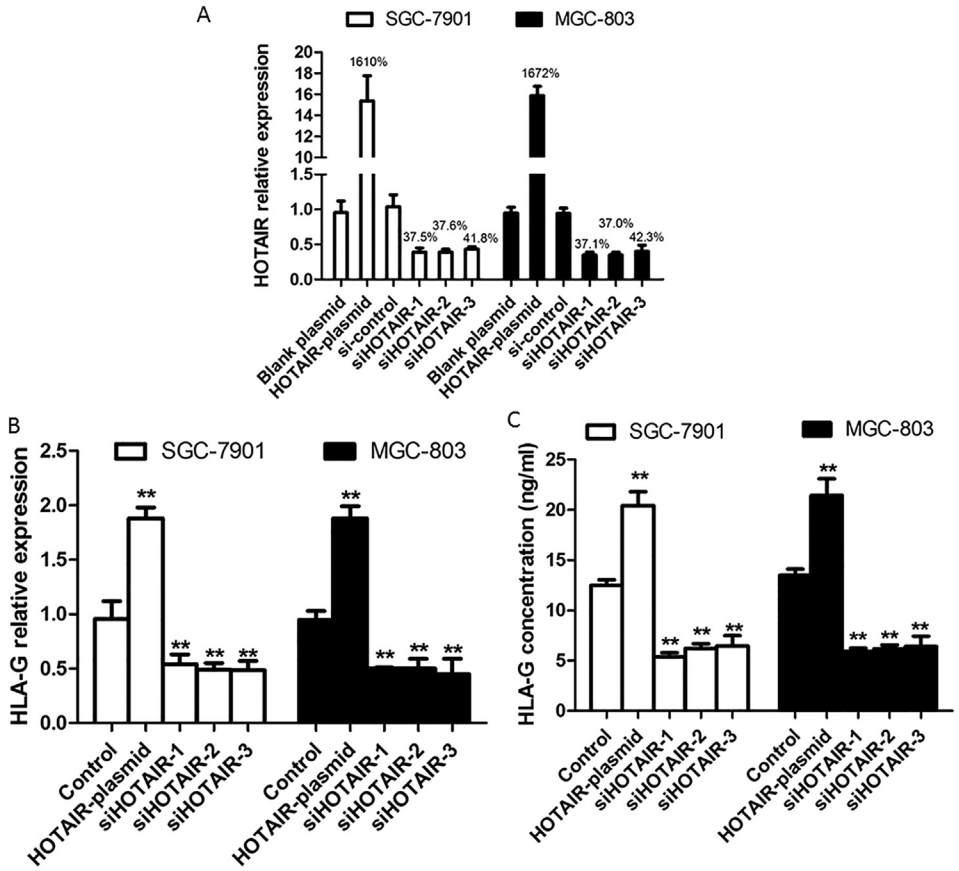

Fig. 2. HOTAIR promoted HLA-G expression in GC cells. A. Detection for HOTAIR expression in SGC7901 and MGC803 cells after transfected with HOTAIR overexpression plasmid (HOTAIR-plasmid) or HOTAIR siRNAs (siHOTAIR-1, siHOTAIR-2 or siHOTAIR-3) for 24 h. B. HLA-G mRNA expression in HOTAIR overexpressing or inhibiting cells after transfection for 24 h. C. HLA-G level detection after HOTAIR plasmid or siRNAs transfection for 48 h using ELISA analysis. **P < 0.01.

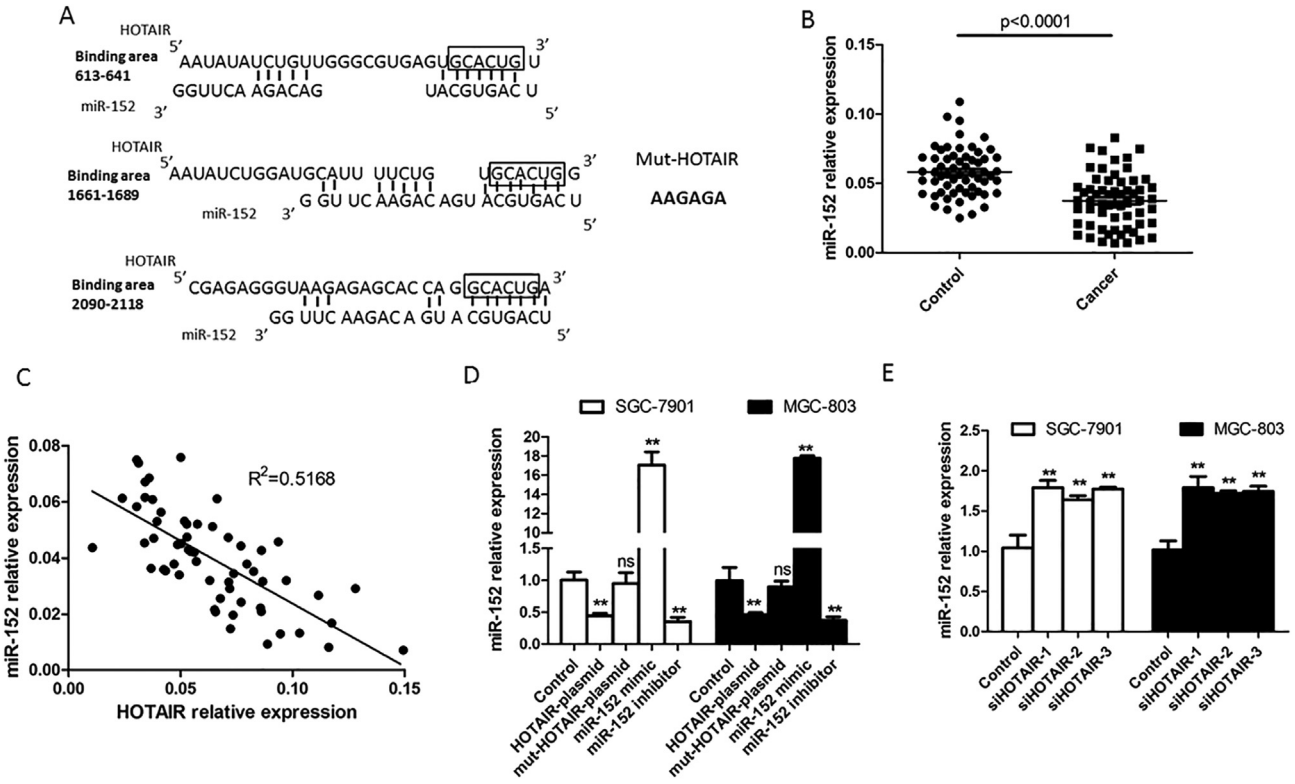

Fig. 3. Bioinformatics analysis for the potential interaction between HOTAIR and miR-152, and HOTAIR regulation on miR-152 expression. A. Three potential binding domains within HOTAIR transcripts through bioinformatics analysis using DIANA TOOLS. And, the common sequences "GCACUG" within binding domains was chosen to be replaced by "AAGAGA" for generating Mut-HOTAIR transcript. B. MiR-152 relative expression in tissues from sixty GC patients (Cancer), as compared to normal ones (Control). $p < 0.0001$. C. Correlation analysis between HOTAIR and miR-152 expression in GC tissues. $R^2 = 0.5168$; $p < 0.001$. D. MiR-152 expression in HOTAIR or Mut-HOTAIR overexpressing cells with mut-HOTAIR-plasmid transfection after transfection for 24 h, with miR-152 mimics or inhibitor transfecting cells as controls. ns: non significance; **$P < 0.01$. E. MiR-152 expression in HOTAIR inhibiting cells after transfection for 24 h **$P < 0.01$.

by miR-152 or HOTAIR respectively, but not modulated by Mut-HOTAIR. Furthermore, miR-152-induced downregulation of HLA-G 3′UTR activity could only be reversed by HOTAIR overexpression (Fig. 4D), while Mut-HOTAIR overexpression also showed no obvious effect. These results showed that HOTAIR-induced downregulation of miR-152 attenuated the post-transcriptional regulation of miR-152 on HLA-G, contributing to HLA-G upregulation.

4. Discussion

In this study, significantly upregulated HOTAIR expression was observed in tissues from GC patients compared with normal paired ones. And, subsequent correlation analysis revealed strong positive relationships between HOTAIR and HLA-G expression at both mRNA and concentration level. Further in vitro studies identified the positive regulation of HOTAIR on HLA-G expression and secretion. As bioinformatics analysis for the interaction with miR-152 showed three potential binding domains within HOTAIR transcript, and correlation analysis also revealed a strong negative relationships between HOTAIR and miR-152 expression, the potential negatively regulation of HOTAIR on miR-152 expression *via* their interactions was then verified in vitro with the assistant of mutation studies. Besides, miR-152 induced abnormal HLA-G expression was also demonstrated in GC cells and this regulation was further proven to be mediated by the regulation of HOTAIR on HLA-G expression.

HOTAIR, named from being transcribed in an antisense manner with canonical HOXC genes, is a 2158 nts ncRNA, residing in the HOXC locus on chromosome 12q13 [12]. Abnormally high expression of HOTAIR in GC tissues was confirmed here which is consistent with results of several recent studies that reveal many important roles of HOTAIR in GC development [17–19]. The high expression of SUZ12 suggests the role of HOTAIR in tumor metastasis through retargeting of PCR2 and trimethylation of H3K27 [20]. Moreover, its role in promoting tumor invasion is explained by HOTAIR inducing the epithelial–mesenchymal transition (EMT) through regulation of Snail and matrix metalloproteinases (MMPs) [21]. Nevertheless, HOTAIR may also function as a competing endogenous RNA (ceRNA), becoming a sink for miR-331-3p, which leads to the regulation of human epidermal growth factor receptor type 2 (HER2) and an additional level of post-transcriptional regulation [22].

Except for the carcinogenic process in GC development, tumor escape mechanisms have also been receiving increasing attentions as immunotherapy developing [3]. Therefore, the potential role of HOTAIR in tumor escape was investigated here on the basis of the fact that HLA-G expression, which shows close links with tumor escape mechanisms [23], also behaves upregulated trends in GC tissues. In this study, both HLA-G expression levels in GC tissues and protein concentration in corresponding peripheral blood samples were detected, as well as subsequent correlation analysis with HOTAIR expression, which showed a strong positive relationship between HOTAIR and HLA-G. Furthermore, HLA-G expression was significantly upregulated or down-regulated in HOTAIR overexpressing or inhibiting GC cells respectively.

Amounting evidence have revealed the posttranscriptional regulation on HLA-G expression by microRNAs [24], among which

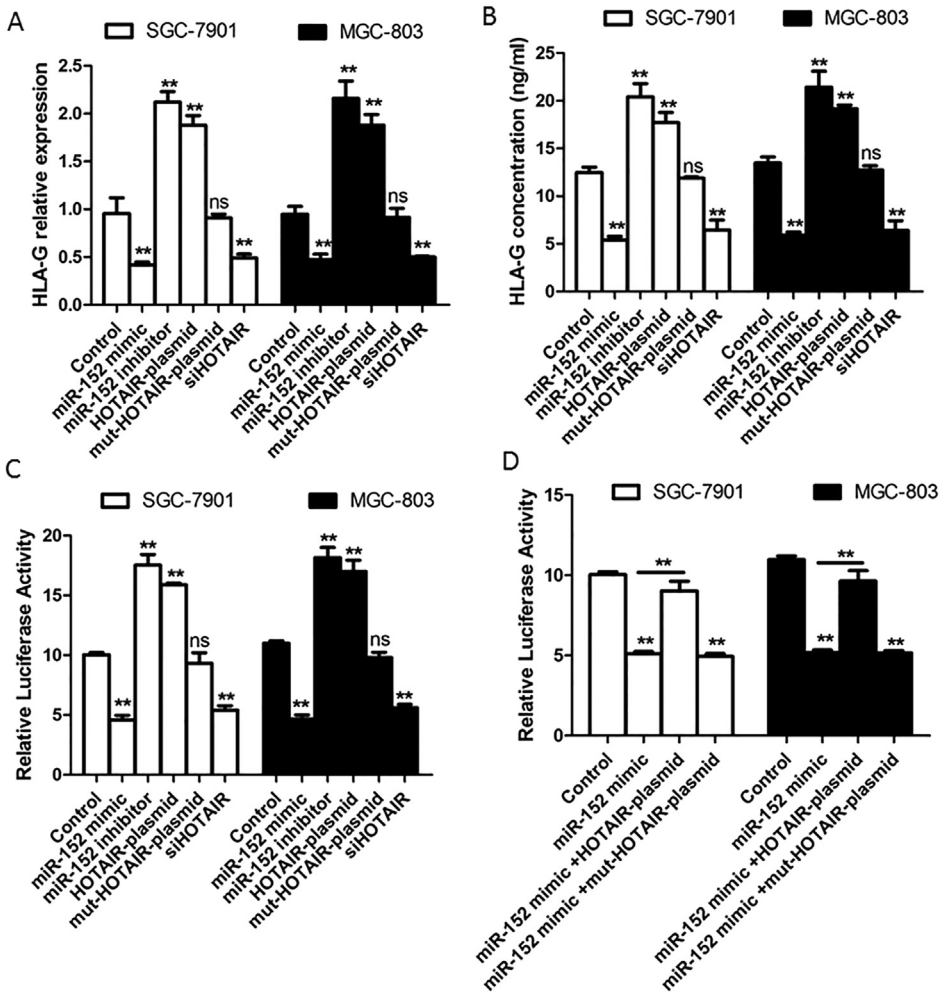

Fig. 4. HOTAIR induced HLA-G up-regulation via inhibiting miR-152 expression. HLA-G mRNA expression (A) was detected in miR-152 mimics or inhibitor transfecting cells after transfection for 24 h, with HOTAIR overexpressing or inhibiting cells as comparison, wherein Mut-HOTAIR overexpression was also introduced and siHOTAIR-3 was chosen as siHOTAIR. And, HLA-G level detection was performed after transfection for 48 h (B), while HLA-G 3′UTR activity analysis was conducted by co-transfecting luciferase reporting plasmid for 24 h in cells with abnormal (Mut-)HOTAIR or miR-152 expression (C), or (Mut-)HOTAIR and miR-152 co-overexpressing cells (D). ns: non significance; $^{**}P < 0.01$.

few are also abnormally expressed in GC tissues or cells. For example, altered miR-152 expression has been reported in GC tissues [25], while its potential targeting on HLA-G expression has also been identified in human trophoblast cell line [26]. Thus, HLA-G expression under microRNA regulation was considered as one possible way to explain the mechanism within the regulation of HOTAIR on HLA-G. As a candidate, miR-152 was subjected to bioinformatics analysis for the interaction with HOTAIR using DIANA TOOLS, with results showing three potential binding domains within HOTAIR transcript. Moreover, miR-152 expression was down-regulated in GC tissues in line with former studies [25,27], and showed strong negative correlation with HOTAIR expression. Therefore, it was hypothesized that HOTAIR may also serve as a ceRNA here to regulate HLA-G expression by sponging miR-152, similar with one underlying molecular mechanisms as described before [22].

In support of this notion, miR-152 expression was detected in HOTAIR overexpressing or inhibiting GC cells, with results showing the negative regulation of HOTAIR on miR-152 expression. For further validation, the posttranscriptional regulation of miR-152 on HLA-G expression in GC cells was also demonstrated through gain- and loss-of-function approaches, with the regulation of HOTAIR on HLA-G expression as comparison. Finally, further experiments showed that miR-152 induced decrease of HLA-G 3′UTR activity could be attenuated by HOTAIR overexpression, indicating the function of HOTAIR as a ceRNA to regulate HLA-G expression by sponging miR-152.

In conclusion, our study revealed the regulation of HOTAIR on HLA-G expression for the first time, indicating a new role of HOTAIR in tumor escape during GC carcinogenic process. Further studies showed that HOTAIR might function as a ceRNA by sponging miR-152, leading to induced HLA-G expression. This study provided new insights for HOTAIR functions in tumor biology, and more researches on the application of HOTAIR in GC immunotherapy could be executed for better prognosis and improved survival.

Conflict of interest

None declared.

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Transparency document

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