Rs145204276 and rs4759314 affect the prognosis of prostate cancer by modulating the GAS5/miR-1284/HMGB1 and HOTAIR/miR-22/HMGB1 signalling pathways

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ABSTRACT
Non-coding RNAs play an important role in the pathogenesis of prostate cancer (PC). This study aims to characterize the role of GAS5 rs145204276 and HOTAIR rs4759314 polymorphisms in the pathogenesis of PC. Both INS allele of GAS5 rs145204276 and A allele of HOTAIR rs4759314 were identified to increase the survival of PC patients. And patients carrying DEL/DEL or AG genotypes tend to present higher levels of HMGB1, GAS5, HOTAIR and lower levels of miR-1284 and miR-22. In addition, the transcription activity of GAS5 promoter was increased by the deletion allele of rs145204276 polymorphism, while the G allele of rs4759314 polymorphism increased the transcription activity of HOTAIR promoter. GAS5 and HOTAIR could bind to miR-1284 and miR-22, respectively, while miR-1284 and miR-22 could bind to the 3’UTR of HMGB1. Compared with the control group, the expressions of miR-1284 or miR-22 were decreased with the presence of GAS5 or HOTAIR, and the expression of HMGB1 was the highest in the GAS5 + HOTAIR group. In summary, the findings of this study demonstrated that both GAS5 rs145204276 and HOTAIR rs4759314 polymorphisms could affect the prognosis of PC by modulating the expression of HMGB1 via modulating the GAS5/miR-1284/HMGB1 and HOTAIR/miR-22/HMGB1 signalling pathways.

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Introduction
As previous reported, prostate cancer (PC) is the second ranked malignancy and the fifth major cause of mortality worldwide, which is attributed to almost 20% of cancers in male patients [1]. Histopathological assessment of tumour tissue samples was usually utilized to confirm the diagnosis of PC. However, biopsy is associated with certain disadvantages such as infection and bleeding [2].

Enriched in chromatin as a non-histone component, high mobility group box 1 (HMGB1) is released from cells into the extracellular environment, where HMGB1 binds to the receptor for advanced glycation end products (RAGE) and subsequently activates several critical cell signalling pathways, including p44/42MAPKs, p38 and NF-κB signalling, to promote cancer development and metastasis [3–9]. In addition, upregulated HMGB1 expression was frequently observed in PC cases, although the value of HMGB1 in the prognosis of PC was only studied in one article, which showed that the expression of HMGB1 protein acted as a prognostic factor for post-radical prostatectomy (RP) survival [9–12]. Furthermore, it is also confirmed that the abnormality in HMGB1 and RAGE expression was involved in the progression of PC. As a result, the co-expression of HMGB1 and RAGE could be used as a better prognostic factor than each protein alone for the progression of PC.

MicroRNAs (miRNAs), which are short ncRNAs of about 22 nucleotides in length, and long noncoding RNAs (lncRNAs), which are usually more than 200 nucleotides in length, can simultaneously regulate the expression of their target genes by modulating the translation and transcription of their target mRNAs. On the other hand, mRNAs also affect the expression of ncRNAs in specific ways [13,14]. For example, the HOX transcript antisense RNA (HOTAIR), which is located on human chromosome 12, encodes a lncRNA of about 2 kb that is enriched in multiple cancers, including laryngeal, pancreatic, liver, colorectal and breast cancer [15,16]. In addition, HOTAIR expression is upregulated in PC cell lines resistant to castration, whereas the silence of HOTAIR expression reduced the viability of PC cells, suggesting that HOTAIR plays an essential role in the growth of PC cells [16].

In past studies, genetic variants in the promoter of lncRNAs were shown to regulate the expression of their host...
IncRNAs through methylation [17,18]. For example, as a 5 bp indel polymorphism (-/AGGCA) located in the promoter of GASS, rs145204276 was reported to increase the risk of hepatocellular carcinoma (HCC) by regulating GASS expression [18]. The potential role of rs145204276 in the prognosis of GC has also been reported. For example, patients carrying the del allele of rs145204276 have significantly better survival and lower rates of cancer progression and metastasis. Therefore, it is likely that rs145204276 plays a protective role in the pathogenesis of GC by regulating GASS expression. Furthermore, it has been shown that the G allele of the rs4759314 polymorphism located in HOTAIR promoter significantly increased the risk of CHD by decreasing the transcription efficiency of HOTAIR promoter and subsequently reducing the expression of HOTAIR.

HMGB1 has been implicated in the pathogenesis and prognosis of PC [19,20]. Meanwhile, it was reported that both miR-1284 and miR-22 function as regulators of HMGB1 expression [21,22]. In addition, GASS and HOTAIR play a role as competing endogenous RNAs against miR-1284 and miR-22, respectively [21,23]. Furthermore, the rs145204276 and rs4759314 polymorphisms located in the promoters of GASS and HOTAIR, respectively, can affect the transcription efficiency of these promoters [24,25]. Therefore, the two polymorphisms may affect the prognosis of PC via modulating the GASS/miR-1284/HMGB1 and HOTAIR/miR-22/HMGB1 signalling pathways. In this study, we collected samples from PC patients to probe the effect of above two polymorphisms on the prognosis of PC.

**Materials and methods**

**Patients and sample collection**

A total of 159 PC patients participated in this study. Clinicopathological information of all subjects, including their age, PSA level, Gleason score, T stage, N stage, and the presence of distant metastasis, were collected and compared, as listed in Table 1. Tissue and peripheral blood samples were collected from all patients, and the patients were then divided into four groups according to their genotypes of rs145204276 polymorphism of GASS (INS/INS, INS/DEL and DEL/DEL) and rs4759314 polymorphism of HOTAIR (AA/AG): a GASS rs145204276 DEL/DEL + HOTAIR rs4759314 AG group (Group 1, N = 26); a GASS rs145204276 DEL/DEL + HOTAIR rs4759314 AA group (Group 2, N = 36); a GASS rs145204276 INS/INS and INS/DEL + HOTAIR rs4759314 AG group (Group 3, N = 42); and a GASS rs145204276 INS/INS and INS/DEL + HOTAIR rs4759314 AA group (Group 4, N = 55). This study was approved by the ethics committee of our institute, and all patients have signed informed consent prior to the start of this study.

**Taqman genotyping**

The genotypes of rs145204276 polymorphism of GASS (INS/INS, INS/DEL and DEL/DEL) and rs4759314 polymorphism of HOTAIR in collected tissue and peripheral blood samples were determined using a Taqman genotyping assay (ABI, Foster City, CA) following the instruction of the manufacturer. The assay was performed on a Roche LightCycler 480 II PCR machine (Roche, Mannheim, Germany) and the results were analysed by Version 1.5 of LightCycler 480 Software (Roche, Mannheim, Germany). Each experiment was repeated three times.

**RNA isolation and real-time PCR**

A miRNeasy Mini kit (Qiagen, Valencia, CA) was used to isolate total RNA from collected tissue and peripheral blood samples following the instruction of the kit. The concentration and quality of isolated RNA were quantified using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Subsequently, 10 μg total RNA of each sample were reversely transcribed into cDNA using a reverse transcription kit (Invitrogen, Carlsbad, CA). The conditions of reverse transcription reactions were: 25 °C for 30 min,
42 °C for 40 min and 85 °C for 5 min. In the next step, the expression of HMGB1 mRNA, GASS, miR-1284, HOTAIR and miR-22 was measured using real-time PCR with specifically designed primers. The conditions of RT-PCR reactions were 94 °C for 10 min, followed by 45 cycles of 94 °C for 15 s and 60 °C for 60 s. Finally, the relative expression levels of HMGB1 mRNA (Forward: 5'- CGAAGAGAACCTGGAGAATGTG-3'; Reverse: 5'- GCATCAAGCTTCTCTTAGCTCG-3'), GASS (Forward: 5'- CCAGAGGAGATAG-3'; Reverse: 5'- ACCAG GAGCAGAACA-3'), miR-1284 (Forward: 5'- TACCAG CCTGCGTTTTT-3'; Reverse: 5'- GAACATCTGCTGTAATTCTC-3'), HOTAIR (Forward: 5'- CCAAGAACGTCGAAAAACCTG-3'; Reverse: 5'- GAGATGATAAGAGAGCAAGAAGG-3'), and miR-22 (Forward: 5'- GTTCTTCAAGGGCGAGC-3'; Reverse: 5'- GAACATGTCTGCTGTAATTCTC-3') were quantified using the 2^(-ΔΔCT) method and GAPDH (Forward: 5'- GTCTCTCTGCTCTTAACAGCG-3'; Reverse: 5'- ACCACCGTCTGCTGTAATTCTC-3') as the internal control. All RT-PCR reactions were run in triplicate and each experiment was repeated at least three times.

**Cell culture and transfection**

LNCaP and PC3 cells were cultured in a Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Thermo Fisher Scientific, Waltham, MA), 4.5 g/L of glucose (Sigma-Aldrich, St. Louis, MO), 1.5 g/L of sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), 4 mM of L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA), 65 units/mL of streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA) and 100 units/mL of penicillin (Gibco, Thermo Fisher Scientific, Waltham, MA). The cells were maintained at 37 °C and saturated humidity in an atmosphere containing 5% CO2 and 95% air. Subsequently, LNCaP and PC3 cells were seeded into 24-well plates at a density of 3 × 10^5 cells/well and then transfected with either wild type or mutant vectors of GASS and HOTAIR, respectively, for 48 h. At 48 h post-transfection, the luciferase activity of transfected cells was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the instruction of the kit. Similarly, in order to assay the regulatory relationships of GASS versus miR-1284, HOTAIR versus miR-22, HMGBl versus miR-1284 and HMGBl versus miR-22, the Quick-Change Site-Directed Mutagenesis Kit was used to introduce mutations in GASS and HMGBl containing the binding sites of miR-1284 and miR-22, respectively, as well as in the sequence of miR-22 containing the binding site of HOTAIR, to generate mutant pGL3 vectors for GASS, miR-22, and HMGBl, respectively. In the next step, LNCaP and PC3 cells were co-transfected with wild type/mutant vectors of GASS/miR-1284/HMGBl and HOTAIR/miR-22/HMGBl, respectively, and the luciferase activity of transfected cells was measured at 48 h post-transfection using the Dual-Luciferase Reporter Assay System. Each experiment described above was carried out in triplicate and repeated for at least three times.

**Western blot analysis**

To measure the protein expression of HMGB1, cell and tissue samples were lysed in a lysis buffer before a Bradford reagent (Bio-Rad Laboratories, Hercules, CA) was used to determine the concentration of isolated protein following a standard protocol. SDS-PAGE was then used to resolve sample proteins, which were subsequently transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) and blocked using Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween20. In the next step, the membrane was incubated at 4 °C for 12 h with primary antibodies against HMGB1 (1:1000 dilution, Abcam, Cambridge, MA) and β-actin (internal control, 1:10,000 dilution, Abcam, Cambridge, MA), followed by incubation with horseradish peroxidase (HPR) labelled secondary antibodies (1:10,000 dilution, Abcam, Cambridge, MA) at room temperature for 1 h. Subsequently, the membrane was visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA) and the relative expression of HMGB1 protein was calculated using β-actin as the internal control. Each experiment was repeated three times.

**Immunohistochemistry**

Tissue samples were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned into 4 μm slices. Subsequently, the sections were incubated in 3% H2O2 for 10 min to block endogenous peroxidase and then incubated with anti-MMP-2 (1:500 dilution, Santa Cruz Biotechnology,
Santa Cruz, CA) and anti-HMGB1 primary antibodies (1:1000 dilution, Abcam, Cambridge, MA) for 12 h at 4°C. Subsequently, horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 dilution, GE Healthcare, Logan, UT) were used to incubate the sections for 2 h at room temperature. A DAB (3, 3-diaminobenzidine) substrate kit (Vector Laboratories Inc., Burlingame, CA) was then used to stain the samples in accordance with the guideline of the supplier, followed by counterstaining with haematoxylin and observation underneath an inverted microscope. All experiments were repeated at least three times.

Statistical analysis

All data were shown as mean±SEM. Student t-tests were used to evaluate the statistical differences between two groups, while the comparisons among multiple groups were performed using one-way ANOVA (Turkey post hoc test). Furthermore, logistic regression analysis was used to analyse the association between the genotypes of polymorphisms and the risk of PC. ROC curves of survival were plotted and analysed by Cox regression and Kaplan–Meier analyses using NCSS statistical software (Kaysville, UT). All statistical analyses were performed using SPSS 21.0 (IBM, Chicago, IL), and a p value of <.05 was considered to be statistically significant.

Results

Clinicopathological characteristics of patients

Student t tests were performed to compare the patient groups and the results revealed no obvious difference among these four groups in terms of above clinicopathological characteristics (Table 1).

Genotypic association between rs145204276/rs4759314 and the survival of PC

Cox regression and Kaplan–Meier analyses were used to assess whether GAS5 rs145204276 and HOTAIR rs4759314 polymorphisms played an important role in the survival of PC. As shown in Figure 1(A), the overall survival of PC was the lowest in Group 1 and the highest in Group 4, indicating that the INS genotype of rs145204276 polymorphism in GAS5 and the A allele of rs4759314 polymorphism in HOTAIR apparently increased the survival rate of PC patients.

Expressions of GAS5, HOTAIR, miR-1284, miR-22 and HMGB1 among different genotypes

Real-time PCR and IHC assays were performed to investigate the effect of GAS5 rs145204276 and HOTAIR rs4759314 polymorphisms on the expression of GAS5, HOTAIR, miR-1284, miR-22 and HMGB1 in patients from the above four groups. As shown in Figure 1(B and C), the levels of HMGB1 mRNA (Figure 1(B)) and protein (Figure 1(C)) were the lowest in Group 4 and the highest in Group 1. In addition, the levels of GAS5 (Figure 1(D)) were higher in Group 1 and Group 2 than those in Groups 3 and 4. On the other hand, the levels of miR-1284 (Figure 1(E)) were lower in Groups 1 and 2 than those in Groups 3 and 4. Furthermore, Groups 1 and 3 showed higher levels of HOTAIR (Figure 1(F)) and lower levels of miR-22 (Figure 1(G)) than Groups 2 and 4.

Interactions among GAS5, HOTAIR, miR-1284, miR-22 and HMGB1

By searching several databases commonly used for target gene prediction (http://targetscan.org/), binding sites for GAS5 and HOTAIR were identified on miR-1284 (Figure 2(A)) and miR-22 (Figure 2(B)), respectively. Meanwhile, the 3’UTR of HMGB1 contained binding sites for both miR-1284 (Figure 2(C)) and miR-22 (Figure 2(D)).

We utilized luciferase assays to examine the effects of GAS5 rs145204276 and HOTAIR rs4759314 polymorphisms on the promoter activity of GAS5 and HOTAIR, respectively. In comparison with the transfection of control constructs, the transfection with the GAS5 constructs carrying the insert or deletion allele of rs145204276 polymorphism apparently increased the luciferase activity of LNCaP (Figure 3(A)) and PC3 (Figure 4(A)) cells. Moreover, compared with the insert allele of rs145204276 polymorphism, the presence of the deletion allele significantly promoted the transcription of GAS5 promoter. Similarly, in comparison with the transfection of control constructs, the transfection with HOTAIR constructs carrying the A or G allele of rs4759314 polymorphism apparently increased the luciferase activity of LNCaP (Figure 3(B)) and PC3 (Figure 4(B)) cells. Moreover, compared with the A allele of rs4759314 polymorphism, the presence of the G allele significantly promoted the transcription of HOTAIR promoter. In addition, the transfection with miR-1284 mimics reduced the luciferase activity of wild-type GAS5 but not that of mutant GAS5 in LNCaP (Figure 3(C)) and PC3 (Figure 4(C)) cells, while the transfection with miR-22 mimics reduced the luciferase activity of wild-type HOTAIR but not that of mutant HOTAIR in LNCaP (Figure 3(D)) and PC3 (Figure 4(D)) cells. Finally, the transfection with miR-1284 (Figures 3(E) and 4(E)) and miR-22 (Figures 3(F) and 4(F)) mimics reduced the luciferase activity of wild-type HMGB1 3’UTR but not that of mutant HMGB1 3’UTR in LNCaP (Figure 3(E and F)) and PC3 (Figure 4(E and F)) cells.

Real-time PCR and Western-blot analysis were then carried out to further confirm the interactions among GAS5, HOTAIR, miR-1284, miR-22 and HMGB1. Compared with the transfection of control vectors, the transfection of GAS5 and GAS5 + HOTAIR decreased the level of miR-1284 in LNCaP (Figure 3(G)) and PC3 (Figure 4(G)) cells. Similarly, compared with the transfection of control vectors, the transfection of HOTAIR and GAS5 + HOTAIR decreased the level of miR-22 in LNCaP (Figure 3(H)) and PC3 (Figure 4(H)) cells. Finally, the transfection with GAS5, HOTAIR and GAS5 + HOTAIR significantly increased the mRNA (Figures 3(I) and 4(I)) and protein (Figure 3(J) and 4(J)) levels of HMGB1 in LNCaP.
Figure 1. Genotypic association between rs145204276/rs4759314 and the survival of PC (Group A: GAS5 rs145204276 DEL/DEL + HOTAIR rs4759314 AG; Group B: GAS5 rs145204276 DEL/DEL + HOTAIR rs4759314 AA; Group C: GAS5 rs145204276 INS/INS&INS/DEL + HOTAIR rs4759314 AG; Group D: GAS5 rs145204276 INS/INS&INS/DEL + HOTAIR rs4759314 AA). (A) The INS genotype of rs145204276 polymorphism in GAS5 and the A allele of rs4759314 polymorphism in HOTAIR apparently increased the survival rate of PC patients; (B) The mRNA level of HMGB1 was the highest in Group 1 and the lowest in Group 4; (C) The level of HMGB1 protein was the highest in Group 1 and the lowest in Group 4; (D) The level of GAS5 mRNA in Groups 1 and 2 was much higher than that in Groups 3 and 4; (E) The level of miR-1284 in Groups 1 and 2 was apparently lower than that in Group 3 and Group 4; (F) The level of HOTAIR mRNA in Groups 1 and 3 was much higher than that in Group 2 and Group 4; (G) The level of miR-22 in Groups 1 and 3 was apparently lower than that in Group 2 and Group 4.
Discussion

In this study, we enrolled 159 PC patients and found that the INS genotype of GAS5 rs145204276 polymorphism and the A allele of HOTAIR rs4759314 polymorphism both apparently increased the survival rate of PC patients.

Growing evidence has shown that HOTAIR can regulate key signalling pathways involved in cancer metastasis and invasion. For example, HOTAIR was shown to increase the metastasis and invasiveness of breast cancer by enhancing the expression of LAMC2, LAMB3 and ABL2 SNAIL, all of which were reported to enhance cancer metastasis [15]. On the other hand, upregulated expression of HOTAIR in HCC may be used as a prognostic factor for post-hepatectomy recurrence of HCC by regulating the expression of VEGF and MMP9 [26]. The above results suggest that HOTAIR plays an essential role in the progression of PC. Moreover, the rs4759314 polymorphism located in the promoter of HOXC11 can affect the expression of HOXC11 by regulating its transcription efficiency [26]. In fact, compared with the A allele of the rs4759314 polymorphism, the G allele was shown to increase the expression of HOTAIR, suggesting that the G allele is associated with a higher risk of carcinogenesis.

It was shown that GAS5 lncRNA can regulate both the apoptosis and proliferation of PC cells when these cells are exposed to a wide range of apoptotic stimuli. Therefore, GAS5 may be closely related to the carcinogenesis of PC and hence may be exploited as a target gene in PC therapy [27].

It was reported that an increased level of GAS5 expression reduced the survival of PC cells in a manner similar to exposing the cells to 22Rv1. Located in the promoter region of GAS5, rs145204276 was associated with the altered expression of GAS5 in vitro [28]. In previous studies, the DEL allele of rs145204276 was shown to increase the expression of GAS5 and hence regulate its...
expression by methylating CpG islands located in the GAS5 promoter. Furthermore, it was also found that gastric cancer patients carrying the del allele of rs145204276 were associated with a remarkably higher level of GAS5 expression in the tumour tissue. In this study, we found that the level of HMGB1 was the highest in Group 1 and the lowest in Group 4. In addition, the level of GAS5 in Groups 1 and 2 was much higher than that in Groups 3 and 4, while the level of miR-1284 in Groups 1 and 2 was much lower than that in Groups 3 and 4. Furthermore, the level of HOTAIR in Groups 1 and 3 was much higher than that in Groups 2 and 4, while the level of miR-22 in Groups 1 and 3 was much lower than that in Groups 2 and 4. Additionally, our results showed that the deletion allele of rs145204276 polymorphism increased the transcription activity of GAS5 promoter, while the G allele of rs4759314 polymorphism increased the transcription activity of HOTAIR promoter. We also found that GAS5 and HOTAIR could directly bind to miR-1284 and miR-22, respectively, while miR-1284 and miR-22 could both target the 3'UTR of HMGB1.

HMGB1 was initially identified as a protein with the chromatin-binding ability. However, HMGB1 has been implicated in the process of cancer cell metastasis, proliferation, and angiogenesis during the development of cancers [11]. In addition, HMGB1 was also shown to play extracellular and intracellular roles by activating critical signalling pathways involved in oncogenesis [11,12]. Moreover, it was demonstrated that the silence of RAGE by siRNA could inhibit HMGB1-induced proliferation of PC cells by reducing the expression of HMGB1 [19]. These studies also showed that the silence of HMGB1 by siRNA could suppress the metastasis of PC cells in an animal model of metastasis.

It has been reported that miR-1284 was down-regulated in osteosarcoma tissues overexpressing HMGB1, indicating the presence of a reverse correlation between the expression of miR-1284 and HMGB1. In addition, miR-1284 can lead to the degradation of HMGB1 by directly binding to its 3'UTR. It was reported that the 3'UTR of HMGB1 contains a binding site of miR-22, and hence the overexpression of miR-22 can suppress the expression of HMGB1 while inhibiting HMGB1-induced autophagy of osteosarcoma cells, and the inhibition of HMGB1 expression by miR-22 in osteosarcoma cells renders the cells more sensitive to the treatments by Cis and Dox by reducing the potential of cancer cell invasion, migration, and proliferation.

Conclusion
In summary, the findings of this study demonstrated the role of HMGB1 in the progression and prognosis of PC. Therefore, the rs145204276 polymorphism located in GAS5 and rs4759314 in HOTAIR may affect the prognosis of PC by modulating the activity of GAS5/miR-1284/HMGB1 and HOTAIR/miR-22/HMGB1 signalling pathways.

Disclosure statement
No potential conflict of interest was reported by the authors.

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