An indel polymorphism in the 3' untranslated region of HMGB1 confers risk for hepatocellular carcinoma by regulating HMGB1 transcriptional activity in a Chinese population

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The present study aimed to assess the association of rs34000982 polymorphism located in the 3' untranslated region (3' UTR) of high mobility group box 1 (*HMGB1*) gene and the risk of hepatocellular carcinoma (HCC), and further to explore the underlying mechanism. Genomic DNA was extracted from peripheral blood of 320 patients with HCC and 360 matched controls. Rs34000982 polymorphism was genotyped by a polymerase chain reaction-polyacrylamide gel electrophoresis assay. The genotype-phenotype association of *HMGB1* mRNA and protein expression in HCC tissues with different genotypes was detected by quantitative (q) PCR assay and western blot. Vectors containing the insertion (ins)/ins or deletion (del)/del genotype of the rs34000982 polymorphism were constructed and the *HMGB1* transcriptional activity affected by the rs34000982 polymorphism was detected by the luciferase assay. It was identified that the ins/ins genotype of rs34000982 significantly increased the risk of HCC compared with the del/del genotype. Further the qPCR results demonstrated that the *HMGB1* mRNA expression level in HCC tissues with ins/ins genotype was 2.24 times that of HCC tissues with ins/del and del/del genotypes and there was a similar trend at protein level. In addition, the insertion allele of rs34000982 disturbed the binding of miR-636 with the 3' UTR of *HMGB1*, thereby increasing *HMGB1* transcriptional activity *in vitro*. These data suggest that the rs34000982 polymorphism may contribute to HCC susceptibility, in full or at least partially through the effect on *HMGB1* transcriptional activity by disturbing the binding of miR-636 with the 3' UTR of *HMGB1*.

Key words: insertion/deletion polymorphism, rs34000982, high mobility group box 1, hepatocellular carcinoma

Liver cancer is the sixth most common cancer globally and the fourth most common cause of cancer-related deaths worldwide in 2018, with about 841,000 new cases and 782,000 deaths annually [1]. Hepatocellular carcinoma (HCC), characterized as an inflammation-related carcinoma, comprising 75–85% of primary liver cancer, whose main risk factors are Hepatitis B virus (HBV) infection and aflatoxin-contaminated foodstuffs, especially in high risk areas including China [2], along with genetic factors also playing important roles in HCC [3]. Despite the intense study of HCC in previous years, the molecular mechanism remains incompletely characterized [4].

High-mobility group box 1 (HMGB1) is a highly conserved and widely expressed nuclear DNA binding protein [5, 6]. Nuclear functions of HMGB1 are essential to life since HMGB1 knockout mice die within 24 hours of birth from hypoglycemia in cells [7]. Once released, extracellular HMGB1 binds to several cell surface receptors including receptor for advanced glycation end product (RAGE) [8, 9], the toll-like receptors [10–13] etc., to activate the downstream signaling pathway. Therefore, HMGB1 has enormous biological functions through interacting with the abovementioned receptors and serves as a key component in many diseases including cancers, such as breast cancer [14], tumors of the gastrointestinal tract [15, 16] and also hepatocellular carcinoma [17].

Because of the important roles of *HMGB1* gene, its expression regulation is critical, and Muller et al. have summarized its expression regulation at different levels, especially at transcriptional level through different mechanisms [18]. The

3' untranslated region (3' UTR) is considered to be an area with abundant microRNA (miRNA) targets and participates in the regulation of gene expression. So genetic polymorphisms in the 3' UTR of genes may affect gene expression through creating new or disturbing the original miRNA bindings. Rs34000982 is a 4-bp indel polymorphism in the 3' UTR of *HMGB1*, in the present study we analyzed the association between rs34000982 polymorphism and HCC susceptibility in a Chinese population, and the potential mechanisms through which the insertion-deletion (indel) polymorphism affects *HMGB1* expression.

Materials and methods

Study populations. Both cases and controls recruited were non-consanguineous ethnic Han Chinese individuals. All 320 HCC cases were diagnosed, hospitalized and treated in the Fifth People's Hospital of Changshu (Changshu, China) and Suzhou Municipal Hospital (Suzhou, China) between May 2010 and January 2017. None of the patients with HCC had been administered any medical treatment prior to providing peripheral blood samples. The diagnosis of the HCC cases, the exclusion and inclusion criteria for all the participants, and the definitions of smokers and drinkers were the same as described previously [19–21]. The diagnosis of these patients was confirmed by a pathological examination combined with positive imaging (magnetic resonance imaging and/or computerized tomography). The matched 360 cancer-free controls were selected during a routine physical examination conducted in the same regions during the same period as the recruitment of the HCC cases. All controls had no history of cancer, and were negative for antibodies to hepatitis C virus, hepatitis D virus, and human immunodeficiency virus. The 60 newly diagnosed and pathologically confirmed HCC tissues were collected following surgical resection without preoperative chemotherapy or radiotherapy. Tumor stages were determined using a modified American Joint Committee on Cancer and International Union Against Cancer system [22]. Written informed consents were gained from all the participants. The design of the study was approved by the Ethics Committee of the Fifth People's Hospital of Changshu (Changshu, China) and Suzhou Municipal Hospital (Suzhou, China).

DNA extraction and genotyping. Peripheral blood DNA was extracted using a DNeasy Blood & Tissue kit (cat no. 69504; Qiagen GmbH, Hilden, Germany). DNA fragments containing the rs34000982 polymorphism were amplified using the following primers: 5'-AAGTAAATGGAAGT-GGGAGGCAAT-3' (forward) and 5'-CCCACAGCACTG-TAACTATCTTG-3' (reverse). The polymerase chain reaction (PCR) was performed in a total volume of 20 μl, containing 2.0 μl 10X PCR buffer, 1.2 μl 25 mM MgCl$_2$, 1 μl 10 mM dNTP Mix, 1 μl 10mM for each primer, 50 ng genomic DNA, and 1.0 U Taq DNA polymerase (GenStar Biosolutions Co., Beijing, China). The amplification protocol consisted of an initial denaturation at 94 °C for 2 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. A 7% non-denaturing polyacrylamide gel electrophoresis (PAGE) and silver staining method was used to analyze the PCR products [23]. The 4-base pair (bp) deletion/insertion (del/ins) allele of rs34000982 yielded bands of 117 and 121 bp, respectively. Genotyping was performed without knowledge of case or control status. The quality control was performed as follows: in order to validate the genotyping method, 15 randomly selected DNA samples were sequenced by Genewiz Inc. (South Plainfield, NJ, USA) following genotyping; to confirm 100% consistency, ~10% of the total DNA samples were randomly selected for genotyping in duplicate by 2 independent technicians.

Quantitative (q)PCR analysis. Total RNA was extracted from 60 HCC tissue samples with different genotypes using an RNeasy Mini Kit (cat no. 74106; Qiagen) and reverse transcribed used the Superscript II reverse transcriptase (cat no. 18064-014; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Next, to quantify the relative mRNA expression level of *HMGB1* in these samples, FastStart Universal SYBR Green Master (Rox; cat no. 04913914001; Roche Diagnostics, Indianapolis, IN, USA) was used, and qPCR was performed on a Roche Light Cycler 480 system. GAPDH was selected as the internal control. The primer sequences used for HMGB1 and GAPDH were as follows: HMGB1-Q forward (F), 5'-TATGGCAAAAGCGGA-CAAGG-3'; HMGB1-Q reverse (R), 5'-CTTCGCAACAT-CACCAATGGA-3'; GAPDH-QF, 5'-CTCTCTGCTCCTCC-TGTTCGAC-3'; GAPDH-QR, 5'-TGAGCGATGTGGCTC-GGCT-3'.

The 20 μl total volume final reaction mixture consisted of: 1 μmol/l for each primer, 10 μl Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 2 μl cDNA. The negative control experiments were performed using distilled H$_2$O as template. The 2$^{-\Delta\Delta Ct}$ algorithm was used to calculate the mRNA expression levels in tissues with different genotypes [24].

Western blot. Total protein was obtained from HCC tissues with different genotypes, and approximately 80 μg of it was separated on a 10% polyacrylamide gel followed by transferring to a polyvinylidene difluoride membrane (cat no. IPVH00010, 0.45 μm, Millipore) and probed with primary antibodies against HMGB1 (cat no. ab77302, 1:10000, Abcam) and glyceraldehydes 3-phosphate dehydrogenase (cat no. ABAB-M-M001, 1:500, Good HERE Biotech Inc., Hangzhou China) and then secondary antibodies (1:1000, Beyotime Biotechnology). Enhanced chemiluminescence system was used to visualize the bands and the intensity of the bands.

Plasmid construction. A DNA fragment of ~300 bp, including the del/del genotype of rs34000982 in the 3' UTR of *HMGB1* was directly synthesized by Genewiz Inc. (South Plainfield, NJ, USA) and cloned into Xba I and Fse

III sites of a pGL3-control expression vector (Promega Corporation, Madison, WI, USA), named pGL3-HMGB1-WT. A QuikChange Lightening Site-Directed Mutagenesis kit (cat. no. 210518, Stratagene; Agilent Technologies, Inc., CA, USA) was used to generate the mutant-type vector (pGL3-HMGB1-MT) including the ins/ins genotype of rs34000982. The sequence and direction of the resulting constructs (pGL3-HMGB1-WT and pGL3-HMGB1-MT) were verified by direct sequencing by Genewiz Inc.

In silico **prediction of miRNAs binding with the 3' UTR of HMGB1 and disturbed by rs34000982.** Mature human miRNA sequences were obtained from miRBase (http://microrna.sanger.ac.uk) in 2016. The putative miRNAs could bind with the 3' UTR of *HMGB1* and disturbed by rs34000982 was analyzed using miRanda (version 3.3a) software with default parameters as described previously [25].

Cell culture and luciferase reporter assay. Both Huh7 and HepG2 cell lines used in the present study were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in Dulbecco's modified Eagle's medium (cat no. 12491-015; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (cat no. 10100147; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin at 37°C in a humidified chamber supplemented with 5% CO_2. Cells were seeded in 24-well plates (cat. no. 3524; Corning Incorporated, Corning, NY, USA) at a density of 1×10^5 cells/well and transfected following culture at 37°C for 16 h using Lipofectamine® 2000 (cat. no. 11668-019; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequently, 500 ng pGL3-HMGB1-WT or pGL3-HMGB1-MT reconstructed vector and 50 ng of pRL-SV40 (Promega Corporation) were co-transfected using Lipofectamine® 2000 with 100 pmol miR-636 mimic or mimic control (Ambion; Thermo Fisher Scientific, Inc.) with ~5×10^6 cells/well. Subsequent to 24 h, cells were harvested immediately following addition of 100 µl passive lysis buffer (cat. no. E1910; Promega Corporation). Dual-luciferase reporter assay (cat. no. E1910; Promega Corporation) was used to measure the firefly luciferase activity in cell lysates in a FilterMax F5, according to the manufacturer's protocol, and Renilla luciferase activity was used to normalize the data. All experiments were repeated at least 3 times with 6 replicates for each group.

Statistical analysis. The Hardy-Weinberg equilibrium was analyzed using the χ^2 test for the genotype distribution in the control group. The association between rs34000982 and HCC risk was analyzed by logistic regression, adjusted for age (mean ± standard deviation), gender, smoking, drinking, tumor stage and HBV infection status. A one-way analysis of variance followed by Dunnett's post-hoc test was used to compare the relative *HMGB1* mRNA expression levels in HCC tissues between different genotypic groups. The difference in luciferase activity was examined using the Student's t-test. Statistical Analysis System software was used for all the statistical analyses (version 8.0; SAS Institute, Cary, NC, USA). A p<0.05 was considered to indicate a statistically significant difference, and all statistical tests were two-sided.

Results

Association of rs34000982 with HCC susceptibility. Table 1 summarizes the demographic characteristics of the patients with HCC and the matched controls. The distribution of age, gender, smoking and drinking status was similar between the patients with HCC and the matched controls. The hepatitis B surface antigen-positive rate was 74.1% and 15.0% in the patients with HCC and the matched controls, respectively. Figure 1 presents the genotyping assays for rs34000982 and the sequencing results. The observed rs34000982 genotypic frequencies in the controls were consistent with the Hardy-Weinberg equilibrium (p>0.05). Table 2 indicates that the ins/ins genotype exhibited a significantly increased risk of HCC compared with the del/del genotype [p=0.001; odds ratio (OR)=2.43; 95% confidence interval (CI): 1.44–4.10]. In addition, the 4-bp insertion allele was associated with a 49% increased risk compared with the deletion allele (p=0.001; OR=1.49; 95% CI, 1.17–1.90). Collectively, these results suggest the association between rs34000982 and HCC susceptibility.

Association between rs34000982 genotype and HMGB1 mRNA expression levels. As demonstrated in Figure 2A, the *HMGB1* mRNA level was higher in the insertion homozygous groups (ins/ins) than that of the heterozygous (ins/del) and

Table 1. Demographic characteristics of hepatocellular carcinoma cases and controls.

Characteristics	Patients, % (n=320)	Control, % (n=360)
Age (mean ± S.D.)	52.9±8.9	54.1±9.7
Sex		
Male	208 (65.0)	238 (66.1)
Female	112 (35.0)	122 (33.9)
Smoking status		
Non-smokers	210 (65.6)	242 (67.2)
Former smokers	52 (16.3)	60 (16.7)
Current smokers	58 (18.1)	58 (16.1)
Drinking status		
Non-drinkers	170 (53.1)	195 (54.2)
Light drinkers	130 (40.6)	145 (40.3)
Heavy drinkers	20 (6.3)	20 (5.5)
Tumor stages		
Ia + Ib	210 (65.6)	
IIa + IIb	80 (25.0)	
IIIa + IIIb	30 (9.4)	
HBsAg		
Positive	237 (74.1)	54 (15.0)
Negative	83 (25.9)	306 (85.0)

HBsAg, Hepatitis B surface antigen.

A

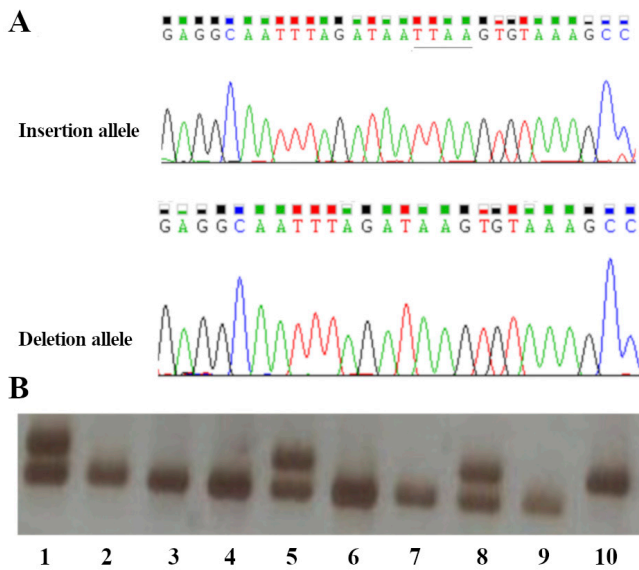Insertion allele

Deletion allele

B

Figure 1. Sequencing and genotyping examples of rs34000982. A) Insertion and deletion allele sequences. The inserted 'TTAA' is underlined. B) Genotyping results using 7% non-denaturing PAGE and silver staining. Lanes 2, 3, 4, 6, 7 and 9 del/del genotype; lanes 1, 5, and 8 ins/del genotype; lanes 10 ins/ins genotype. Ins, insertion; del, deletion.
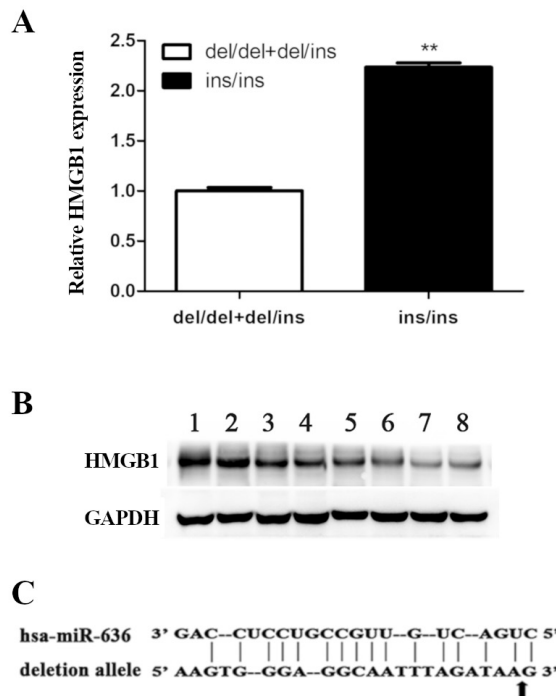
A

B

C

Figure 2. A) *HMGB1* mRNA expression levels in HCC tissues with different genotypes. The *HMGB1* mRNA expression level in HCC tissues with ins/ins genotype was 2.24 times that of the del/del and ins/del genotype (**p<0.01 vs. del/del+ ins/del). ins/ins, n=7, ins/del, n=14, del/del, n=39. B) *HMGB1* protein expression levels in HCC tissues with different genotypes. The *HMGB1* protein expression levels in HCC tissues with different genotypes. (Lanes 1 and 2 ins/ins genotype; Lanes 3, 4 and 5 ins/del genotype; Lanes 6, 7 and 8 del/del genotype. C) miRNA-*HMGB1* mRNA binding model and position of the insertion allele (arrow). *HMGB1*, high mobility group box 1; miRNA, microRNA.

Table 2. Genotype distributions of the rs34000982 polymorphism in patients with HCC and healthy controls.

Genotype	HCC (n=320) n (%)	Controls (n=360) n (%)	OR (95% CI)[a]	p-value
del/del	160 (50.0)	211 (58.6)	1 (reference)	
ins/del	112 (35.0)	123(34.2)	1.20 (0.86–1.68)	0.28
ins/ins	48 (15.0)	26 (7.2)	2.43 (1.44–4.10)	0.001
del allele	432 (67.5)	545 (75.7)	1 (reference)	
ins allele	208 (32.5)	175 (24.3)	1.49 (1.17–1.90)	0.001

Ins, insertion; del, deletion; HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval; [a]Adjusted for age, gender, smoking status, drinking status, tumor stage and HBV infection.

deletion homozygous group (del/del). The average *HMGB1* mRNA expression level of the insertion homozygous group was 2.24 times that of the heterozygous and deletion homozygous group. The data of this part in the present study suggested an association between genotypes and *HMGB1* mRNA expression levels *in vivo*.

Association between rs34000982 genotype and HMGB1 protein expression levels. As demonstrated in Figure 2B, the HMGB1 protein level was the highest in the HCC tissues with insertion homozygous (ins/ins) and then the heterozygous (ins/del) and deletion homozygous (del/del). The data of this part of the study suggested an association between genotypes and HMGB1 protein expression levels *in vivo*.

rs34000982 polymorphism affects HMGB1 transcription activity by regulating its binding with miR-636 *in vitro*. The bioinformatics analysis indicated that rs34000982 lies within the predicted human miR-636 binding site, and the insertion allele of rs34000982 may disrupt the binding of miR-636 with the 3' UTR of *HMGB1* (Figure 2C). To assess this prediction, two vectors, pGL3-HMGB1-WT (with del/del genotype) and pGL3-HMGB1-MT (with ins/ins genotype) were constructed. In the pGL3-HMGB1-MT group, the expression level of *HMGB1* mRNA, indicated by firefly luciferase activity, in the miR-636 mimic-transfected group was similar to that of the mimic control group. However, for the pGL3-HMGB1-WT group, the expression of *HMGB1* mRNA in the miR-636 mimic transfected group was significantly lower in the mimic control group (Figure 3). These results suggest that miR-636 may bind with the 3' UTR of *HMGB1* with del/del wild genotype and negatively regulate *HMGB1* transcription, and that the insertion allele may weaken its regulatory effect by disrupting the binding of miR-636 to the 3' UTR of *HMGB1*. Therefore, the *in vitro* experiments suggest that the polymorphism rs34000982 affects *HMGB1* expression by altering the binding of miR-636 to the 3' UTR of *HMGB1*.

Discussion

In the present study we set out to examine the association between polymorphisms in the 3' UTR of *HMGB1* and HCC susceptibility. Through extensive analyses we found that the
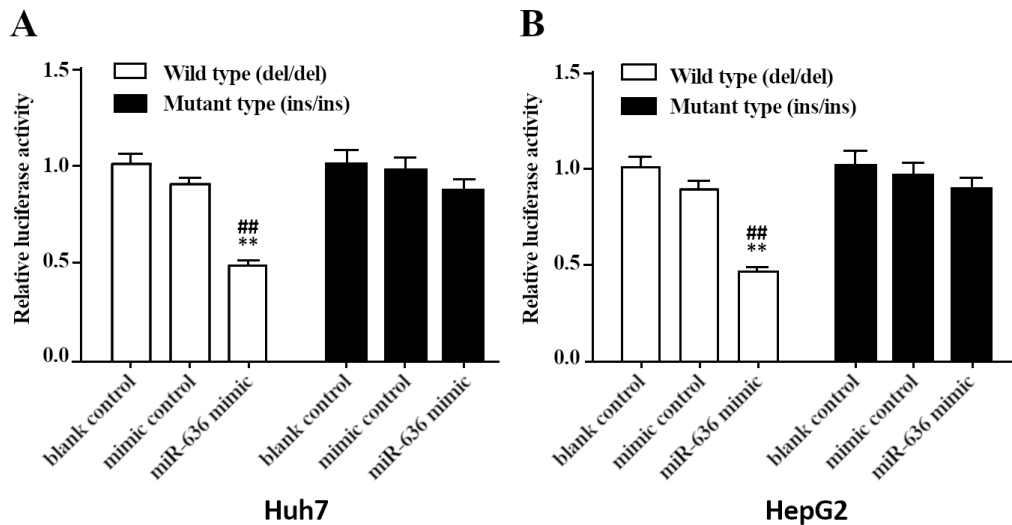
Figure 3. *HMGB1* transcriptional activity affected by rs34000982 and determined by luciferase reporter assay. The changes of the relative luciferase activity of Huh7 and HepG2 cells that were co-transfected with miR-636 mimic or mimic control and pGL3-HMGB1-WT or pGL3- HMGB1-MT vectors. The pGL3-HMGB1-WT and miR-636 mimic transfected group exhibited a significantly lower luciferase activity than the mimic control transfected group (**p<0.01; ##p<0.01). The Renilla luciferase vector was selected as an internal control. ** represents the difference between the blank control group and the miR-636 mimic group in the pGL3-HMGB1-WT transfected group is significant, and ## represents the difference between the miR-636 mimic group transfected with pGL3-HMGB1-WT and pGL3- HMGB1-MT is significant.

ins/ins genotype of rs34000982 in the 3' UTR of *HMGB1* was significantly associated with an increased risk of HCC. The following phenotype-genotype association experiment indicated that the expression level of *HMGB1* at both mRNA and protein level was the highest in the HCC tissues with insertion homozygous (ins/ins), followed by the heterozygous (ins/del) and deletion homozygous (del/del). In addition, the luciferase reporter assay suggested that the ins/ins genotype potentially worked in full, or at least partially, by disturbing the binding of miR-636 with the 3' UTR of HMGB1 to regulate *HMGB1* expression. To the best of our knowledge, this is the first study to investigate the association between the indel polymorphism in the *HMGB1* 3'UTR and the risk of HCC, and the potential mechanism involved.

HMGB1, discovered as a non-histone chromosomal protein in 1973, is one of the HMGB family members (HMGB 1, 2, 3 and 4) with a molecular weight of 25 to 30 kDa [26]. Serum HMGB1 levels are increased in patients with HCC and the expression of HMGB1 in the liver closely correlates with proliferation, invasion, pathological grade, distant metastases, and drug resistance of liver cancer [27–32]. In addition, HMGB1 released from the hypoxic tumor microenvironment can bind to TLR4 and RAGE, which in turn mediate HCC invasion and metastasis by activating inflammasome, NF-κB, and AKT pathways [8, 12, 17, 33, 34]. Moreover, therapeutic targeting of HMGB1 inhibits HMGB1 expression, release, and activity, which in turn suppresses tumor growth in liver metastasis models of colon cancer [8, 35–37]. These findings suggest that HMGB1 plays a critical role in the pathogenesis and treatment of HCC. All of these

data are consistent with our results that the risk insertion allele with a high level of *HMGB1* mRNA and protein expression level may increase the risk of HCC development.

As described above, abnormal expression of *HMGB1* participates in the progression of HCC, and a number of previous studies have indicated that polymorphisms in the 3' UTR of genes disturb their binding with miRNAs or reveal novel binding sites for miRNAs [38, 39]. This affects the expression level of target genes and may be involved in numerous types of diseases, including cancer [39, 40]. Gao et al. found that an indel polymorphism at a miRNA-122-binding site in the 3' UTR of interleukin (IL)-1α conferred increased risk for HCC [38], and Wang et al. demonstrated that rs56288038 in the 3' UTR of *interferon regulatory factor 1* (*IRF-1*) regulated by miR-502-5p promoted the development of gastric cancer [39]. Certain previous studies have also indicated that single nucleotide polymorphisms or indels in miRNA binding sites of their target genes may participate in the development or progression, or be associated with fluorouracil resistance of HCC by affecting the binding of miRNAs with the target genes [40, 41]. To explore the possible mechanism through which rs34000982 is involved in HCC susceptibility, miRNAs that can bind with the 3' UTR of *HMGB1* and be affected by rs34000982 were predicted, and it was identified that the ins/ins genotype could disturb the bind of miR-636 with the 3' UTR of *HMGB1*, and consequently up-regulated the expression of *HMGB1*, thus increasing the risk of HCC.

In 2013, Jang and colleagues firstly reported that miR-636 might function as a tumor suppressor miRNA through the

ANT2/miR-636/Ras axis in HCC [42]. The roles miRNAs in cancer are dependent on their target genes, and the results of the present study are consistent with the previous study on HCC that miR-636 serves as a tumor suppressor miRNA through targeting HMGB1. Due to the complicated mechanism of HCC development, additional functional analyses are required.

The present study contained multiple limitations: The subjects in the study were all ethic Han, and the numbers were limited, so additional large-scale studies in different populations are required. An additional limitation is that the possible mechanism of action of the indel was predicted by software, further experimental validation is required.

In summary, the present study demonstrated that the rs34000982 polymorphism in the 3' UTR of *HMGB1* may contribute to HCC susceptibility, possibly working in full, or at least partially, through an effect on *HMGB1* transcriptional activity, by disturbing its binding with miR-636.

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