An indel polymorphism in the 3' untranslated region of *JAK1* confers risk for hepatocellular carcinoma possibly by regulating *JAK1* transcriptional activity in a Chinese population

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Abstract. The purpose of the present study was to assess whether the rs112395617 polymorphism located in the Janus kinase 1 (*JAK1*) 3' untranslated region (3' UTR) was associated with the risk of hepatocellular carcinoma (HCC), and to explore the potential mechanism of action. Genomic DNA was extracted from peripheral blood of 290 patients with HCC and 320 controls. A polymerase chain reaction-polyacrylamide gel electrophoresis assay was used to genotype the rs112395617 polymorphism. Quantitative (q)PCR was used to detect the genotype-phenotype association between HCC tissues and different genotypes. Vectors containing the insertion (ins)/ins or deletion (del)/del genotype of the rs112395617 polymorphism were constructed, and the luciferase assay was used to detect the *JAK1* transcriptional activity affected by the rs112395617 polymorphism. It was identified that, when compared with the ins/ins genotype, the del/del and del/ins genotypes of rs112395617 were significantly associated with a decreased risk of HCC. The qPCR results demonstrated that the *JAK1* mRNA expression level with ins/ins and ins/del genotypes was increased by 3.36 and 1.75-fold compared with the del/del genotype in human HCC tissue samples. In addition, the 'AATT' insertion allele of rs112395617 disrupted the binding site for microRNA (miR)-431-5p, thereby increasing *JAK1* transcription *in vitro*. These data suggest that the rs112395617 polymorphism may contribute to HCC susceptibility, in full or at least partially through an effect on *JAK1* transcriptional activity by disrupting its binding with miR-431-5p.

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Introduction

Hepatocellular carcinoma (HCC) is a common liver cancer and its mortality rate ranks third among cancer-associated mortalities worldwide in 2008 (1,2). A total of >80% of HCC cases are from the Asian and African continents, >50% of which are from mainland China (3). HCC is a complicated type of cancer, involving numerous risk factors in its occurrence and development: Hepatitis B virus (HBV) infection and genetic factors also serve key roles in HCC carcinogenesis (4). Despite intense study of the molecular carcinogenic mechanism of HCC in previous years, it remains incompletely characterized (5).

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal pathway plays a key role in HCC (6). Up to 45.5% of cases of HCC harbor genomic alterations in the JAK-STAT signal pathway (6) and a number of anti-HCC drugs also serve their roles by blocking the JAK-STAT signal pathway. For example, Murphy *et al* (7) suggested that interferon-α (IFN-α) serves an anti-cancer role in HCC through the JAK-STAT signal pathway, and Subramaniam *et al* (8) identified that that emodin suppressed *STAT3* activation by modulating the activation of the upstream kinases, including Janus kinase 1 *(JAK1)*, in HCC. There are 4 *JAKs* in mammals, *JAK1*, *JAK2*, *JAK3* and Tyrosine kinase 2. *JAK1*, located on chromosome 1p31.3, was first identified in 1991 (9). It is a crucial component of diverse signal pathways, and polymorphisms in *JAK1* may be functional and serve a role in human cancer development (10,11). Xie *et al* (12) demonstrated that mutations in *JAK1* may contribute to the development of HCC, and Yang *et al* (13) revealed that $JAK1^{S703I}$ was an activating mutation for the JAK-STAT signaling pathway, which may represent a novel therapeutic approach for HCC.

In view of the important roles served by *JAK1* in HCC, the present study analyzed the association between the 4-bp indel polymorphism rs112395617 in the 3' untranslated region (3'UTR) of *JAK1* and HCC susceptibility in a Chinese population, and the potential mechanisms through which the insertion-deletion (indel) polymorphism affects *JAK1* expression.

Materials and methods

Study populations. Genomic DNA was extracted from the peripheral blood of patients with newly-diagnosed HCC, together with controls, subsequent to gaining written informed consent. The cases and controls recruited were non-consanguineous ethnic Han Chinese individuals. All 290 HCC cases were diagnosed, hospitalized and treated in the Suzhou Municipal Hospital (Suzhou, China) and the First Affiliated Hospitals of Soochow University (Suzhou, China) between May 2009 and January 2016. 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 (14-16). Briefly, patients were excluded if they had: (a) primary or secondary biliary cirrhosis or Budd-Chiari syndrome, (b) autoimmune hepatitis or toxic hepatitis, (c) recurrence of HCC, (d) tumors other than HCC or (e) liver disease due to parasitosis, diabetes, fatty liver, metabolism disorders or severe cardiovascular diseases. The diagnosis of these patients was confirmed by a pathological examination combined with positive imaging (Magnetic resonance imaging and/or computerized tomography). The 320 cancer-free controls were selected during a routine physical examination conducted in the same regions during the same period as the recruitment of the 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 70 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 (17). The design of the study was approved by the Ethical Committee of Suzhou Municipal Hospital and Soochow University (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 rs112395617 polymorphism were amplified using the following primers: 5'-CAGGTTCTGGGAATGAGT-3' (forward) and 5'-TGAGAAAGCTGGTTCTACAT-3' (reverse). The polymerase chain reaction (PCR) was performed in a total volume of 20 μl, containing 2.0 μl 10X PCR buffer, 1.5 mmol/l $MgCl_2$, 0.25 mmol/l for each dNTP, 0.5 mmol/l of each primer, 50 ng genomic DNA, and 1.0 U Taq DNA polymerase (Tiangen Biotech Co., Ltd., Beijing, China). The amplification protocol consisted of an initial denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 56˚C for 40 sec and extension at 72˚C for 48 sec, followed by a final extension at 72˚C for 5 min. A 7% percent non-denaturing PAGE and silver staining method was used to analyze the PCR products (18). The 4-base pair (bp) deletion/insertion (del/ins) allele of rs112395617 yielded bands of 127 and 131 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, 30 randomly selected DNA samples were sequenced by Genewiz Inc. (South Plainfield, NJ, USA) following genotyping and the results were all validated; to confirm 100% consistency with the PCR results, ~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 70 HCC tissue samples with different genotypes using a 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 *JAK1* 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 *JAK1* and *GAPDH* were as follows: JAK1-Q forward (F), 5'-CCACTACCGGATGAGGTTCTA-3'; JAK1-Q reverse (R), 5'-GGGTCTCGAATAGGAGCCAG-3'; GAPDH-QF, 5'-CTCTCTGCTCCTCCTGTTCGAC-3'; GAPDH-QR, 5'-TGAGCGATGTGGCTCGGCT-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_2O as template. The $2^{-\Delta\Delta Cq}$ algorithm was used to calculate the mRNA expression levels in tissues with different genotypes (19).

Plasmid construction. A DNA fragment of ~300 bp, including the ins/ins genotype of rs112395617 in the 3' UTR of JAK1 (sequence, TCTAGAAAATGACTGTATTCTCTCACCAGTAGGACTTAAACTTTGTTTCTCCAGTGGCTTAGCTCCTGTTCCTTTGGGTGATCACTAGCACCCATTTTTGAGAAAGCTGGTTCTACATGGGGGGATAGCTGTGGAATAGATAATTTGCTGCATGTTAATTCTCAAGAACTAAGCCTGTGCCAGTGCTTTCCTAAGCAGTATACCTTTAATCAGAACTCATTCCCAGAACCTGGATGCTATTACACATGCTTTTAAGAAACGTCAATGTATATCCTTTTATAACTCTACCACTTTGGGGCAAGCTATTCCAGGCCGGCC), 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-JAK1-WT. A QuikChange Lightening Site-Directed Mutagenesis kit (cat. no. 210518, Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) was used to generate the mutant-type vector (pGL3-JAK1-MT) including the del/del genotype of rs112395617 (sequence, TCTAGAAAATGACTGTATTCTCTCACCAGTAGGACTTAAACTTTGTTTCTCCAGTGGCTTAGCTCCTGTTCCTTTGGGTGATCACTAGCACCCATTTTTGAGAAAGCTGGTTCTACATGGGGGGATAGCTGTGGAATAGATAATTTGCTGCATGTTCTCAAGAACTAAGCCTGTGCCAGTGCTTTCCTAAGCAGTATACCTTTAATCAGAACTCATTCCCAGAACCTGGATGCTATTACACATGCTTTTAAGAAACGTCAATGTATATCCTTTTATAACTCTACCACTTTGGGGCAAGCTATTCCAGGCCGGCC). The sequence and direction of the resulting

constructs (pGL3-JAK1-WT and pGL3-JAK1-MT) were verified by direct sequencing by Genewiz Inc.

In silico prediction of microRNA (miRNA/miR) binding to rs112395617. Mature human miRNA sequences were obtained from miRBase (http://microrna.sanger.ac.uk) in 2016. A 27-bp region comprising rs112395617 was analyzed for hybridization of putative miRNAs using miRanda (version 3.3a) software with default parameters as described previously (20).

Cell culture and luciferase reporter assay. The Sk-Hep-1 cell line used in the present study was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The Sk-Hep-1 cells 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 $1x10^5$ 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-JAK1-WT or pGL3-JAK1-MT reconstructed vector and 50 ng of pRL-SV40 (Promega Corporation) were co-transfected using Lipofectamine® 2000 with 100 pmol miR-431-5p mimic or mimic control (Ambion; Thermo Fisher Scientific, Inc.) with (~$5x10^6$ 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 rs112395617 and HCC risk was analyzed by logistic regression, adjusted for age (mean ± standard deviation), sex, 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 *JAK1* mRNA expression levels in HCC tissues among 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). $P<0.05$ was considered to indicate a statistically significant difference, and all statistical tests were two-sided.

Results

Association of rs112395617 with HCC susceptibility. Table I summarizes the demographic characteristics of the patients with HCC and the controls. The distribution of age, sex, smoking and drinking status was similar between the patients with HCC and controls. The Hepatitis B surface antigen-positive rate was 70 and 10% in the HCC patients and controls, respectively, supporting the assumption that HBV infection is a risk factor for HCC (4). Fig. 1 presents the genotyping assays for rs112395617. The observed rs112395617 genotypic frequencies in the controls were consistent with the Hardy-Weinberg equilibrium ($P>0.05$). Table II indicates that the ins/del or del/del genotype exhibited a significantly decreased risk of HCC compared with the ins/ins genotype [$P=0.00019$; Odds ratio (OR)=0.52; 95% confidence interval (CI): 0.36-0.74; and $P=0.004$; OR=0.48; 95% CI, 0.28-0.82, respectively). In addition, the 4-bp deletion allele was associated with a 36% decreased risk compared with the insertion allele ($P=0.00016$; OR=0.64; 95% CI, 0.50-0.81). Collectively, these results suggest an association between rs112395617 and HCC susceptibility.

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

Characteristics	Patients, % (n=290)	Control, % (n=320)
Age (mean ± standard deviation)	53.5±9.6	52.4±10.1
Sex		
Male	187 (0.64)	208 (0.65)
Female	103 (0.36)	112 (0.35)
Smoking status		
Non-smokers	203 (0.70)	218 (0.68)
Former smokers	44 (0.15)	48 (0.15)
Current smokers	43 (0.15)	54 (0.17)
Drinking status		
Non-drinkers	145 (0.50)	166 (0.52)
Light drinkers	119 (0.41)	128 (0.40)
Heavy drinkers	26 (0.09)	26 (0.08)
Tumor stages		
Ia + Ib	200 (0.69)	
IIa + IIb	61 (0.21)	
IIIa + IIIb	29 (0.10)	
HBsAg		
Positive	203 (0.70)	31 (0.10)
Negative	87 (0.30)	289 (0.90)

HBsAg, Hepatitis B surface antigen.

Association between rs112395617 genotype and JAK1 mRNA expression levels. As demonstrated in Fig. 2, the *JAK1* mRNA level was highest in the AATT insertion homozygous group (ins/ins), followed by the heterozygous (ins/del) and AATT deletion homozygous groups (del/del). The average *JAK1* mRNA expression levels of the heterozygous and AATT insertion homozygous group were increased by 1.75- and 3.36-fold, respectively, compared with that of the AATT deletion homozygous group. The data of the present study suggests an association between different genotypes and *JAK1* mRNA expression levels *in vivo*.

Table II. Genotype distributions of the rs112395617 polymorphism in patients with HCC and healthy controls.

Genotype	HCC (n=290) n (%)	Controls (n=320) n (%)	OR (95% CI)[a]	P-value
ins/ins	146 (50.4)	107 (33.4)	1 (reference)	
ins/del	112 (38.6)	162 (50.6)	0.52 (0.36-0.74)	0.0002
del/del	32 (11.0)	51 (16.0)	0.48 (0.28-0.82)	0.004
ins allele	404 (69.7)	376 (58.8)	1 (reference)	
del allele	176 (30.3)	264 (41.2)	0.64 (0.50-0.81)	0.0002

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

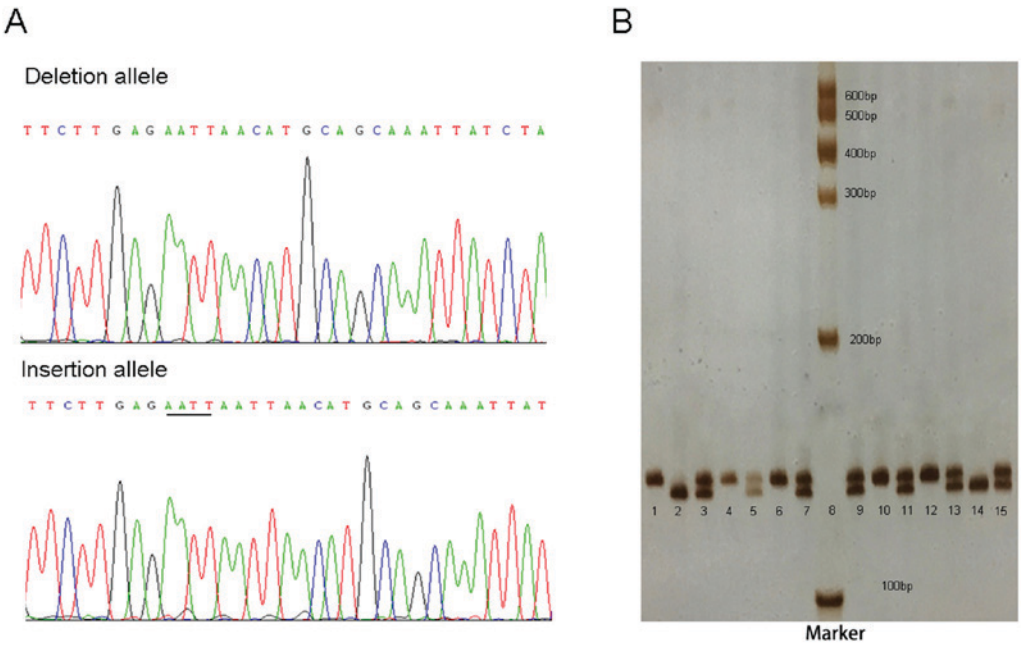

Figure 1. Sequencing and genotyping example of rs112395617. (A) Deletion and insertion allele sequences. The inserted 'AATT' is underlined. (B) Genotyping results using 7% non-denaturing PAGE and silver staining. Lanes 1, 4, 6, 10 and 12 ins/ins genotype; lanes 3, 5, 7, 9, 11, 13 and 15 ins/del genotype; and lanes 2 and 14, del/del genotype; lanes 8 DNA marker. Ins, insertion; del, deletion.

rs112395617 polymorphism affects JAK1 transcription activity by regulating its binding with miR-431-5p in vitro. The bioinformatics analysis indicated that rs112395617 lies within the predicted human miR-431-5p binding site, and the insertion allele of rs112395617 may disrupt the binding of miR-431-5p with the 3' UTR of *JAK1* (Fig. 3). To assess this prediction, 2 vectors, pGL3-JAK1-WT and pGL3-JAK1-MT, were constructed. In the pGL3-JAK1-WT group, the expression of *JAK1* mRNA, indicated by firefly luciferase activity, in the miR-431-5p mimic-transfected group was similar to that of the mimic control group. However, for the pGL3-JAK1-MT group, the expression of *JAK1* mRNA in the miR-431-5p mimic transfected group was significantly lower compared with that of the mimic control group (Fig. 4). These results suggest that miR-431-5p may bind with the del/del mutant 3' UTR of *JAK1* and negatively regulate *JAK1* transcription, and that the AATT insertion allele may weaken its regulatory effect by disrupting the binding of miR-431-5p to the 3' UTR of *JAK1*. Therefore, the *in vitro* study suggests that the polymorphism rs112395617 affects *JAK1* expression by altering the binding of miR-431-5p to the 3' UTR of *JAK1*.

Discussion

Although the association between polymorphisms in the *JAK1* exon and the risk of HCC or other types of cancer has been investigated (10-13), to the best of our knowledge, no study concerning the effect of polymorphisms in the 3' UTR of *JAK1* on HCC has been performed. The present study assessed the effect of the rs112395617 polymorphism in the 3' UTR of *JAK1* on the prevalence of HCC, and the potential mechanism of action. It was identified that the del/del and del/ins genotypes of rs112395617 were significantly associated with a decreased risk of HCC. The following phenotype-genotype association experiment indicated that the level of *JAK1* mRNA expression in HCC tissues with the del/del or del/ins genotype was significantly lower compared with that in HCC tissues with the ins/ins genotype. In addition, the luciferase reporter assay suggested

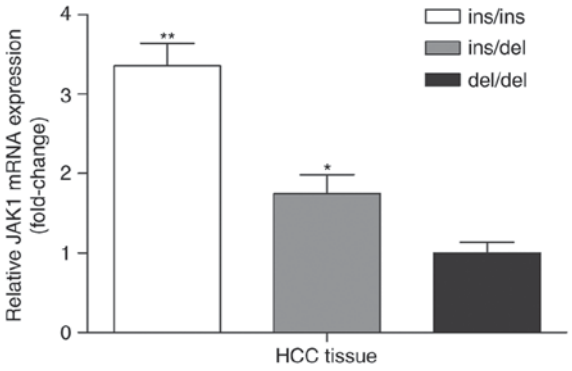

Figure 2. JAK1 mRNA expression levels in HCC tissues with different genotypes. The JAK1 mRNA expression level in HCC tissues with ins/del and ins/ins geno›type was 1.75- and 3.36-fold higher compared with that of the del/del genotype, respectively (*P<0.05 and **P<0.01 vs. del/del). ins/ins, n=33, ins/del, n=29, del/del, n=8. JAK1, Janus kinase 1; HCC, hepatocellular carcinoma; ins, insertion; del, deletion.

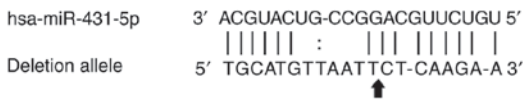

Figure 3. miRNA-JAK1 mRNA binding model and position of the insertion allele (arrow). Symbol ':' indicates that G pairs with U but not as precise as G and C or A and T. JAK1, Janus kinase 1; miRNA, microRNA.

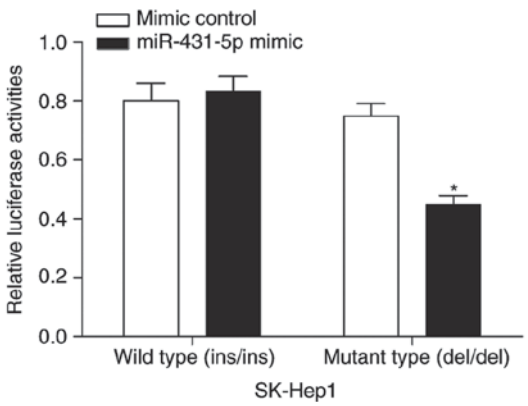

Figure 4. JAK1 transcriptional activity affected by rs112395617 and determined by luciferase reporter assay. The changes of the relative luciferase activity of SK-Hep1 cells that were co-transfected with miR-431-5p mimic or mimic control and pGL3-JAK1-WT or pGL3-JAK1-MT vectors. The pGL3-JAK1-MT and miR-431-5p mimic transfected group exhibited a significantly lower luciferase activity compared with the mimic control transfected group (*P<0.05). The Renilla luciferase vector was selected as an internal control. miRNA, microRNA; ins, insertion; del, deletion; JAK1, Janus kinase 1; WT, wild type; MT, mutant.

that the del/del or del/ins genotype potentially worked in full, or at least partially, by miR-431-5p mediated down-regulation of *JAK1* mRNA expression. To the best of our knowledge, this is the first study to investigate the association between the indel polymorphism in the *JAK1* 3' UTR and the risk of HCC.

JAK1 is a member of the Janus kinase family, which mediates the growth factor and cytokine-induced STAT signal pathway and has effects on immunity, cell growth, and differentiation (21). It has been demonstrated that the JAK-STAT signal pathway serves a key role in the development of HCC, and up to 45.5% of HCC cases harbor genomic alterations in the JAK-STAT signal pathway (6). *JAK1* expression in cancer cells may enable individual cells to contract and metastasize to other parts of the body (22). Activating mutations in *JAK1* have been identified in acute leukemia and myeloproliferative neoplasms (23). All of these data are consistent with the results demonstrated that the protective deletion allele with a low level of *JAK1* mRNA expression may decrease the risk of HCC development.

As described above, abnormal activation of *JAK1* 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 (24-28). This affects the level of expression of target genes and may be involved in numerous types of diseases, including cancer (25-27). Gao *et al* (24) demonstrated that an indel polymorphism at a miRNA-122-binding site in the 3' UTR of interleukin (IL)-1a conferred increased risk for HCC, and Wang *et al* (25) demonstrated that rs56288038 in the *IRF-1* 3' UTR regulated by miR-502-5p promoted the development of gastric cancer. 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 binding with the target genes (26-28). To explore the possible mechanism through which rs112395617 is involved in HCC susceptibility, miRNAs that would be able to bind with the *JAK1* 3' UTR and be affected by rs112395617 were predicted, and it was identified that miR-431-5p was able to bind with the del/del genotype, but not the ins/ins genotype, and consequently down regulated the expression of *JAK1*, thus reducing the risk of HCC.

miR-431, formerly hypothesized to be a nervous system-specific miRNA (29), has been identified to have a role as a novel tumor-related miRNA (30-33). Elevated expression of miR-431 was demonstrated to be able to act as a biomarker in differentiating patients with colorectal cancer (CRC) from healthy controls (30), suggesting an oncogenic role in CRC. Studies on medulloblastoma and glioblastoma have confirmed that miR-431 serves a role in the inhibitory effect of human IFN-β on cell viability by modulating the JAK-STAT signal pathway (31), indicating its anti-tumor role in these types of cancer. A previous study on HCC identified that the versican 3' UTR may promote HCC cell proliferation, migration, invasion and colony formation by binding and arresting miR-431 functions (32). An additional study of HCC indicated that miR-431 may inhibit HCC cell migration and invasion by targeting *ZEB1* (33). The roles played by miR-431 in cancer are dependent on the type of cancer, and the results of the present study are consistent with the previous studies on medulloblastoma, glioblastoma and HCC. Due to the bidirectional effects of miR-431 on different types of cancer, and 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; additional experimental validation is required.

To conclude, the present study demonstrated that the rs112395617 polymorphism in the *JAK1* 3' UTR may contribute to HCC susceptibility, possibly working in full, or at least partially, through an effect on *JAK1* transcriptional activity, by disturbing its binding with miR-431-5p.

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Availability of data and materials

All the datasets generated in the present study are included in the present manuscript and they are available upon reasonable request from the corresponding author.

Authors' contributions

The experiments were designed by QY and WC; the samples was collected by WQ; DNA extraction and genotyping was performed by JW; qPCR was conducted by YW; plasmid construction, *in silico* prediction of miRNA binding to rs112395617, cell culture and luciferase reporter assay were conducted by JZ; data analysis was performed by QY and was also the major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The design of the study was approved by the Ethical Committee of Suzhou Municipal Hospital (Suzhou, China).

Consent for publication

Patients provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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