Interleukin-6 and rs1800796 locus single nucleotide polymorphisms in response to hypoxia/reoxygenation in hepatocytes

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Abstract. Ischemia-reperfusion injury due to hypoxia/reoxygenation (H/R) is one of the main causes of liver damage during liver surgery. Donor interleukin-6 (*IL-6*) rs1800796 single nucleotide polymorphisms (SNPs) affect the metabolism of tacrolimus following liver transplantation-related hepatic H/R. This study investigated the response of *IL-6* and its promoter polymorphisms to hepatic H/R in liver parenchymal cells. The association between *IL-6* rs1800796 SNPs and IL-6 expression was measured in 84 disease-free liver tissues using tissue microarrays and immunohistochemistry. Subsequently, LO2G, LO2C and NC-LO2 cells were successfully constructed via stable lentivirus-mediated transfection. The effects of *IL-6* and its SNPs on the biological function of LO2 cells were examined using a cell model of H/R. Our results revealed that IL-6 was mainly expressed in hepatocytes. The intermediate IL-6 expression rate in genotype CC carriers was higher than that in genotype CG/GG carriers (P=0.006), which was subsequently verified at the *IL-6* mRNA level (P=0.002). The concentrations of alanine aminotransferase in the LO2G cells were significantly higher than those in the LO2C cells following H/R for 6 h and H/R for 24 h (P<0.05). The viability of the LO2C cells was higher than that of the LO2G cells (P<0.05). Furthermore, the expression of *IL-6* and its downstream molecules was significantly increased in the LO2C cells compared with the LO2G cells (P<0.05). Therefore, the sequence variants of rs1800796 SNPs (G→C) exhibit an increased *IL-6* transcription efficiency in liver parenchymal cells. In addition, the increased expression of *IL-6* protects the hepatocytes following hepatic H/R injury.

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Introduction

Ischemia-reperfusion injury (IRI) caused by hypoxia/reoxygenation (H/R) in the liver is a common cause of liver damage during liver resection, liver transplantation and hepatic failure (1-4). It has been widely demonstrated that interleukin-6 (IL-6) is a vital mediator, and IL-6/signal transducer and activator of transcription 3 (STAT3) signaling is of major importance in restoring normal hepatic function following liver IRI (5-7). Studies have found that IL-6 is transported to the liver in an endocrine manner from immunocytes at the wound site, or in a paracrine manner from hepatic immunocytes within the liver (8,9). However, the biological mechanisms of *IL-6* synthesis in parenchymal hepatic cells, not hepatic immunocytes, has previously been overlooked.

Additionally, the prevalence of rs1800795 (-174G/C) and rs1800797 locus polymorphisms in the *IL-6* gene promoter is extremely rare, whereas the allele C at the *IL-6* rs1800796 locus is frequently observed in East Asian populations (10-13). Although studies have reported that *IL-6* rs1800796 (also designated as -572C/G or -634G>C) single nucleotide polymorphisms (SNPs) affect *IL-6* transcriptional activity, their conclusions were not always consistent among similar populations (13,14). The rs1800796 locus SNPs may be present in a regulatory DNA binding site, leading to altered affinity with the regulatory protein, and may subsequently influence *IL-6* transcription efficiency (15). Furthermore, only donor *IL-6* rs1800796 SNPs affect the metabolism of tacrolimus, an immunosuppressive drug, mainly by altering liver function and regeneration in response to hepatic H/R following liver transplantation (16). Therefore, we hypothesized that *IL-6* and its rs1800796 SNPs in parenchymal hepatic cells may protect liver function during H/R, and that the sequence variants of rs1800796 SNPs (G→C) may increase the transcription efficiency of *IL-6*. Thus, in order to confirm our hypothesis, in the present study, we investigated the response of *IL-6* and its rs1800796 locus SNPs to hepatic H/R stimulus in parenchymal liver cells.

Materials and methods

Collection of liver tissue samples. Disease-free liver tissues were collected from 84 male Chinese volunteers at the

Shanghai Jiao Tong University Affiliated First People's Hospital (Shanghai, China) after obtaining written informed consent. The mean age of the subjects was 35.8±8.3 years. The subjects had no hepatic disease, cardiovascular or cerebrovascular disease that affected the serum IL-6 levels. Each sample was immediately cryopreserved in liquid nitrogen, and then transferred to refrigerator at -80˚C. All the surgical protocols were conducted similarly. This study was conducted according to the Declaration of Helsinki and its amendments, and was approved by the Ethics Committee of the Medical Faculties of Shanghai Jiao Tong University.

Materials. Rosewell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM), TRIzol reagemt and the SuperScript VILO cDNA Synthesis kit were purchased from Invitrogen (Carlsbad, CA, USA). Wild-type overexpressed *IL-6* vectors named p-IL-6(WT), negative control *IL-6* overexpression vectors, HG transgene reagent, all lentivectors, and the Lentiviral Packaging Plasmid Mix (Lenti-HG Mix) were obtained from GenomeDitech Co., Ltd. (Shanghai, China). Restriction endonucleases (*Bam*HI, *Cla*I and *Eco*RI) were purchased from MBI Fermentas (MBI Fermentas, Burlington, ON Canada). The QuickChange™ Site-Directed Mutagenesis kit was obtained from Stratagene (La Jolla, CA, USA). The RNeasy mini kits and the AllPrep DNA/RNA Mini kits were obtained from Qiagen (Venlos, The Netherlands). The SuperScript VILO cDNA synthesis kits were obtained from Invitrogen. The cell counting kit-8 (CCK-8) and alanine aminotransferase (AST) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies used were as follows: anti-*IL-6* and anti-*STAT3* polyclonal antibodies (ab6672 and ab68153; Abcam, Cambridge, UK). Ampicillin, puromycin, or blasticidin were obtained from MP Biomedicals (Irvine, CA, USA). The secondary antibody [goat anti-rabbit immunoglobulin G (ZB-2301)] was obtained from Golden Bridge Biotechnology Co., Ltd. (Beijing, China). The human normal liver cell line LO2 (17) and 293T cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China) and the cells were cultured in DMEM supplemented with 10% FBS. All normal cultures were maintained at 37˚C in saturated humidified incubator with 5% CO_2.

Stable cell transfection with IL-6 short hairpin RNA. Four IL-6-targeted short hairpin RNAs (IL-6-shRNAs), as well as one negative control mismatch sequence (IL-6-NC-shRNA), were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The targeting shRNA sequences containing recognition sites for *Bam*HI in the forward primer and *Eco*RI in the reverse primers are shown in Table I (Primers 1-5). Subsequently, the pGMLV-SC7-IL-6-shRNA lentiviral vectors were constructed using double restriction enzyme digestion and gene recombination. The sequence of these lentivectors was confirmed using direct sequencing. Subsequently, 293T cells were transiently transfected with the recombinant lentivectors, Lenti-HG Mix and HG transgene reagent as described in the manufacturer's manual. The packaging virus was collected from the cell supernatant through lysis after 3 consecutive freeze-thaw cycles. The viral titer was approximately 1x10^9 TU/ml, which was tested using end-point dilution assay. The LO2 cells were then steadily transfected with lentivirus particles at a multiplicity of infection (MOI) of 20, and the green fluorescence protein in the cells was observed using a laser fluorescence microscope (serial no. N-STORM; Nikon, Tokyo, Japan). Following transfection, the cells were subjected to drug screening with the corresponding antibiotics.

Subsequently, *IL-6* expression was measured by RT-qPCR as described in the section entitled 'RNA isolation and quantitative RT-PCR'. The primer sequences of the target gene (*IL-6*) and housekeeping gene (*GAPDH*) are listed in Table I (Primers 6 and 8). Compared with the control group, the mRNA expression of *IL-6* was reduced by 73, 80, 78 and 74% in the cells transfected with *IL-6*-shRNA 1, 2, 3 and 4, respectively, when measured at 48 h post-transfection (Fig. 3A). Therefore, the lentiviral particles harboring *IL-6*-shRNA2 were selected for use in the subsequent experiments.

Stable cell transfection and clone selection. First, total DNA was extracted from the LO2 cells using an AllPrep DNA/RNA mini kit according to the manufacturer's instructions. A 751-bp sequence (-635 to +116) of the *IL-6* promoter region was amplified from the genomic DNA of LO2 homozygous for the -572G allele at the rs1800796 locus (18). Specific primer sequences for the 751-bp amplification contained recognition sites for *Cla*I in the forward primer and *Eco*RI in the reverse primer. The 751-bp fragments containing the -572G allele represented the wild-type promoters derived from the LO2 cells. The fragments carrying the -572G allele and the pGMLV-PA2 lentiviral vectors were doubly digested with restriction endonuclease *Eco*RI and *Cla*I. The cytomegalovirus promoter was removed during this process in the linear lentiviral vector. Subsequently, the linear lentivectors and 751-bp fragments were ligated with T4 DNA ligase. The resulting lentivectors were named p-ProG, which carried the -572G allele. The G allele at the *IL-6* rs1800796 locus was site-specifically mutated to C with the template of wild-type promoter and the QuickChange™ Site-Directed Mutagenesis kit, as previously described (18). The p-ProC plasmids were synthesized as the p-ProG ones. Specific primer sequences and the plasmid profile for constructing p-ProG and p-ProC are listed in Table I (Primers 9 and 10) and Fig. 1A and B. The sequence of p-ProG and p-ProC was confirmed using direct DNA sequencing.

Second, nonsense mutations of *IL-6*-overexpressing fragments were obtained based on the *IL-6*-shRNA2 sequence and the template of p-IL-6(WT) using the QuickChange™ Site-Directed Mutagenesis kit, as previously described (18). The resulting fragments were named p-NM-IL-6(WT), and guaranteed that *IL-6* overexpression was not reduced by *IL-6*-shRNA2 in the LO2 cells. In particular, NM of p-NM-IL-6(WT) refers to nonsense mutations. Specific primers used in this process contained recognition sites for *Cla*I in the forward primer and *Eco*RI in the reverse primer (Table I; Primers 11 and 12). Later, the p-ProG lentivectors and p-NM-IL-6(WT) were doubly digested with restriction endonucleases *Eco*RI and *Bam*HI. Subsequently, the linear p-ProG vectors and *IL-6*-overexpressing fragments were ligated with T4 DNA ligase. The resulting lentiviral vector was named p-ProG-IL-6(MT) (Fig. 1C). Subsequently, the p-ProC-IL-6(MT) lentiviral vector (Fig. 1D) was constructed as was the p-ProG-IL-6(MT). The altered sequences were verified using direct sequencing before stable cell transfection.

Third, the negative control *IL-6*-overexpressing lentivirus particles (no expression of IL-6) and *IL-6*-overexpressing lentivirus particles [carrying ProG-IL-6(MT) or ProC-IL-6(MT)] were packaged as pGMLV-SC7-shRNA-IL-6 described in the previous section. Finally, the LO2 cells with an 80% suppression of basic *IL-6* were cultured in a 6-well culture plate at a density of approximately 2×10^5 cells/well for 24 h, and then stably transfected with *IL-6*-overexpressing lentivirus particles at an efficiency of approximately 70%. These stably transfected cells were named LO2C and LO2G, respectively. Similarly, the LO2 cells stably transfected with *IL-6*-NC-shRNA were again transfected with the negative control *IL-6*-overexpressing lentivirus particles. The resulting cells were designated as NC-LO2 (negative control LO2 cells). Following transfection, the cells were subjected to drug screening with the corresponding antibiotics (ampicillin, puromycin, or blasticidin).

Cell model of H/R. To induce H/R after ischemia reperfusion in the liver during liver transplantation and resection, the cell model of H/R was constructed as follows: cellular hypoxia was maintained by flushing the incubator set with a continuous gas flow comprising a mixture of 95% N_2 and 5% CO_2, as previously described (19,20). Re-oxygenation was ensured by continuously flushing with humidified a 95% air and 5% CO_2 gas mixture. This method resulted in an oxygen content of 0.1% in the chamber. Subsequently, during the logarithmic growth phase of the LO2C cells, LO2G cells, normal LO2 cells (control LO2) and NC-LO2 cells were inoculated into 96-well plates or 3.5 cm^2 culture dishes, and incubated with normal medium (DMEM supplemented with 10% FBS) under normoxic conditions (at 37°C in a saturated humidified incubator with 5% CO_2 and 95% air). Each of the cell types was prepared in 4 groups. Following overnight culture, the medium of these 4 cell types was changed to serum-free Krebs-Henseleit-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, as previously described (19), and subjected to hypoxia for 6 h. Subsequently, all the cells were transferred to fresh, warm, oxygenated medium and returned to reoxygenation conditions for a further 1, 6, 12 and 24 h, represented as H/R1h, H/R6h, H/R12h and H/R24h, respectively. Additionally, another 4 normoxic groups of cells, as the controls, were incubated for 12 h along with these H/R-exposed cells. Each group mentioned above was treated in triplicate.

Other methods
Genomic DNA isolation and genotype determination. Genomic DNA was extracted from the 84 liver specimens with an AllPrep DNA/RNA Mini kit according to the manufacturer's instructions. These samples were previously stored at -80°C until genotyping. Genotyping of the SNPs was performed using a Sequenom MassARRAY SNP genotyping platform, as previously described (21). The protocols mainly included DNA and primer preparation, PCR amplification, primer extension, spotting of primer extension products on the SpectroCHIP, and detection of the primer extension products by mass spectrometry.

Construction of tissue microarray and immunohistochemistry assay. Tissue microarray (TMA) was constructed from disease-free liver tissues (n=84) in collaboration with Shanghai Biochip (Shanghai, China) as previously described (22). Immunohistochemistry assay was then performed using the primary anti-*IL-6* polyclonal antibody (1:600). The secondary antibody was goat anti-rabbit immunoglobulin G. As the negative controls for all the experiments, the primary antibody was omitted.

Immunohistochemical scoring was performed independently in a blinded manner by two pathologists. The scoring standard has been previously described (23).

RNA isolation and quantitative RT-PCR (RT-qPCR). Total RNA from the 84 disease-free liver tissues and all types of LO2 cells was extracted using TRIzol reagent and the mini RNeasy® kit. First, single-stranded cDNAs were synthesized using a SuperScript VILO cDNA Synthesis kit. Subsequently, RT-qPCR was performed using 2X SYBR-Green qPCR mix and a Mastercycler® Ep Realplex. The fold change of relative mRNA copies was expressed as the relative quantification calculated using the $2^{-\Delta\Delta Ct}$ method for each group and calculated as previously described (23). Namely, the data were normalized to a housekeeping gene (*β-actin* or *GAPDH*). The primer sequences of the target gene and housekeeping gene are shown in Table I (Primers 6 to 8, and 13 and 14).

Cell counting kit-8 assay. A total of 2×10^3 LO2C cells, LO2G cells, normal LO2 cells (control LO2 cells) and NC-LO2 cells in the logarithmic growth phase were seeded into 96-well plates, and each group was allocated at least 3 wells. Subsequently, the cells were incubated with normal culture medium and subjected to H/R, and the effects of different H/R conditions on the cell growth or cell viability were evaluated using the CCK-8 assay according to the instructions of the manufacturer. The cells were incubated with CCK-8 (10 μl/well) at 37°C for 3 h. The absorbance (value A) of individual wells was determined at a 450 nm wavelength. The formula for calculating cell viability was (value A of treatment group - value A of blank group)/ (value A of control group - value A of blank group) x100.

ELISA for determining the AST level. After being subjected to different H/R conditions or normal culture, the degree of H/R injury in the LO2 cells was directly quantified by measuring the concentrations of AST in the cell supernatants using ELISA kits according to the manufacturer's instructions. Inter- and intra-detection coefficients of variation were <10% and <5%, respectively.

Western blot analysis. The expression of *STAT3* was determined by western blot analysis as previously described (4). In brief, total cellular protein was extracted and separated by electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were then washed, blocked and incubated with the primary antibodies (1:1,000 dilution for *STAT3*; 1:1,000 dilution for *β-actin*) overnight at 4°C, and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000). Signals were detected by chemiluminescence using ECL luminous fluid and scanned by Quantity One software. The protein expression of STAT3 was determined by normalization of the gray value of the *STAT3* gene to that of the control housekeeping gene (*β-actin*). Then, the resting relative level of STAT3 protein was calculated using the blank control group as a reference sample.

Statistical analysis. All data were analyzed using SPSS version 19.0 statistical software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 6.02 (GraphPad Software, San Diego, CA, USA). Continuous variables were reported as

Table I. Primer sequences used in this study.

No.	Name	Primer sequences
Primer 1	IL-6-shRNA1	F: 5'-**gatcc**GCCCTGAGAAAGGAGACATGTTTCAAGAGAACATGTCTCCTTTCTCAGGGCTTTTTT**g**-3'
		R: 5'-**aattc**AAAAAAGCCCTGAGAAAGGAGACATGTTCTCTTGAAACATGTCTCCTTTCTCAGGGC**g**-3'
Primer 2	IL-6-shRNA2	F: 5'-**gatcc***GGATGCTTCCAATCTGGATTC*TTCAAGAGAGAATCCAGATTGGAAGCATCCTTTTTT**g**-3'
		R: 5'-**aattc**AAAAAAGGATGCTTCCAATCTGGATTCTCTCTTGAA*GAATCCAGATTGGAAGCATCC***g**-3'
Primer 3	IL-6-shRNA3	F: 5'-**gatcc**GGAGTTTGAGGTATACCTAGATTCAAGAGATCTAGGTATACCTCAAACTCCTTTTTT**g**-3'
		R: 5'-**aattc**AAAAAAGGAGTTTGAGGTATACCTAGATCTCTTGAATCTAGGTATACCTCAAACTCC**g**-3'
Primer 4	IL-6-shRNA4	F: 5'-**gatcc**GCAGGACATGACAACTCATCTTTCAAGAGAAGATGAGTTGTCATGTCCTGCTTTTTT**g**-3'
		R: 5'-**aattc**AAAAAAGCAGGACATGACAACTCATCTTCTCTTGAAAGATGAGTTGTCATGTCCTGC**g**-3'
Primer 5	IL-6-NC-shRNA	F: 5'-**gatcc**GTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAACTTTTTTACGCGT**g**-3'
		R: 5'-**aattc**ACGCGTAAAAAAGTTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAC**g**-3'
Primer 6	Cell IL-6	F: 5'-CAGACAGCCACTCACCTCTTCAG-3'
		R: 5'-CTGCCAGTGCCTCTTTGCTG-3'
Primer 7	Cell STAT3	F: 5'-CTTTGAGACCGAGGTGTATCACC-3'
		R: 5'-GGTCAGCATGTTGTACCACAGG-3'
Primer 8	Cell GAPDH	F: 5'-GTCTCCTCTGACTTCAACAGCG-3'
		R: 5'-ACCACCCTGTTGCTGTAGCCAA-3'
Primer 9	p-ProG	F: 5'-GGCC**atcgat**TCTAAGTGGGCTGAAGCAGGTGAAG-3'
		R: 5'-GCCG**gaattc**TGGGCTCCTGGAGGGGA-3'
Primer 10	p-ProC	F: 5'-TCTACAACAGCC**C**CTCACAGGGAGAGC-3'
		R: 5'-GCTCTCCCTGTGA**G**GGGCTGTTGTAGA-3'
Primer 11	IL-6(WT)	F: 5'-CCG**gaatcc**GCCACC ATG AACTCCTTCTCCACAAGCGC-3'
		R: 5'-CCG**ggatcc**CTACATTTGCCGAAGAGCCCTCA-3'
Primer 12	IL-6(MT)	F: 5'-CCAAAGATGGCTGAAAAAGAT*GG**C**TG**T**TT**T**CA**G**AGCGG**C**TTT*AATGAGGAGA-3'
		R: 5'-CACCAGGCAAGTCTCCTCATT*AAAGCC**G**CTCTG**A**AAA**C**AGCC*ATCTTTTTCA-3'
Primer 13	IL-6 in liver tissue	F: 5'-GACCCAACCACAAATGCCAG-3'
		R: 5'-ACATTTGCCGAAGAGCCCTC-3'
Primer 14	β-actin in liver tissue	F: 5'-CTCCATCCTGGCGCTGT-3'
		R: 5'-GCTGTCACCTTCACCGTTCC-3'

*Bam*HI, *Cla*I, *Eco*RI and nonsense mutations sites and polymorphism position were introduced into the sense and antisense primer as indicated by the underlined text. IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse. Bold text indicates base sequences. Italic letters highlight the bases of nonsense mutations different from IL-6-shRNA2. Restriction enzymes identify the lower case letters.

Table II. IL-6 expression in human disease-free liver tissues in the TMA assay.

Groups	No. of case	IL-6 expression		P-value
		Weak (%)	Intermediate (%)	
CC	39	11 (28.2)	28 (71.8)	0.006
CG/GG	45	26 (57.8)	19 (42.2)	

IL-6, interleukin-6; TMA, tissue microarray.

the means ± standard deviation (SD) and examined using the Student's t-test or ANOVA. Categorical data were analyzed using the Chi-square test. Two-sided probability values were used for all statistical tests. Significance was established at the P<0.05 level.

Results

Distribution of genotypes and alleles. The frequencies of genotypes CC, CG and GG were 46.4, 47.6 and 6.0%, respectively. The allele C and G frequencies were 70.2 and 29.8% (data not shown). The genotype CG/GG carriers were pooled into one group, as previously described (16) (Table II). There were no significant differences between the CC group and CG/GG group with regard to race, gender, age distribution and body mass index (P>0.05; data not shown).

Expression of IL-6 in human disease-free liver tissues. The expression of *IL-6* in the 84 human disease-free liver tissues was evaluated using the TMA. The panels in Fig. 2A1-A3 and B1-B3
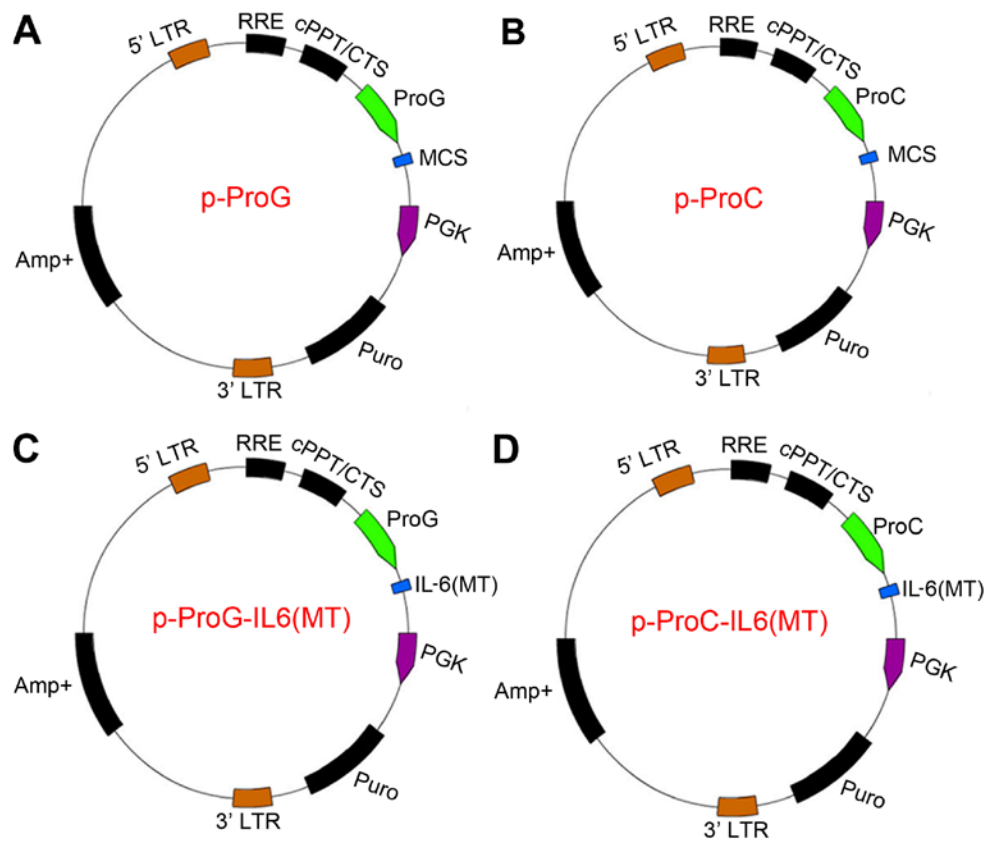
Figure 1. Lentiviral vector profile constructed and used in the present study. (A) Lentivector profile of completed construction containing -572G at rs1800796 locus. (B) Lentivector profile of completed construction containing -572C. (C) Lentivector profile of completed construction containing -572G and *IL-6* overexpression fragment. (D) Lentivector profile of completed construction containing -572C and *IL-6* overexpression fragment. IL-6, interleukin-6.

represent the intermediate and weak immunostaining profiles of IL-6 protein in hepatocytes. Among the 45 genotype (CG/GG) carriers, 57.8% (26/45) of the specimens exhibited weak staining and 42.2% (19/45) were intermediately immunoreactive for IL-6 (Table II). However, of the 39 genotype CC carriers, there was differential *IL-6* expression, with intermediate staining in 71.8% (28/39) of the samples and weak staining in 28.2% (11/39) of the samples (P=0.006) (Table II). Subsequently, RT-qPCR analysis was undertaken to further determine the *IL-6* mRNA levels in the 84 liver tissue samples. The ΔCt value for the genotype CC carriers was less than that for the CG/GG carriers (6.7±1.2 vs. 7.6±1.3, P=0.002) (Fig. 2C), confirming the results of immunostaining analysis. Further, when comparing the results of RT-qPCR between all the intermediate samples and all weak staining samples, the difference was more obvious (P<0.001). The protein (staining intensity) and *IL-6* mRNA expression were well correlated.

Construction of LO2 cell lines. The LO2C cells and LO2G cells, stably transfected with IL-6-shRNA2 lentivirus particles and *IL-6* overexpression lentivirus particles, were successfully constructed. Therefore, the LO2C and LO2G cells produced ectopic *IL-6* overexpression and approximately 20% of basic *IL-6*. Similarly, the NC-LO2 cells were constructed by stable transfection with IL-6-NC-shRNA and negative control *IL-6*-overexpressing lentiviral particles. The foregoing stable transfection protocol was described in the Materials and methods. Notably, the NC-LO2 cells served as the negative

controls. Normal LO2 cells were used as blank controls. The LO2G cells contained the wild-type promoter derived from the LO2 cells, and the WT promoter for the control compared with the LO2C cells. The double transfection of lentiviral particles did not alter the cell morphology of the LO2 cells significantly compared with the normal LO2 cells (Fig. 3B and C) at 48 h. Additionally, fluorescence analysis of the LO2 cells revealed an infection lentiviral transduction efficiency of approximately 70% in accordance with the manufacturer's instructions. There were no cells in the blank zone, and the fluorescence was 100% after 0.5 µg/ml puromycin selection (Fig. 3D).

H/R injury level of LO2 cells. Compared with the normoxic group cells, the concentrations of AST (ng/ml) in the LO2 cells increased significantly in all the H/R-exposed groups ranging from 30.18 to 78.98%, suggesting varying degrees of damage in the LO2 cells (Fig. 4A). In addition, the concentrations of AST in the LO2G cells were much higher than those in the LO2C cells at H/R6h, H/R12h and H/R24h (P=0.003, P=0.041 and P<0.001, respectively) (Fig. 4A). The differences between the experimental groups (LO2C and LO2G) and the control groups (control LO2 and NC-LO2) were more significant at all H/R exposure time points (P<0.01). However, there was no obvious difference between the control LO2 and NC-LO2 cells, which suggested that the lentiviral particles had a decreased effect on H/R injury in LO2 cells. This difference between the LO2C and LO2G cells was mainly caused by the sequence variants of the IL-6 rs1800796 locus SNPs (G→C).
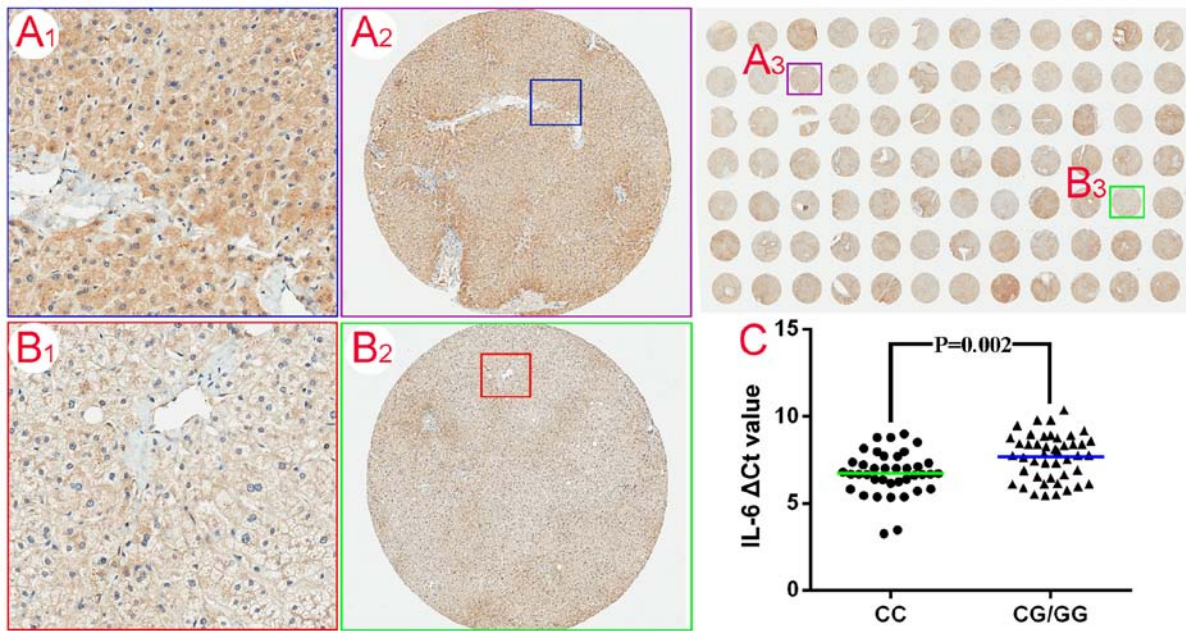
Figure 2. IL-6 expression in normal human liver tissues (n=84). (A1-A3) Intermediate staining of IL-6 in normal liver tissues (original amplification, x200, x40, x4, respectively). (B1-B3) Weak staining of IL-6 in normal liver tissues (original amplification, x200, x40, x4, respectively). (C) *IL-6* mRNA expression was determined using RT-qPCR according to genotypes of the 84 disease-free tissues. IL-6, interleukin-6.
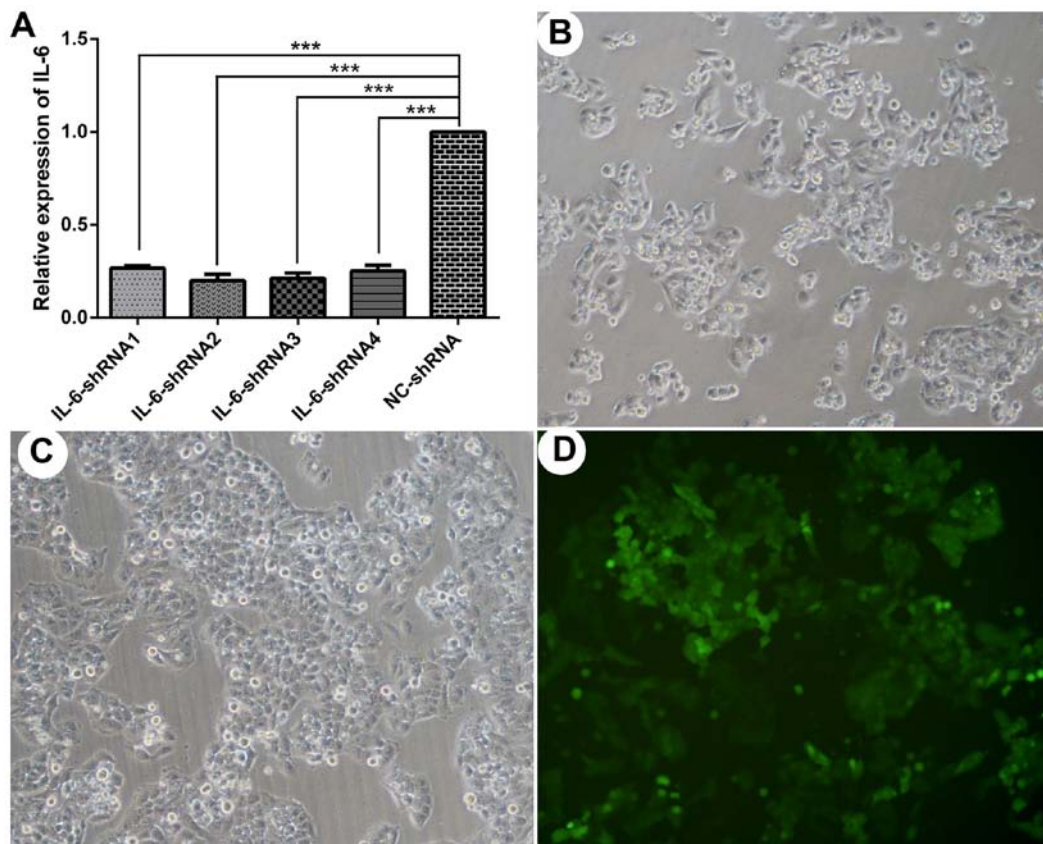

Figure 3. Relative expression of *IL-6* and images of LO2 cells (original amplification, x10). (A) Relative expression of *IL-6* examined by RT-qPCR after transfection of the LO2 cells with lentiviral particles of control (*IL-6*-NC-shRNA) and *IL-6*-shRNAs. (B) Bright-field images of LO2 cells before lentiviral transduction. (C) Bright-field images of LO2 cells at 48 h after lentiviral transduction. (D) Fluorescent image of LO2 cells at 48 h after lentiviral transduction. All data are expressed as the means ± SD (n=3 experiments). ***P<0.001 compared with control group. IL-6, interleukin-6; NC-shRNAs, negative control-short hairpin RNAs.

Viability of LO2 cells. Following H/R injury, the rate of survival in the 4 groups of cells was significantly decreased

by approximately 45% compared with the normoxic group of cells. In addition, the viability of the LO2C cells was signfi-
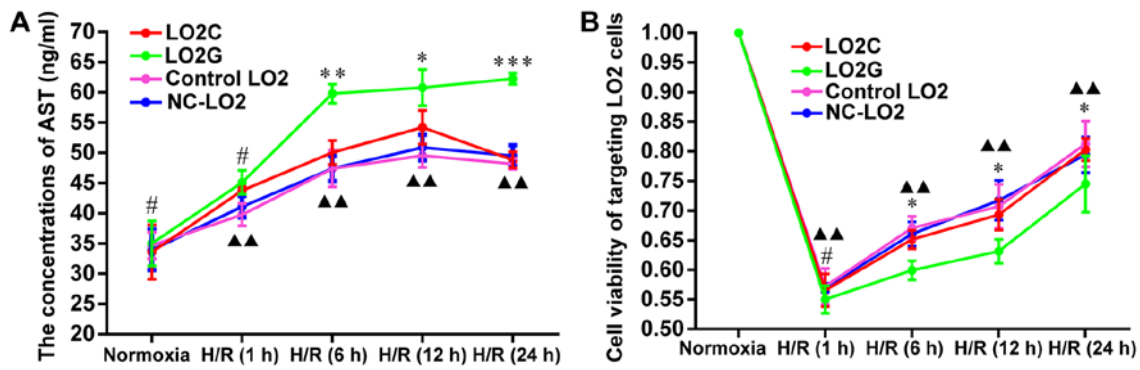
Figure 4. Role of *IL-6* rs1800796 SNPs in LO2 cells. (A) Concentrations of AST in the supernatant of LO2 cells in normoxic and H/R groups. (B) Cell viability of LO2 cells in in normoxic and H/R groups. All data are expressed as the means ± SD (n=3 experiments). ***P<0.001, **P<0.01, *P<0.05 and #P>0.05 between LO2G and LO2C groups and ▲▲P<0.01 between the experimental group and the control groups. AST, alanine aminotransferase; H/R, hypoxia/reoxygenation; *IL-6*, interleukin-6; SNP, single nucleotide polymorphism.
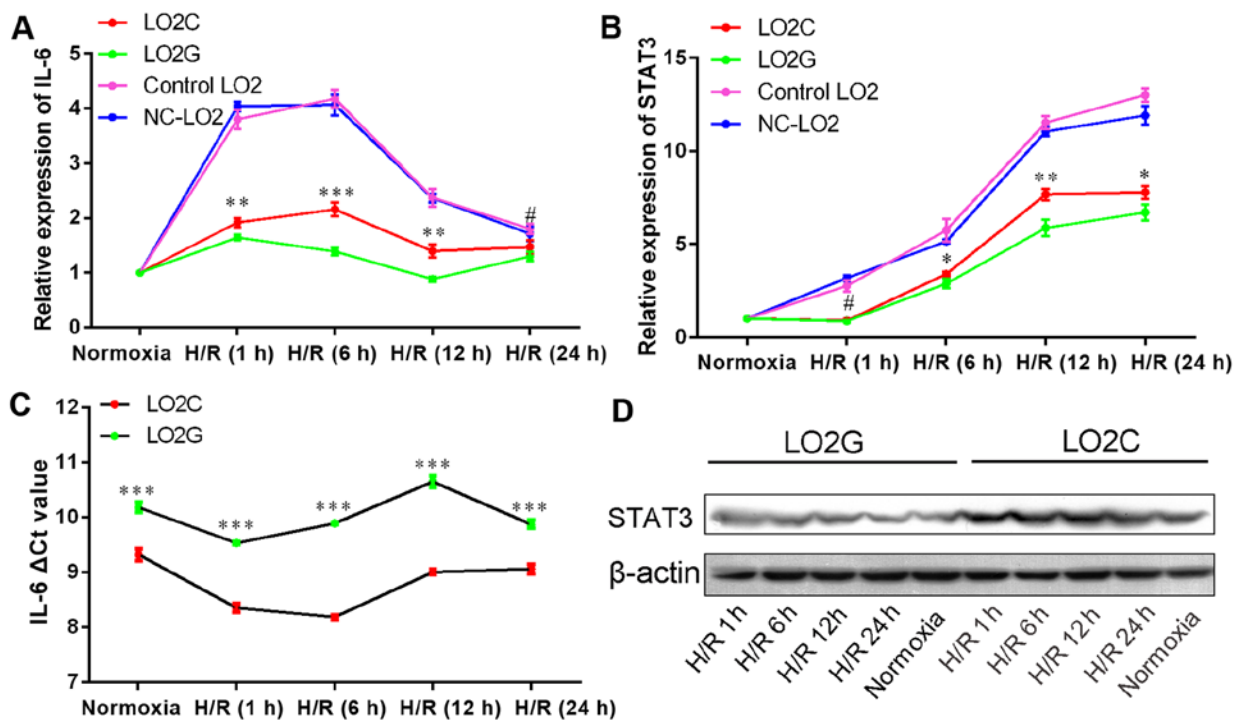

Figure 5. Effects of *IL-6* rs1800796 SNPs on the expression of *IL-6* and *STAT3* in normoxic and H/R groups. (A) Relative expression of *IL-6* detected using RT-qPCR. (B) Relative expression of *STAT3* detected using RT-qPCR. (C) ΔCt values of *IL-6*. (D) Levels of expression of STAT3 protein were determined by western blot analysis. All data are expressed as the means ± SD (n=3). ***P<0.001, **P<0.01, *P<0.05 and #P>0.05 comparing LO2G and LO2C groups. H/R, hypoxia/reoxygenation; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription; SD, standard deviation; SNP, single nucleotide polymorphism.

cantly increased compared to that of the LO2G cells from H/R6h to H/R24h (P=0.034, P=0.016 and P=0.031) (Fig. 4B). In addition, there were significant differences between the experimental group and the control group at all H/R exposure time points (P<0.01) (Fig. 4B). There were no obvious differences in cell viability between the control LO2 and NC-LO2 cells, which suggested that the lentiviral particles had less prominent effects on the biological activity of the LO2 cells. The results were consistent with the those obtained for the AST levels.

Expression of IL-6 and STAT3 in LO2 cells. Compared with *IL-6* expression in the normoxic groups, the increase in the mRNA expression of *IL-6* in the LO2C cells was greater than that in

the LO2G cells from H/R1h to H/R24h (P=0.009, P<0.001, P=0.002 and P<0.05, respectively) (Fig. 5A). Similarly, *STAT3* expression also increased from H/R1h to H/R24h (P<0.05, P=0.034, P=0.004 and P=0.027) (Fig. 5B). Additionally, relative to the housekeeping gene (*GAPDH*), *IL-6* expression was lower in the LO2G cells than in the LO2C cells under normoxic conditions (P<0.001) (Fig. 5C). This result was consistent with that of *IL-6* expression in human disease-free liver tissues. We also investigated whether STAT3 protein expression was increased under H/R conditions by conducting subsequent western blot analysis, which confirmed that the STAT3 level was also higher at all time points. Additionally, STAT3 expression was higher in the LO2C cells compared with the LO2G cells (Fig. 5D).

The result was confirmed by densitometry (integral optical density, IOD) to measure the STAT3 levels from H/R1 to H/R24 (P<0.05).

Discussion

Hepatic IRI caused by H/R is a common pathological phenomenon during liver surgery, liver transplantation and severe liver trauma (1-4). The simultaneous activation and interaction of multiple cytokines, particularly multifunctional IL-6, regulate cell injury and death during liver regeneration. *IL-6* plays an essential role in triggering liver regeneration during the priming phase (24). Furthermore, protective *IL-6/STAT3* signaling is initiated within minutes to hours after partial liver hepatectomy (4,25). Recently, the *IL-6* rs1800796 locus SNPs were thoroughly investigated in a healthy population (13), and under several clinical conditions (26-28). It has been reported that only donor *IL-6* rs1800796 SNPs affect the metabolism of tacrolimus, mainly by influencing liver function and regeneration following liver transplantation (16). Therefore, in this study, we examined IL-6 expression in 84 disease-free liver tissues using TMA. The IL-6 level was higher in genotype CC carriers than in genotype CG/GG carriers, due to a higher intermediate expression rate (P=0.006) (Fig. 2A and B and Table II). Subsequently, it was verified at the mRNA level (Fig. 2C). Therefore, allele C promotes *IL-6* transcription efficiency, which is consistent with a study involving basic promoter experiments using a reporter gene system (18). As shown in Fig. 2A and B, the IL-6 was mainly expressed in the cytoplasm of hepatocytes. It has been reported that approximately 70% of liver cells are hepatocytes (29). Furthermore, studies have suggested that primary rodent and LO2 hepatocytes synthesize and secrete IL-6 (9,30,31). Therefore, the biological mechanisms of *IL-6* and its rs1800796 locus SNPs, in human hepatocytes, warrant further investigation.

The immortalized human normal liver cell line, LO2, was derived from primary normal human hepatocytes, and still retains the biological characteristics of normal adult hepatocytes (17). Additionally, the current third-generation lentivirus used in this study, proved that human immunodeficiency virus-based vectors are valid manufacturing tools for stable genetic modification (32,33). The LO2G cells and LO2C cells successfully generated via lentivirus-mediated method stably produced ectopic IL-6 overexpression and approximately 20% of endogenic IL-6. The LO2G and LO2C cells, respectively, carried allele G or C at the *IL-6* rs1800796 locus. The NC-LO2 cells, as the negative control group, were also successfully constructed. Furthermore, cell morphology was not altered significantly following double lentivirus transfection (Fig. 3B and C). Subsequently, based on the LO2C cells, the LO2G cells, normal LO2 cells (blank control group) and NC-LO2 cells, a cell model of H/R was successfully established in order to examine H/R injury (19,20).

In this study, the concentrations of AST were higher in the LO2C cells than in the LO2G cells from H/R6h to H/R24h (P<0.05) (Fig. 4A). Similarly, there were also marked differences between the LO2C and LO2G cells as regards cell viability from H/R6h to H/R24h (P<0.05) (Fig. 4B). The differences in the AST data or cell viability between the experimental and the control groups were significant at all H/R time-points (P<0.01) (Fig. 4). Interestingly, these data did not differ between the control LO2 and NC-LO2 cells, which suggested that the lentiviral particles exerted less prominent effects on the biological properties of LO2 cells. Therefore, the differences between LO2C and LO2G cells were mainly attributed to allele G mutating into C at the rs1800796 locus.

The relative increase in IL-6 expression in the LO2C cells was higher than that in the LO2G cells from H/R1h to H/R12h (Fig. 5A). LO2C cells expressed greater IL-6 than LO2G cells in the normoxia group (P<0.001) (Fig. 5C). The expression of STAT3, downstream signal molecules of IL-6, in LO2C cells increased higher than in LO2G cells, from H/R6h to H/R24h (P<0.05) (Fig. 5B). Similarly, it was also found that the STAT3 level was higher at all time-points under H/R conditions. This phenomenon was more obvious in LO2C cells compared with LO2G cells (Fig. 5D) and confirmed by grey level of STAT3 from H/R1 to H/R24 (P<0.05).

The results suggest that LO2 cells synthesized IL-6, and the subsequent IL-6/STAT3 signaling pathway was triggered within minutes to hours following H/R injury. IL-6 exerted hepatoprotective effects and decreased cell activity through the upregulation of IL-6/STAT3 signaling. Although IL-6 production by non-parenchymal cells, such as Kupffer cells may play a role (8,9), these findings suggested that IL-6 produced in hepatocytes promoted the activation of the IL-6/STAT3 signaling pathway and also exerted hepatoprotective effects in an autocrine manner.

It was observed that *IL-6* rs1800796 SNPs affected *IL-6* transcription efficiency under normoxic or H/R conditions. Furthermore, the allele G→C substitution accelerated the production of IL-6 and attenuated H/R injury in hepatocytes. These SNPs were present in the *IL-6* promoter region, and altered the affinity with the regulatory protein, and subsequently influenced *IL-6* transcription (15). For example, SNPs in the osteopontin promoter region have been shown to alter DNA binding affinity to transcription factors SP1/SP3 (34).

The present study has several limitations. First, although the use of TMAs enables the analysis of 84 samples in a relatively short period of time, the results may be unreliable due to the small sample size. Thus, these results need to be confirmed with a larger population. Second, *in vitro* studies using the LO2 cell line, but not adult hepatocytes, may be confounded by the cell type and culture conditions. Therefore, further *in vivo* studies are warranted in order to elucidate the function of *IL-6* gene and SNPs. Third, this study mainly investigated the changes in STAT3 and IL-6 RNA or protein levels. Although many studies have fully established the association between IL-6 protein and mRNA expression, its functional significance requires further analysis. Nevertheless, our results provide insight into the potential role of *IL-6* and its SNPs in human hepatocytes.

In conclusion, this study, to the best of our knowledge, was the first of its kind to demonstrate that sequence variants of *IL-6* rs1800796 locus SNPs (G→C) increase the transcription efficiency of the *IL-6* gene and protect hepatocytes following H/R. H/R injury triggered the synthesis of IL-6 and STAT3 in the liver parenchymal cells. Our results facilitate efficient liver regeneration and restoration following liver surgery. Additionally, these data establish the framework for exploring the role of the *IL-6* gene and its polymorphisms in other H/R conditions, such as those affecting the brain and kidneys.

Acknowledgements

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