INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours all over the world. The hepatitis B virus (HBV) appears to be an indispensable element in the progression of almost all cases of HCC. HBV infection is highly endemic in China, but only a small number of exposed individuals develop cancer, indicating that additional host factors are involved in carcinogenesis. Other factors, such as environmental factors, genetic susceptibility or alterations of immunological factors, increase the incidence of HBV-related lesions, including liver cancer. Indeed, differences in genetic susceptibility and host immune responses have been found to increase the incidence of HBV-related lesions and thus cancer.

CTLA-4 is an inhibitory molecule expressed on activated T cells and plays critical roles in the balance between the pro- and anti-immune responses via down-regulation of T cell signalling. A large number of genetic association studies suggest that CTLA-4 is an important susceptibility locus.
for autoimmune diseases and certain types of cancer; however, the mechanism by which polymorphism of CTLA-4 acts to inhibit cancer remains unclear. Recently, several studies focused on the association of CTLA-4 single nucleotide polymorphisms (SNPs) with susceptibility to various types of cancer, such as gastric cancer, cervical cancer, colorectal cancer and lung cancer. Also, a few case-control studies focused on the association of CTLA-4 SNPs with susceptibility to HCC. However, due to the small sample size and the limited amount of data, the relationship between CTLA-4 SNPs and risk of HCC was not fully understood.

There are various factors associated with the development of HCC, and the host immune response has been highlighted as a genetic biomarker for the disease with the production of several cytokines. Cytokines released from a variety of activated T cells play key roles in the regulation of immune responses, and SNPs in the regulatory sequences of genes are presumed to be associated with the production of cytokines. Therefore, polymorphisms in genes coding cytokines can also influence the production and function of these proteins, susceptibility or disease progression. Among them, TNF-α and IL-10 are of particular interest, as the genes for these cytokines are located in the central major histocompatibility complex (MHC).

Tumour necrosis factor alpha (TNF-α) is one of the major pro-inflammatory cytokines involved in tumour proliferation, invasion and metastasis. Furthermore, it was reported that the expression of TNF-α is regulated at the transcriptional level and various SNPs in TNF have been associated with TNF-α level. In particular, the polymorphisms at position −238 and −308 in the promoter region have been commonly studied.

As an anti-inflammatory cytokine, IL-10 is produced by Th2 lymphocytes, monocytes and B lymphocytes. It inhibits macrophage-dependent cytokine synthesis by Th1 cells and thus regulates balance between cellular and humoral immune responses. The IL 10 presents its transcript governed by SNPs in the promoter region of the gene. Numerous polymorphic sites have been identified in the IL 10 gene, including −592 C/G and −819 C/T, which appear to be mostly related to SNPs in the promoter and might influence the circulating IL-10 levels.

Host immune responses are known determinants of cancer susceptibility. Cancer-bearing hosts have different antitumour responses, whereas the underlying mechanisms are not fully understood. In the current study, we hypothesized that SNPs in CTLA-4, TNF and IL 10 can interfere with T cell function, which might be a genetic susceptibility factor for HCC. To test this hypothesis, we investigate the possible associations of CTLA-4, TNF and IL 10 genotypes with the susceptibility to HCC. We also functionally characterized the effects of these SNPs by analysing T cell proliferation, cytotoxicity and cytokine production using PBMCs with diverse genotypes. Additionally, the synergistic effects of these genotypes were also determined.

### Materials and Methods

#### Study populations

A total of 277 patients with HCC were enrolled between September 2014 and August 2016 at Sichuan Provincial People’s Hospital (Table 1). Diagnosis of HCC was confirmed by routine histopathological examinations. Meanwhile, 306 ethnically and geographically matched healthy individuals were randomly selected as controls, with evidence of negative serological tests for HBsAg, anti-HBc; no individual history of infectious diseases or cancer and treatment of immune diseases; and no history of diabetes mellitus, hyperthyroidism, rheumatoid arthritis or other autoimmune diseases within the past two years. Information about tobacco use, alcohol use and HBV infection status was obtained from the patient’s

| Gene | SNPs | Primer pairs | Product length (bp) |
|------|------|--------------|---------------------|
| CTLA-4 | −318 T/C | Forward: AGGATGGTGCTTCACAGAT; reverse: AGCCAATCCATGGATGGA | 288 |
| | rs5472909 | | |
| CT60 | G/A | Forward: TGCAAGTCATTCTTGGAAG; reverse: CTGTGATAGTTGAGCTGA | 210 |
| | rs30807243 | | |
| TNF | −238 G/A | Forward: AGACCCCCCTCGGAATC; reverse: ATCTGGAGGAAGCGGTAGTG | 149 |
| | rs361525 | | |
| | −308 G/A | Forward: GCAATAGGTTTTGAGGGGCAT; reverse: TCCCTGCTCCCGATTTCCG | 102 |
| | rs1800629 | | |
| IL10 | −592 C/A | Forward: GGTGAGCACTACCTGACTAGC; reverse: CTCAGGTACACGTGACCTG | 412 |
| | rs1800872 | | |
| | −819 C/T | Forward: CCAGATATCTGAAGAAGTCCTG; reverse: TGGGGGAAGTGGGTAAGAGT | 559 |
| | rs1800871 | | |
medical history. Patients and controls were Chinese, enrolled from Sichuan province of China. Written informed consent was obtained from all participants during recruitment.

### 2.2 DNA extraction and genotyping

Genomic DNA of all subjects was extracted from peripheral leucocytes using QIAamp DNA mini Kit (Qiagen GmbH, Hilden, Germany). DNA purity and concentration were determined by an ultraviolet spectrophotometer and then kept at −20°C refrigerator.

Genotyping for polymorphisms in *CTLA-4* (−318 T/C, CT60 G/A), *TNF* (−238 G/A, −308 G/A) and *IL 10* (−592 C/A, −819 C/T) was carried out using PCR, with a 25 μL reaction solution containing 100 ng genomic DNA, 1 × PCR buffer, 2 mmol/L MgCl₂, 200 μmol/L dNTPs, 2 unit DNA polymerase (Takara, Japan) and 1 μmol/L of specific primer mix (Shenggong, China). The reaction conditions were as follows: initial denaturation for 2 minutes at 95°C, followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes. The primer pairs employed were listed in Table 1. SNP genotyping was performed using a 3730 DNA sequencer (Applied Biosystems).

### 2.3 Peripheral blood mononuclear cells isolation and culture

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized venous blood using Ficoll-Hypaque
density-gradient centrifugation, washed and resuspended in 
RPMI 1640 medium containing 10% foetal bovine serum 
(Gibco). Then, we incubated PBMCs with or without 25 μg/
ml phytohemagglutinin (PHA; Sigma) for 6 hours.

2.4 | 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium 
bromide (MTT) assay was done to determine cell prolifera-
tion. 500 μL PBMC suspension (10^4 cells/mL) was seeded in 
each well of a 96-well plate. After PBMCs were stimulated 
with or without PHA for 6 hours, 20 μL of MTT solution 
(5 mg/mL) was added into each well. After incubation for 
another 4 hours at 37°C, PBMCs were centrifuged at 350 g
for 5 minutes and resuspended in 150 μL DMSO. Absorbance
was read at 570 nm with a BIO-RAD microplate reader 
(Hercules, CA).

2.5 | Cytotoxic assay

The cytotoxic assays were done by using a non-radioactive 
cytotoxicity assay kit (Promega) according to the manufac-
turer’s instructions and as described previously. PBMCs 
treated with PHA were used as effector cells, and HepG2 
liver cancer cells were used as target cells.

2.6 | ELISAs

Enzyme-linked immunosorobt assay (ELISAs) were em-
ployed to detect the cytokine levels (IL-2, IL-4, TGF-β, 
IL-10 and TNF-α) in undiluted serum samples and PBMC

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### TABLE 3  Genotype frequencies of CTLA-4 gene in the patients with HCC and controls

| Genotype | Frequency, No. (%) | OR (95% CI) | P value |
|----------|--------------------|-------------|---------|
|          | Patients (n = 554) | Controls (n = 612) |         |
|         | Codominant model   |             |         |
| CC      | 360 (65.0)         | 466 (76.1)  | Reference |
| TC      | 170 (30.7)         | 134 (21.9)  | 1.624 (1.260, 2.140) <.01 |
| TT      | 24 (4.3)           | 12 (2.0)    | 2.589 (1.277, 5.247) .006 |
| Dominant model |  |             |         |
| CC      | 360 (65.0)         | 466 (76.1)  | Reference |
| TC + TT | 194 (35.0)         | 146 (23.9)  | 1.720 (1.333, 2.220) <.01 |
| Recessive model |  |             |         |
| CC + TC | 530 (95.7)         | 600 (98.0)  | Reference |
| TT      | 24 (4.3)           | 12 (2.0)    | 2.264 (1.121, 4.572) .019 |
| Alleles |                  |             |         |
| C       | 445 (80.3)         | 532 (86.9)  | Reference |
| T       | 109 (19.7)         | 80 (13.1)   | 1.629 (1.189, 2.232) .002 |
| CT60, G/A | Codominant model   |             |         |
| AA      | 200 (36.1)         | 240 (39.2)  | Reference |
| AG      | 238 (43.0)         | 274 (44.8)  | 1.042 (0.807, 1.346) .751 |
| GG      | 116 (20.9)         | 98 (16.0)   | 1.420 (1.023, 1.972) .036 |
| Dominant model |  |             |         |
| AA      | 200 (36.1)         | 240 (39.2)  | Reference |
| AG + GG | 354 (63.9)         | 372 (60.8)  | 1.142 (0.901, 1.448) .273 |
| Recessive model |  |             |         |
| AA + AG | 438 (79.1)         | 514 (84.0)  | Reference |
| GG      | 116 (20.9)         | 98 (16.0)   | 1.389 (1.032, 1.871) .030 |
| Alleles |                  |             |         |
| A       | 319 (57.6)         | 378 (61.8)  | Reference |
| G       | 235 (42.4)         | 234 (38.2)  | 1.190 (0.941, 1.504) .146 |
culture supernatants. Each cytokine was detected in triplicate according to the manufacturer’s instructions (Beyotime).

2.7 | Statistical analysis

The genotype frequencies in patients and controls were tested for conformity with Hardy-Weinberg equilibrium using chi-squared test. The homozygous form of the most common allele was used as the reference variant. For the sake of easy calculations, the frequencies of genotypes and alleles in Tables 3, 4, 5 and 6 were calculated in double, and the frequencies between the two groups were compared using chi-squared test. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to evaluate the relative disease risk. Student’s t test or one-way analysis of variance (ANOVA) was used to compare differences in lymphocyte proliferation, cytokine concentrations and cytolytic activity between the different genotypes. All statistical analyses and graphics were conducted using SPSS 22.0 software (SPSS Inc) and GraphPad Prism 5.0 (GraphPad Inc). P value < .05 was considered statistically significant.

3 | RESULTS

3.1 | Participant characteristics

Table 2 summarizes the main characteristics of the two groups. There was statistical difference of distribution on alcohol consuming between patients and controls ($P < .05$). No significant differences were observed between the two groups in regard to age, sex distributions and smoking status ($P = .518$, $P = .468$ and $P = .059$).
### TABLE 5 Genotype frequencies of IL-10 gene (IL10) in the patients with HCC and controls

| Genotype | Frequency, No. (%) | OR (95% CI) | P value |
|----------|-------------------|-------------|---------|
|          | Patients (n = 554) | Controls (n = 612) |         |
| −592, C/A |                    |             |         |
| Codominant model |                 |             |         |
| CC       | 160 (28.9)        | 214 (34.9)  | Reference |
| CA       | 282 (50.9)        | 288 (47.1)  | 1.310 (1.007, 1.703) | .044 |
| AA       | 112 (20.2)        | 110 (18.0)  | 1.362 (0.976, 1.901) | .069 |
| Dominant model |                 |             |         |
| CC       | 160 (28.9)        | 214 (34.9)  | Reference |
| CA + AA  | 394 (71.1)        | 398 (65.1)  | 1.324 (1.034, 1.696) | .026 |
| Recessive model |                |             |         |
| CC + CA  | 442 (79.8)        | 502 (82.0)  | Reference |
| AA       | 112 (20.2)        | 110 (18.0)  | 1.156 (0.863, 1.549) | .330 |
| Alleles  |                   |             |         |
| C        | 301 (54.3)        | 358 (58.5)  | Reference |
| A        | 253 (45.7)        | 254 (41.5)  | 1.185 (0.939, 1.494) | .152 |
| −819, T/C |                    |             |         |
| Codominant model |                 |             |         |
| CC       | 182 (32.8)        | 250 (40.8)  | Reference |
| CT       | 304 (54.9)        | 306 (50.0)  | 1.365 (1.065, 1.749) | .014 |
| TT       | 68 (12.3)         | 56 (9.2)    | 1.668 (1.116, 2.493) | .012 |
| Dominant model |                 |             |         |
| CC       | 182 (32.8)        | 250 (40.8)  | Reference |
| CT + TT  | 372 (67.2)        | 362 (59.2)  | 1.412 (1.111, 1.794) | <.01 |
| Recessive model |                |             |         |
| CC + CT  | 486 (87.7)        | 556 (90.8)  | Reference |
| TT       | 68 (12.3)         | 56 (9.2)    | 1.774 (1.238, 2.543) | .084 |
| Alleles  |                   |             |         |
| C        | 334 (60.5)        | 403 (65.8)  | Reference |
| T        | 220 (39.5)        | 209 (34.2)  | 1.270 (1.001, 1.612) | .049 |

### TABLE 6 Association of combination distributions of CTLA-4, TNF and IL10 with HCC susceptibility

| Genotype | Frequency, No. (%) | OR (95% CI) | P value |
|----------|-------------------|-------------|---------|
|          | Patients (n = 554) | Controls (n = 612) |         |
| CTLA-4 −318/ TNF −238 |                                   |         |
| CC/AA    | 14 28             | Reference   |         |
| TC or TT/AA | 16 36             | 0.889 (0.372, 2.124) | .791 |
| CC/GG or GA | 336 400           | 1.680 (0.870, 3.243) | .119 |
| TC or TT/GG or GA | 188 148         | 2.541 (1.291, 4.998) | <.01 |
| CTLA-4 −318/IL10 −819 |                               |         |
| CC/CC    | 124 192           | Reference   |         |
| TC or TT/CC | 116 112           | 1.604 (1.137, 2.262) | <.01 |
| CC/CT or TT | 226 202           | 1.732 (1.290, 2.327) | <.01 |
| TC or TT/CT or TT | 88 106         | 1.285 (0.895, 1.846) | .173 |
**TABLE 7** Associations of *CTLA-4*, *TNF* and *IL 10* polymorphisms with serum cytokine levels

| Genotypes | IL-2 |       | IL-4 |       | TGF-β |       | IL-10 |       | TNF-α |       |
|-----------|------|-------|------|-------|-------|-------|-------|-------|-------|-------|
|           | HCC  | Control | HCC  | Control | HCC  | Control | HCC  | Control | HCC  | Control |
| **CTLA-4** |      |         |      |         |      |         |      |         |      |         |
| −318      |      |         |      |         |      |         |      |         |      |         |
| CC        | 9.38 ± 0.61 | 5.05 ± 0.42 | 7.36 ± 0.50 | 4.80 ± 0.58 | 5.56 ± 0.50 | 4.60 ± 0.51 | 5.16 ± 0.44 | 5.21 ± 0.38 | 5.22 ± 0.31 | 5.28 ± 0.34 |
| TC        | 7.95 ± 0.53 | 5.32 ± 0.47 | 7.09 ± 0.49 | 4.62 ± 0.48 | 5.83 ± 0.47 | 4.96 ± 0.49 | 5.31 ± 0.52 | 5.31 ± 0.29 | 5.01 ± 0.42 | 5.21 ± 0.32 |
| TT        | 6.12 ± 0.55 | 4.58 ± 0.44 | 5.93 ± 0.46 | 4.95 ± 0.42 | 6.11 ± 0.49 | 5.02 ± 0.52 | 5.54 ± 0.57 | 5.07 ± 0.30 | 4.99 ± 0.49 | 5.11 ± 0.30 |
| t         | 4.520 | 1.550 | 2.452 | 0.270 | 2.101 | 1.905 | 0.676 | 0.164 | 1.941 | 1.434 |
| p         | <.01  | .128  | .027  | .817  | .048  | .092  | .536  | .871  | .062  | .162  |
| **TNF −238** |      |         |      |         |      |         |      |         |      |         |
| GG        | 5.21 ± 0.33 | 4.41 ± 0.22 | 5.61 ± 0.54 | 5.41 ± 0.47 | 5.62 ± 0.47 | 4.82 ± 0.45 | 4.63 ± 0.27 | 4.91 ± 0.54 | 5.61 ± 0.54 | 5.77 ± 0.42 |
| GA        | 5.41 ± 0.42 | 4.50 ± 0.31 | 5.36 ± 0.40 | 5.25 ± 0.39 | 5.21 ± 0.30 | 4.77 ± 0.56 | 4.44 ± 0.36 | 4.77 ± 0.48 | 5.45 ± 0.43 | 5.63 ± 0.55 |
| AA        | 5.48 ± 0.38 | 4.49 ± 0.29 | 5.21 ± 0.46 | 5.22 ± 0.33 | 5.09 ± 0.35 | 4.54 ± 0.53 | 4.27 ± 0.29 | 4.53 ± 0.51 | 5.68 ± 0.39 | 5.61 ± 0.36 |
| t         | 2.348 | 1.111 | 2.393 | 1.207 | 1.674 | 1.435 | 2.038 | 1.883 | 1.437 | 1.472 |
| p         | .026  | .277  | .023  | .237  | .105  | .163  | .052  | .071  | .203  | .151  |
| **IL 10** |      |         |      |         |      |         |      |         |      |         |
| −819      |      |         |      |         |      |         |      |         |      |         |
| CC        | 4.81 ± 0.32 | 4.71 ± 0.44 | 4.76 ± 0.47 | 4.66 ± 0.51 | 5.45 ± 0.46 | 5.33 ± 0.34 | 5.38 ± 0.50 | 5.47 ± 0.30 | 5.41 ± 0.56 | 5.01 ± 0.44 |
| CT        | 4.69 ± 0.44 | 4.69 ± 0.55 | 4.88 ± 0.52 | 4.70 ± 0.38 | 5.74 ± 0.51 | 5.11 ± 0.39 | 5.71 ± 0.47 | 5.52 ± 0.46 | 5.59 ± 0.51 | 5.16 ± 0.36 |
| TT        | 4.56 ± 0.52 | 4.67 ± 0.40 | 4.92 ± 0.45 | 4.55 ± 0.42 | 6.21 ± 0.52 | 5.19 ± 0.33 | 5.94 ± 0.22 | 5.31 ± 0.54 | 5.78 ± 0.49 | 5.21 ± 0.29 |
| T         | 1.700 | 0.145 | 0.757 | 0.919 | 4.702 | 1.723 | 3.015 | 0.413 | 1.493 | 1.730 |
| p         | .101  | .885  | .455  | .367  | <.01  | .095  | <.01  | .682  | .145  | .094  |

*Note:* All values are given in pg/mL.

For −318 T/C, the difference between TC + TT and CC was calculated. For −238 G/A, the difference between GG and GA + AA was calculated. For −819 T/C, the difference between CC and CT + TT was calculated.
3.2 Prevalence of CTLA-4 polymorphisms in patients with HCC

CTLA-4 −318 T/C, CT60 G/A allele and genotype frequencies in patients with HCC and controls are shown in Table 3. Distributions of the polymorphisms in case and control groups were consistent with Hardy-Weinberg equilibrium (P > .05). For CTLA-4 −318 T/C, the TC and TT genotypes had significant associations with HCC; respective ORs were 1.624 (95% CI: 1.260-2.140) and 2.589 (95% CI: 1.277-5.247) for the codominant model, confirmed in the dominant model TC + TT with an OR of 1.720 (95% CI: 1.333-2.220), P < .05 and recessive model TT with an OR of 2.264 (95% CI: 1.121-4.572), P = .019. In assessing the association for alleles, a significant association with HCC was observed for the T allele, with an OR of 1.629 (95% CI: 1.189-2.232), P = .002.

For CT60 G/A, only GG genotype had association with HCC, with an OR of 1.420 (95% CI: 1.023-1.972), P = .036, in the codominant model. This result was confirmed in the recessive model GG with an OR of 1.389 (95% CI: 1.032-1.871), P = .030. No significant association with HCC was found for the G allele.

3.3 Prevalence of TNF and IL 10 polymorphisms in patients with HCC

Distributions of −238 G/A, −308 G/A polymorphisms in TNF and −592 C/A, −819 T/C polymorphisms in IL10 were in Hardy-Weinberg equilibrium in case and control groups (P > .05). Tables 4 and 5 show the association analysis of the above genotypes and HCC risk as ORs. For −238 G/A, we found that in comparison with healthy controls, patients with HCC had significantly lower AA genotype with an OR of 0.483 (95% CI: 0.279-0.836), P < .05 for the codominant model. This result was confirmed in the dominant model GA + AA with an OR of 0.724 (95% CI: 0.557-0.941), P = .016, and recessive model AA with an OR of 0.508 (95% CI: 0.295-0.877), P = .013. For alleles, a strong negative association with HCC was observed for the A allele, with an OR of 0.699 (95% CI: 0.509-0.961), P = .027. Nevertheless, no significant associations were found between genotypes or allele frequencies of −308 G/A and HCC risk (P > .05).

Table 5 shows a marginal positive association between the genotype CA in −592 C/A and HCC, with an OR of 1.310 (95% CI: 1.007-1.703), P = .044, in the codominant model. The combination of CA and AA remained positively associated with HCC, with an OR of 1.324 (95% CI: 1.034-1.696), P = .026, in the dominant model. For −819 T/C, in the codominant model, both CT and TT genotypes were positively associated with HCC; respective ORs were 1.365 (95% CI: 1.065-1.749) and 1.668 (95% CI: 1.116-2.493), P < .05. This result was confirmed in the dominant model CT + TT with an

FIGURE 1 T lymphocyte activation and proliferation in PBMCs from healthy individuals carrying CTLA-4 −318 T/C (A), TNF −238 G/A (B) and IL 10 −819 C/T (C) genotypes. Cell proliferation was assessed using the MTT assay, which showed that, when stimulated with PHA, T lymphocytes carrying CTLA-4 −318 TT and TC genotypes had significantly lower rates of proliferation than those carrying −318 CC, while T lymphocytes carrying IL 10 −819 CC genotype had significantly higher rates of proliferation than those carrying CT/TT genotypes. The line inside each box is the median, while the upper and lower limits of the box are the 75th and 25th percentiles, respectively. The vertical bars above and below the box indicate the maximum and minimum values, respectively. *TT + TC vs CC, P < .05. #CC vs CT + TT, P < .05.
OR of 1.412 (95% CI: 1.111-1.794), P < .05. For the alleles, a marginal positive association was observed between T allele with HCC risk, with an OR of 1.270 (95% CI: 1.001-1.612), P = .049.

### 3.4 Prevalence of CTLA-4 and TNF or IL 10 polymorphism combinations in patients with HCC

In order to investigate whether CTLA-4 and TNF or IL10 polymorphisms could act together to have an additive effect, the associations of combinations of these genotypes with HCC were analysed (Table 6). Statistically significant models with the strongest positive odds ratios from Tables 3, 4 and 5 were used in the calculations. For the combination of CTLA-4 −318 T/C with TNF −238 G/A, only CTLA-4 −318 TC or TT and TNF −238 GG or GA had significant association with HCC, with an OR of 2.541 (95% CI: 1.291-4.998), P < .01. For CTLA-4 −318 T/C with IL10 −819 T/C, the frequency of combinations of CTLA-4 −318 TC or TT and IL10 −819 CC, CTLA-4 −318 CC and IL10 −819 CT or TT in patients with HCC was of statistical significance (P < .01).

### 3.5 Association of CTLA-4, TNF and IL 10 polymorphisms with serum cytokine levels

The serum IL-2, IL-4, TGF-β, IL-10 and TNF-α levels in patients with HCC and controls were analysed using the same model mentioned above. As shown in Table 7, the CTLA-4 −318 T/C, TNF −238 G/A and IL 10 −819 T/C genotypes had no associations with IL-2, IL-4, TGF-β, IL-10 and TNF-α levels in the healthy controls (P > .05). However, for patients with HCC, compared with CC genotype, the TC and TT genotypes in −318 were significantly associated with a decrease in serum IL-2 (P < .01) and IL-4 (P = .027), and an increase in serum TGF-β (P = .048) levels. For TNF −238 G/A, the GG genotype was significantly associated with a decreased IL-2 level (P = .026) and an increased IL-4 level (P = .023) compared with GA and AA genotypes. Similarly, the CT and TT genotypes in IL 10 −819 were significantly associated with increase in TGF-β (P < .01), IL-10 (P < .01) levels compared with CC genotype. Together, we can conclude from these results that CTLA-4 −318 TC/TT, TNF −238 GG and IL 10 −819 CT/TT genotypes are associated with HCC, possibly through modulating the shift from Th1/Th2 to Th3-type cytokines.

### 3.6 Effects of CTLA-4, TNF and IL 10 polymorphisms on T cell proliferation and cytokine production

MTT assay was performed to evaluate the proliferation of T lymphocytes (Figure 1), while cytokine levels (IL-2, IL-4, TGF-β, IL-10 and TNF-α) in the cell culture supernatants were detected by ELISAs (Figure 2). MTT assays showed that PBMCs carrying CTLA-4 −318 TC/TT genotypes had significantly lower cell proliferation rates after stimulation with PHA (P < .05), while PBMCs with IL 10 −819 CC had significantly higher rate of proliferation rate. Analysis of IL-2, IL-4, TGF-β, IL-10 and TNF-α levels in cell culture supernatant showed that PBMCs with CTLA-4 −318 TC/TT genotypes secreted significantly lower IL-2, IL-4 but higher TGF-β (P < .05). Similarly, for IL 10 −819 T/C, the CC genotype had association with lower levels of TGF-β, IL-10 (P < .05). These results mirror what is seen in the serum and clearly suggest that CTLA-4 −318 TC/TT and IL 10 −819 CC genotypes have significant effects on cell proliferation and/or cytokine production.
3.7 | The cytolytic activities of PBMCs with CTLA-4, TNF and IL 10 genotypes on liver cancer cells

For cytotoxic assay, the effector and target cells were PBMCs carrying different CTLA-4, TNF, IL 10 genotypes and HepG2 cells, respectively. As shown in Figure 3, a significant decrease in tumour lysate of HepG2 cancer cells was observed in PBMCs with CTLA-4 −318 TC/TT compared with −318 CC genotype ($P < .05$). Similarly, for IL 10 −819 T/C, PBMCs with −819 CC genotype exhibited a significant increase in tumour lysate of HepG2 cancer cells compared with PBMCs with −819 CT/TT ($P < .05$). However, no significant change was found between the genotypes in TNF −238.

3.8 | Association of CTLA-4, TNF and IL 10 genotypes with clinicopathologic characteristics

The genotype frequencies for CTLA-4 (−318 T/C), TNF (−238 G/A) and IL 10 (−819 T/C) in HCC patients with different clinicopathologic stages are shown in Tables 8 and 9. We found that there were strong associations between CTLA-4 −318 T/C or IL 10 −819 T/C polymorphisms and the pathologic stages of HCC. For the genotype combinations of CTLA-4 −318 T/C with IL 10 −819 T/C, the combinations of CTLA-4 −318 CC and IL10 −819 CT or TT, CTLA-4 −318 TC or TT and IL10 −819 CT or TT were significantly associated with the pathologic stages of HCC. However, for the combinations of CTLA-4 −318 T/C with TNF −238 G/A, no significant associations were found with clinicopathologic stages of HCC.

4 | DISCUSSION

The multifactorial natures of HCC are fully recognized, but genetic factors are considered strong determinants of these diseases, which has encouraged scientists to search for the genes responsible. Many studies have confirmed a critical role of T cell response in the antitumour effect, and therefore, genes encoding molecules involved in T cell response may potentially affect the carcinogenesis of cancers. The present study aims to assess the associations of CTLA-4, TNF and IL 10 polymorphisms with susceptibility to HCC.

CTLA-4 is a structural homolog for CD28 and expressed on activated T cells. CTLA-4 can function as a negative costimulatory regulator of T cell activation and modulate immune responses of the body. CTLA-4 is polymorphic, and several important SNPs in CTLA-4 have been reported to be associated with a range of different malignancies. Although a large number of studies suggest that +49 G/A polymorphism in CTLA-4 is significantly associated with HCC, only few studies have been conducted to investigate the associations between −318 T/C, CT60 G/A variants and HCC risk. Thus, we performed a comprehensive case-control study to explore the association of CTLA-4 −318 T/C and CT60 G/A polymorphisms with HCC risk in a Chinese Han population. The results showed that patients with HCC had obviously higher frequencies of −318 TC/TT genotypes and −318 T alleles, which implied that −318 TC/TT in CTLA-4 were positively associated with HCC risk. To our knowledge,
this is the first study about the association of CTLA-4 −318 T/C and CT60 G/A SNPs with susceptibility to HCC, which may contribute to our understanding of the role of CTLA-4 SNPs in the pathogenesis of HCC.

TNF-α is a powerful pro-inflammatory factor involved in several biological processes such as macrophage activation, recruitment of inflammatory cells and amplification of pro-inflammatory cytokines. Polymorphisms in the promoter of TNF may increase its transcription level and thus cytokine production. Many studies have reported the associations of −238 G/A and/or −308 G/A polymorphisms in TNF with susceptibility to HCC, but the results are conflicting. Our findings indicated that TNF −238 GG genotype was associated with increased risk of HCC. These findings are consistent with some previous reports but not with others. Polymorphisms in IL10, another immunomodulatory gene, have also been implicated in HCC. Two polymorphisms (−592 C/A and −819 C/T) in IL 10 were examined to find possible associations with HCC. Our results showed that −592 CA genotype was marginally associated with HCC. Also, we found that −819 CT/TT genotypes were significantly associated with HCC. This is consistent with previous studies, but contradicts that reported in other works. These inconsistent results may be partly due to small sample sizes, racial and ethnic differences, and publication bias.

Next, in order to explore whether the interaction of the above SNPs had an additive effect, association of combinations of CTLA-4 and TNF or IL10 genotypes with HCC risk was analysed. We found that combinations of CTLA-4 −318 TC or TT and TNF −238 GG or GA; CTLA-4 −318 TC or TT and IL10 −819 CC; CTLA-4 −318 CC and IL10 −819 CT or TT were strongly associated with susceptibility to HCC. The increase in the odds ratios compared with the single SNPs alone suggests that there may be an additive effect of having high-risk genotypes at multiple loci.

Effective antitumour responses require CD4+ Th cells and CD8+ cytotoxic T lymphocytes (CTLs). To further characterize the function of these polymorphisms, the associations of polymorphisms in CTLA-4 −318 T/C, TNF −238 G/A and IL 10 −819 C/T with T cell activation and cytokine secretion were investigated. Our analyses showed that PBMCs carrying CTLA-4 −318 TC/TT and IL10 −819 CT/TT genotypes showed lower proliferation rates and cytolytic activities. The cytokine profile in vitro was consistent with serum cytokine profiles in patients with HCC. Furthermore, CTLA-4 −318 TC/TT and IL10 −819 CT/TT genotypes showed lower proliferation rates and cytolytic activities. The cytokine profile in vitro was consistent with serum cytokine profiles in patients with HCC. Furthermore, CTLA-4 −318 TC/TT had significant associations with lower IL-2 (Th1-type cytokine), IL-4 (Th2-type cytokine) and higher TGF-β levels (Th3-type cytokine), and IL 10 −819 CT/TT had significant associations with higher TGF-β and IL-10 (Th2-type cytokine) levels. This shift from Th1/Th2 to Th3 might be responsible for facilitating tumour progression by subverting various cellular immune surveillance mechanisms. Moreover, the present study provides evidence that CTLA-4 −318 T/C and IL 10 −819 T/C variants were associated with the severity of HCC. Therefore, we may reasonably expect that individuals who carry the CTLA-4 −318 TC/TT and IL 10 −819 CT/TT genotypes might negatively associated with

| Stage | Cases (n) | CTLA-4−318 T/C | TNF−238 G/A | IL10−819 T/C |
|-------|-----------|----------------|-------------|-------------|
| I     | 36        | 28 8 0         | 25 7 4      | 16 13 7     |
| II    | 91        | 67 23 1        | 73 16 2     | 36 38 17    |
| III   | 118       | 71 43 4        | 91 25 2     | 25 83 10    |
| IV    | 32        | 14 11 7        | 23 7 2      | 14 18 0     |

| Genotype | I   | II  | III | IV  | χ²  | P   |
|----------|-----|-----|-----|-----|-----|-----|
| CTLA-4−318/TNF−238 |
| CC/AA    | 3   | 2   | 1   | 1   | 1   | .966|
| TC or TT/AA | 3   | 2   | 2   | 1   | 0.268 | .966|
| CC/GG or GA | 18  | 54  | 79  | 17  | 7.501 | .058|
| TC or TT/GG or GA | 12  | 33  | 36  | 13  | 5.079 | .166|
| CTLA-4−318/IL10−819 |
| CC/CC    | 20  | 19  | 15  | 8   | Reference |
| TC or TT/CC | 8   | 16  | 21  | 13  | 7.465 | .058|
| CC/CT or TT | 4   | 46  | 57  | 6   | 34.757 | <.01|
| TC or TT/CT or TT | 4   | 10  | 25  | 5   | 13.999 | <.01|
T cell proliferation and function, which might be likely the underlying mechanisms conferring HCC susceptibility.

Cumulatively, these data demonstrate that CTLA-4 −318 T/C and IL 10 −819 T/C can affect susceptibility to HCC by altering the immune status of an individual. Additional studies using a larger sample size and haplotype analysis with other SNPs may lead to better understanding of these variants in the pathogenesis of HCC.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS

JJW wrote the manuscript. JJW and ZBW carried out the experiments. TCT designed the whole study and performed the statistical analysis. All authors reviewed the manuscript.

ETHICAL APPROVAL

Written informed consent was obtained from all participants during recruitment. The project was approved by the Ethics Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (Chengdu, China).

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