Association between hepatocellular carcinoma and tumor necrosis factor alpha polymorphisms in South Korea

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Data sharing statement: All the dataset are available from the author at nkkim@cha.ac.kr

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Abstract

AIM: To investigate associations between the tumor necrosis factor alpha (TNF-α) -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A polymorphisms and HCC in Korea.

METHODS: Hepatocellular carcinoma (HCC) cases were diagnosed at CHA Bundang Medical Center from June 1996 to August 2008. The association between TNF-α polymorphisms and HCC was analyzed in 157
HCC patients and 201 controls using a polymerase chain reaction-restriction fragment length polymorphism assay. We investigated five TNF-α polymorphisms, which are TNF-α -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A. The TNF-α genotype frequencies, genotype combinations and haplotypes were analyzed to disclose the association with HCC.

RESULTS: None of the TNF-α polymorphisms was significantly associated with HCC. However, nine genotype combinations had associations with increased likelihood of HCC. Among them, TNF-α -1031/-857/-238 TT/CC/GA (AOR = 18.849, 95%CI: 2.203-161.246, $P = 0.007$), TNF-α -1031/-308/-238 TT/ GG/GA (AOR = 26.956, 95%CI: 3.071-236.584, $P = 0.003$), and TNF-α -1031/-238 TT/GA (AOR = 21.576, 95%CI: 2.581-180.394, $P = 0.005$) showed marked association with HCC. There were five haplotypes of TNF-α polymorphisms which were significantly associated with HCC. They are TNF-α -1031/-863/-857/-308/-238 T-C-C-G-A (OR = 25.824, 95%CI: 1.491-447.223, $P = 0.0005$), TNF-α -1031/-857/-308/-238 T-C-G-A (OR = 12.059, 95%CI: 2.747-52.950, $P < 0.0001$), TNF-α -1031/-857/-238 T-C-A (OR = 10.696, 95%CI: 2.428-47.110, $P = 0.0001$), TNF-α -1031/-308/-238 T-G-A (OR = 7.556, 95%CI: 2.173-26.280, $P = 0.0002$) and TNF-α -1031/-238 T-A (OR = 10.865, 95%CI: 2.473-47.740, $P = 0.0001$). Moreover, HCC Okuda stage III cases with the TNF-α -1031 CC genotype had better survival than those with the TT genotype (AOR = 5.795, 95%CI: 1.145-29.323).

CONCLUSION: Although no single TNF-α polymorphism is associated with HCC in this study, some TNF-α genotype combinations and haplotypes are associated with HCC. In addition, HCC Okuda stage III cases with the TNF-α -1031 TT genotype may have a better prognosis than those with the CC genotype.

Key words: Tumor necrosis factor-alpha; Polymorphism; Single nucleotide; Carcinoma; Hepatocellular

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Core tip: We genotyped five single nucleotide polymorphisms [Tumor necrosis factor alpha (TNF-α) -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A] in hepatocellular carcinoma (HCC) patients and control subjects. A number of genotype combinations and some haplotypes had association with HCC. In addition, HCC Okuda stage III cases with the TNF-α -1031 TT genotype had a better prognosis than those with the CC genotype.

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INTRODUCTION

Primary liver cancer is the fifth most common cancer (fourth in men, sixth in women) in South Korea, and the second most common cause of cancer-related deaths[5]. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. HCC carcinogenesis is a multistep, multifactorial process. However, hepatitis B virus (HBV) and hepatitis C virus (HCV) are the best-known risk factors for HCC. Approximately 70%-80% of HCCs are related to these persistent viral infections[12-5]. Various inflammatory mediators related to chronic inflammation are known as cofactors in carcinogenesis[6]. Among these, tumor necrosis factor alpha (TNF-α) is the most important cytokine in inflammation-associated tumorigenesis[7].

TNF-α is a potent, pleiotropic, proinflammatory cytokine. It is produced mainly by macrophages, but also by a broad variety of other cell types, including lymphoid cells, mast cells, endothelial cells, fibroblasts, and neurons[8]. The TNF-α gene lies in the class III region of the major histocompatibility complex (MHC) and is located on human chromosome 6p21.3[9]. Some single nucleotide polymorphisms (SNPs) in the TNF promoter have been identified, and they are thought to affect TNF-α production[10]. Past research has shown that the TNF-α -308 GG genotype[11] and TNF-α -857 T polymorphism[12] are associated with gastric cancer, the TNF-α -308 G>A polymorphism[13] is associated with vascular invasion of breast tumors, the TNF-α -308 G/A polymorphism[14] is associated with lung cancer, the TNF-α -308 G>A polymorphism[15] is associated with prostate cancer, the TNF-α -308 G>A polymorphism[16] is associated with oral squamous cell carcinoma, the TNF-α -238 G>A polymorphism[17] is associated with bone cancer, and the TNF-α -308 G>A polymorphism[18] is associated with invasive cervical cancer.

The results of past studies on the association between TNF-α polymorphisms and HCC have had conflicting results. A meta-analysis of 10 case-control studies reported that the TNF-α -308 GG genotype is associated with a modest decrease in HCC risk[19]. Similarly, a meta-analysis by Yang et al[20] reported that the TNF-α -308 G>A polymorphism is associated with increased HCC risk and that the TNF-α -238 G>A polymorphism is not associated with HCC. However, these meta-analyses did not investigate other TNF-α polymorphisms. Wei et al[21] performed a meta-analysis of 17 relevant studies and found that the TNF-α -308 G>A, -238 G>A, and -863 C>A polymorphisms are associated with HCC among Asians, while the TNF-α -857 C>T and -1031 T>C polymorphisms are not related to HCC risk. This meta-analysis did not
investigate the association between TNF-α haplotypes and HCC risk, however.

We investigated the association between the TNF-α -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A polymorphisms and HCC in Korea.

MATERIALS AND METHODS

Study population
A total of 157 HCC cases diagnosed at CHA Bundang Medical Center from June 1996 to August 2008 were enrolled. The control group consisted of 201 individuals randomly selected from a health screening program with age and gender matched to HCC patients. Exclusion criteria included a history of other cancer or other severe medical conditions. Individuals with only hypertension and diabetes were not excluded. The grade of hepatic impairment was classified by Child-Pugh scores. HCC clinical stage was evaluated on the basis of TNM classification and Okuda stage. The present study was approved by the Institutional Review Board of CHA Bundang Medical Center, and written informed consent was obtained from all case and control subjects in the study.

**DNA extraction and genotyping**
Genomic DNA was extracted from leukocytes using a G-Dex II Genomic DNA Extraction Kit (iNtRON Biotechnology, Seongnam, South Korea). The TNF-α -238 G>A (rs361525), TNF-α -308 G>A (rs1800629), TNF-α -857 C>T (rs1799724), TNF-α -863 C>A (rs1800630), and TNF-α -1031 T>C (rs1799964) polymorphisms were analyzed using a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay. The -238 G>A polymorphism was analyzed using the forward primer 5′-AGA AGA CCC CCC TCG GAA CC and the reverse primer 5′-ATC TGG AGG AAG CGG TAG TG, and PCR products were cut using the restriction enzyme MspI. Genotyping of the -308 G>A polymorphism was performed using the forward primer 5′-AGG CAA TAG GTT TTG AGG GCC AT, and reverse primer 5′-CTC TGG GAA TGT GGT GCC AT, and PCR products were cut using the restriction enzyme NcoI. Genotyping of the -857 C>T polymorphism was performed using the forward primer 5′- AAG TCG AGT ATG GGG ACC CCC CGT TAA and the reverse primer 5′- CCA CAG TGT GGG ATG CTC GCC AT, and PCR products were cut using the restriction enzyme MspI A1. To genotype the -863 C>A polymorphism, the forward primer 5′- GGC TCT GAG GAA TGG GTT, and reverse primer 5′- CTG CTA CAT GCC CCT GTC TTG AGC were used, and PCR products were cut using the restriction enzyme TaqI.

Table 2 Baseline characteristics of study sample n (%) | n | Cases | Controls | P value
---|---|---|---|---
**Variable** | | | | |
N | 157 | 201 | | 0.061
Age (yr, mean ± SD) | 56.00 ± 11.16 | 54.00 ± 11.22 | | 0.871
Sex (male, %) | 120 (76.4) | 148 (73.6) | | 0.012
Hypertension | 19 (12.1) | 20 (49.9) | | 0.195
Diabetes mellitus | 30 (19.1) | 42 (21.6) | | 1.000
BMI > 25 kg/m² | 40 (25.5) | 51 (24.9) | | 0.274
Smoking | 84 (53.5) | 78 (38.6) | | 0.016
Drinking | 90 (57.3) | 95 (46.3) | | 0.241
Tumor size | < 5 cm | 68 (43.3) | | 0.567
≥ 5 cm | 89 (56.7) | | |
Portal vein thrombosis | No | 92 (58.6) | | -
Yes | 65 (41.4) | | -
Surgical resection | No | 145 (92.4) | | -
Yes | 12 (7.6) | | -
CTx/R Tx | No | 27 (17.2) | | -
Yes | 130 (82.8) | | -
TNM stage | I | 34 (21.7) | | -
II | 37 (23.3) | | -
III | 51 (32.5) | | -
IV | 35 (22.3) | | -
Okuda stage | I | 46 (29.3) | | -
II | 68 (43.3) | | -
III | 43 (27.4) | | -
CTP class | A | 83 (52.9) | | -
B | 36 (22.9) | | -
C | 38 (24.2) | | -

1 All of the restriction enzymes were available from New England Biolabs (MA, United States) and we used the reaction conditions recommended by the instructions. The underlined bases in the primer were mismatched with the wild-type sequence in order to introduce the restriction enzyme site.

1 Derived from χ² tests for categorical data and Mann-Whitney tests for continuous data. BMI: Body mass index; CTx: Chemotherapy; RTx: Radiotherapy; CTP: Child-Turcotte-Pugh.
restriction enzyme BbsI. These primers and restriction enzymes are shown in Table 1.

The five polymorphic regions were amplified using the following PCR conditions: 95 °C for 5 min, 38 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Digestion with the appropriate restriction enzymes was performed at 37 °C for 16 h, as described by the manufacturer (New England Biolabs, Beverly, MA, United States).

**Statistical analysis**

We used $\chi^2$ tests to analyze baseline categorical variables and Mann-Whitney tests to analyze baseline continuous variables. Associations between TNF-α polymorphisms and HCC were estimated using adjusted odds ratios (AORs) and 95% CIs obtained from multiple logistic regression models adjusted for age, sex, hypertension, diabetes mellitus, obesity, smoking status, and drinking status.

The informations about hypertension, diabetes, smoking status and drinking status were obtained by questionnaires.

Survival time was calculated from the date of HCC diagnosis to the date of death or last follow-up. Survival analysis was performed using the Kaplan-Meier method, the log-rank test, and the Cox-proportional hazards regression model. The statistical significance level of all tests was set at $P < 0.05$. Analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, United States) and Medcalc version 11.1.1.0 (Medcalc Software, Mariakerke, Belgium).

**RESULTS**

**Study sample**

Baseline characteristics of HCC cases and controls are shown in Table 2. The mean age of HCC cases was 56 years, and 120 were male (76.4%). HCC cases and
controls were matched on age and sex ($P = 0.069$ and 0.570, respectively). Compared with controls, HCC cases were less likely to have hypertension ($P = 0.002$). However, no significant differences in obesity (BMI > 25 kg/m²) or in the prevalence of diabetes mellitus, smoking, or drinking were identified between cases and controls. HBV and HCV infections were identified in 25 kg/m² 0.570, respectively). Compared with controls, HCC controls were matched on age and sex ($P = 0.069$ and 0.570, respectively). Compared with controls, HCC cases were less likely to have hypertension ($P = 0.002$). However, no significant differences in obesity (BMI > 25 kg/m²) or in the prevalence of diabetes mellitus, smoking, or drinking were identified between cases and controls. HBV and HCV infections were identified in 125 (79.6%) and 16 (10.2%) HCC cases, respectively. Of the HCC cases, 12 (7.6%) underwent surgical resection. The Okuda stage distribution was as follows: stage I, 46 (29.3%); stage II, 68 (43.3%); and stage III, 43 (27.4%).

**Genotype frequencies of the TNF-α -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A polymorphisms**

The genotype frequencies of the TNF-α -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A polymorphisms in HCC cases, HCC cases with HBV, and controls are shown in Table 3. We found no significant differences in the genotype frequencies of the TNF-α -1031 T>C, -863 C>A, -857 C>T, -308 G>A, or -238 G>A polymorphisms between cases and controls. We also found no significant difference in genotype frequencies of these polymorphisms between HCC cases with HBV and controls.

**Associations between combinations of TNF-α polymorphisms and HCC**

Next, we evaluated the combined effect of TNF-α polymorphisms on HCC risk. Using multiple logistic regression models, we found nine combined TNF-α genotypes associated with increased likelihood of HCC, as shown in Table 4. Some combinations showed marked association with HCC. The combinations were TNF-α -1031/-857/-238 TT/CC/CA (AOR = 18.849, 95%CI: 2.203-161.246, $P = 0.007$) TNF-α -1031/-308/-238 TT/CC/CA (AOR = 26.956, 95%CI: 3.071-236.584, $P = 0.003$) and TNF-α -1031/-238 TT/CC (AOR = 21.576, 95%CI: 2.581-180.394, $P = 0.005$).

**Haplotype frequencies of TNF-α polymorphisms**

We then calculated haplotype frequencies to attain more comprehensive information about the association between TNF-α and HCC. TNF-α -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A haplotype frequencies in HCC cases and controls are shown in Table 5. The TNF-α -1031/-863/-857/-308/-238 T-C-C-G-A haplotype was significantly associated with HCC (OR = 25.824, 95%CI: 1.491-447.223, $P = 0.0005$). Other haplotypes significantly associated with HCC included TNF-α -1031/-857/-308/-238 T-C-G-A (OR = 12.059, 95%CI: 2.747-52.950, $P < 0.0001$), TNF-α -1031/-857/-238 T-G-A (OR = 10.865, 95%CI: 2.173-55.56, $P < 0.0001$), TNF-α -1031/-308/-238 T-G-A (OR = 7.556, 95%CI: 2.173-26.280, $P = 0.0002$), and TNF-α -1031/-238 T-A (OR = 6.593, 95%CI: 2.173-21.280, $P = 0.0002$).

**Cox regression analysis of HCC case survival**

A stepwise Cox regression analysis of HCC-related survival is shown in Table 6. Survival in HCC cases was associated with sex, history of chemotherapy or radiotherapy, portal vein thrombosis, and TNF-α -1031 polymorphism status.

**TNF-α polymorphisms and HCC case survival stratified by stage**

TNF-α polymorphism-related survival was analyzed in HCC cases stratified by TNM and Okuda stage. Survival of cases with the TNF-α -1031 wild type (TT) genotype or a single T>C polymorphism was significantly better than those with the CC genotype (regression coefficient = 1.772, HR = 5.881, 95%CI: 1.408-24.554, $P = 0.0002$), as shown in Figure 1 and Table 7. Other TNF-α polymorphisms did not affect HCC case survival at any TNM or Okuda stage.

**DISCUSSION**

We performed this case-control study to investigate the associations between the TNF-α -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A polymorphisms and HCC. Furthermore, we analyzed TNF-α polymorphism combinations and haplotypes. In this study, the TNF-α -1031 T>C, -863 C>A, -857 C>T, -308 G>A, and -238 G>A polymorphisms were not associated with HCC risk. This result is inconsistent with the findings of previous meta-analyses. An
Table 5  $\text{TNF-} \alpha-1031$ $\text{T>C}$, $-863$ $\text{C>A}$, $-857$ $\text{C>T}$, $-308$ $\text{G>A}$, and $-238$ $\text{G>A}$ haplotypes in hepatocellular carcinoma cases and controls$^1$

| Haplotype                        | Overall   | Control | Case         | OR (95%CI)       | $P$-value |
|---------------------------------|-----------|---------|--------------|-----------------|-----------|
| $\text{TNF-} \alpha-1031/-863/\text{-857/-238}$ | 0.5266    | 0.5399  | 0.5139       | 1.000 (reference) |           |
| $\text{TNF-} \alpha-1031/-863/\text{-857/-308}$ | 0.0822    | 0.0908  | 0.0897       | 1.000 (reference) |           |
| $\text{TNF-} \alpha-1031/-863/\text{-857/-238}$ | 0.0197    | 0.0210  | 0.0209       | 1.000 (reference) |           |
| $\text{TNF-} \alpha-1031/-863/\text{-857/-308}$ | 0.0020    | 0.0025  | 0.0026       | 1.000 (reference) |           |
| $\text{TNF-} \alpha-1031/-863/\text{-857/-238}$ | 0.0010    | 0.0005  | 0.0011       | 1.000 (reference) |           |
| $\text{TNF-} \alpha-1031/-863/\text{-857/-308}$ | 0.0000    | 0.0005  | 0.0005       | 1.000 (reference) |           |
| $\text{TNF-} \alpha-1031/-863/\text{-857/-238}$ | 0.0042    | 0.0099  | 0.0084       | 1.000 (reference) |           |
| $\text{TNF-} \alpha-1031/-863/\text{-857/-308}$ | 0.1631    | 0.1638  | 0.1646       | 1.000 (reference) |           |

$^1$Only data for haplotypes significantly associated with hepatocellular carcinoma are presented in the table. Haplotypes with frequencies of less than zero among cases or controls are not shown.

To the best of our knowledge, no study has reported an association between HCC prognosis and TNF-$\alpha$ polymorphisms. TNF-$\alpha$ acts like a double-

Table 6  Results of stepwise Cox regression analysis of hepatocellular carcinoma survival

| Covariate                     | $\beta$  | SEM | HR (95%CI)       | $P$-value |
|-------------------------------|----------|-----|-----------------|-----------|
| Sex                           | -1.318   | 0.480 | 0.258 (0.105-0.682) | 0.006     |
| Chemotherapy or radiotherapy  | -1.914   | 0.500 | 0.148 (0.056-0.391) | 0.0001    |
| Portal vein thrombosis        | 1.334    | 0.478 | 3.795 (1.495-9.631) | 0.005     |
| TNF-$\alpha$ -1031 TT or CC   | 1.772    | 0.733 | 5.881 (1.408-24.554) | 0.016     |

An insufficient number of HCC cases and controls in our study could be the reason for this discordance; for example, we were able to analyze only 18 TNF-$\alpha$ -308 GA controls and 23 cases and 2 TNF-$\alpha$ -308 AA controls and 1 HCC case.

Past studies have analyzed combined genotypes or haplotypes to attain a more comprehensive understanding of the association between various polymorphisms and diseases$^{[22,23]}$. In this study, we found significant associations between some TNF-$\alpha$ genotype combinations and HCC risk. All of the statistically significant genotype combinations included the GA genotype at TNF-$\alpha$ -238 or -308. The TNF-$\alpha$ -863 CA genotype accompanied by the TNF-$\alpha$ -238 GA genotype was also associated with increased HCC risk in some combinations. This result suggests that the TNF-$\alpha$ -238 GA and -308 GA genotypes increase HCC risk in specific genotype combinations. The TNF-$\alpha$ -863 CA genotype accompanied by the TNF-$\alpha$ -238 GA genotype was associated with increased HCC risk, but the TNF-$\alpha$ -863 CA genotype was not associated with increased HCC risk when accompanied by other genotype combinations. This suggests that the TNF-$\alpha$ -863 polymorphism increases the risk of HCC only in the presence of the TNF-$\alpha$ -238 GA genotype.

Associations between TNF-$\alpha$ haplotypes and various medical conditions, such as polycystic ovary syndrome (PCOS)$^{[24]}$, bladder cancer$^{[25]}$, gastric carcinoma$^{[26,27]}$, peptic ulcer$^{[28]}$, and anemia$^{[29]}$ have been reported. Chen et al$^{[30]}$ reported that the TNF-$\alpha$ -1031/-863/-B 57/-308/-238 T-C-C-G-A haplotype may account for decreased susceptibility to HCC. However, we found this haplotype was more common in HCC patients. All haplotypes significantly associated with HCC contained the TNF-$\alpha$ -238 A allele, suggesting it may play a role in HCC development. The HCC odds ratio for TNF-$\alpha$ haplotype -1031/-863/-B 57/-308/-238 T-C-C-G-A was 0.800, while the OR for haplotype T-A-C-G-A, in which the -238 allele changed from G to A, was 4.716. This result supports the hypothesis that the -238 A allele is associated with HCC risk, though the HCC odds ratio for haplotype T-A-C-G-A was not statistically significant.

To the best of our knowledge, no study has reported an association between HCC prognosis and TNF-$\alpha$ polymorphisms. TNF-$\alpha$ acts like a double...
In conclusion, although we found no direct association between TNF-α polymorphisms and HCC risk, some TNF-α genotype combinations and haplotypes were associated with HCC. In addition, HCC patients with the TNF-α -1031 TT genotype had a better prognosis than those with the CC genotype.

An important limitation of our study was the small sample size, which prevents us from drawing definitive conclusions. Therefore, our results should be interpreted cautiously. However, to the best of our knowledge, ours is the first study to analyze the association between these five TNF-α polymorphisms, as well as their combinations and haplotypes, and HCC case status and prognosis. Our findings provide a more comprehensive understanding of TNF-α polymorphisms and HCC. Further studies with sufficient sample size are needed to clarify the role of TNF-α polymorphisms in HCC risk and prognosis.

**Table 7** TNF-α genotypes and survival in Okuda stage III hepatocellular carcinoma cases

| Genotype     | Validation set     | Adjusted HR (95% CI) | P-value |
|--------------|--------------------|----------------------|---------|
|              | Cases (n = 43)     | Deaths (n = 41)      |         |
| TT           | 29 (67.4)          | 27 (65.9)            | 1.000 (reference) | 0.621 |
| TC           | 11 (25.6)          | 11 (26.8)            | 1.314 (0.448-3.856) | 0.201 |
| CC           | 3 (7.0)            | 3 (7.3)              | 5.795 (1.145-29.323) | 0.035 |
| Dominant model (TT + TC) | 40 (93.0) | 34 (82.9) | 3.761 (0.869-16.265) | 0.078 |
| Recessive model (TT + CC) | 3 (7.0) | 7 (17.1) | 1.805 (0.733-4.442) | 0.201 |

1Adjusted for age, sex, hypertension, diabetes mellitus, drinking status, smoking status, portal vein thrombosis, tumor size, surgical resection, and chemotherapy or radiotherapy.

**Figure 1** Survival curves for hepatocellular carcinoma Okuda stage III cases with the TNF-α-1031 (rs1799964) TT genotype (reference) and the CC genotype.

Edged sword via its two distinct receptors: TNF receptor 1 (TNFR-1) and receptor 2 (TNFR-2)\(^{[31,32]}\). The major difference between the two receptors is the death domain in TNFR-1 absent in TNFR-2. On the one hand, TNFR-1 plays an important role in apoptotic cell death. On the other hand, TNF-α can stimulate the growth, proliferation, and angiogenesis of cancer cells by activating nuclear factor NF-κB\(^{[32]}\). Nakazaki et al\(^{[33]}\) reported higher levels of TNF-α in patients with recurrent HCC than in patients without HCC recurrence or patients with metastatic liver carcinoma or gastrointestinal carcinoma. Liu et al\(^{[34]}\) have reported that TNF-α overexpression is related to poor differentiation and microvascular invasion in HCC. Our study raises the possibility that specific TNF-α polymorphisms are related to HCC patient prognosis. The TNF-α polymorphism associated with treatment outcomes in our study (TNF-α -1031) may potentially be used in the clinic as a surrogate biomarker of prognosis.

In conclusion, although we found no direct association between TNF-α polymorphisms and HCC risk, some TNF-α genotype combinations and haplotypes were associated with HCC. In addition, HCC patients with the TNF-α -1031 TT genotype had a better prognosis than those with the CC genotype.
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