Tumor necrosis factor-alpha gene −308G > A polymorphism alters the risk of hepatocellular carcinoma in a Han Chinese population

Hua Feng1, Jing-hua Kuai2, Ming-yan Zhang1, Guang-chuan Wang1, Yong-jun Shi1 and Jun-yong Zhang1,3*

Abstract
Background: The aim of the present study was to evaluate the influence of tumor necrosis factor-alpha (TNF-α) −308 G > A polymorphism on hepatocellular carcinoma (HCC) risk.

Methods: The present case control study was conducted in a Han Chinese population consisting of 753 HCC patients and 760 controls from May 2010 to March 2013. The −308 TNF-a promoter polymorphisms were detected. Conditional logistic regression was performed to analyze the association between TNF-α −308 G > A polymorphism and the risk of HCC, which were estimated by odds ratios (ORs) and their 95% confidence intervals (95% CIs).

Results: The genotypic frequencies in the cases were not similar to that of the controls, differences being statistically significant (P = 0.002). Using the GG genotype as the reference genotype, AA was significantly associated with increased risk of HCC (adjusted OR = 5.12, 95% CI = 2.31 – 7.82). Similarly, AG + AA genotype showed 5.59-fold increased HCC risk in a dominant model. Furthermore, we found A allele was significantly associated with increased risk of HCC, compared with G allele (OR = 4.18, 95% CI = 1.76 – 6.97).

Conclusion: The present study showed that TNF-α −308 G > A polymorphism was associated with increased HCC risk in a Han Chinese population. Further prospective studies on large and different ethnic populations will be necessary to confirm our findings and elucidate the underlying molecular mechanism for the development of HCC.

Virtual Slides: The virtual slide(s) for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/vs/13000_2014_199

Keywords: Hepatocellular carcinoma, TNF-α, Genetic variant, Susceptibility, Risk

Background
Hepatocellular carcinoma (HCC) is one of the common malignant tumors globally, which is the fifth most prevalent cancer and the third cause of cancer-related deaths worldwide [1]. Approximately 650,000 cases die from HCC each year, and >75% of these cases occur in the Asia-Pacific region [2]. It is indicated that China has a very high incidence with about 55% of annual new cases of HCC worldwide [3]. HCC has been one of the most common causes of cancer-related deaths in China. Although chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, aflatoxin B1, alcohol and nonalcoholic steatohepatitis are regarded as the main carcinogenic mechanism, only a few of these patients with these risk factors develop HCC during their lifetime, suggesting the etiology of HCC is not well clarified [4]. Thus some genetic factors may contribute to the carcinogenic mechanism.

Tumor necrosis factor-alpha (TNF-α) encodes a pro-inflammatory cytokine that is secreted primarily by macrophages and plays critical roles in the pathogenesis of inflammatory autoimmune and malignant diseases. The TNF-α protein induces the expression of adhesion molecules, facilitating the invasion of metastatic tumor cells [5], and high levels of endogenous TNF-α have been observed in the blood of some cancer patients. Several polymorphisms in the promoter region of TNF-α have
been associated with different TNF-α expression levels [6]. Of these, the TNF-α −308 G > A polymorphism is the best studied. It involves the substitution of a guanine (G) by an adenine (A) and is associated with an increase in TNF-α expression levels [7]. TNF-α −308G > A polymorphism has been reported to alter the risk of several types of cancers, such as breast cancer, lung cancer, non-Hodgkin lymphomas, and prostate cancer [8-11]. However, the association between TNF-α −308 G > A polymorphism and risk of HCC is controversial [12-15]. The present case control study was performed to assess the association of HCC risk and TNF-α −308 G > A polymorphism in a Han Chinese population.

Methods
Study population
The present case control study consisted of 753 HCC patients and 760 cancer-free controls from May 2010 to March 2013 at the Digestive Disease Department, Shandong Provincial Hospital Affiliated to Shandong University. All subjects were unrelated Han Chinese living in China. Health subjects were randomly selected from health screening program participants to exclude those with a history of cancer and other medical diseases. All HCC patients were diagnosed on the basis of histology or the combination of typical radiological findings of HCC, and underwent surgery in Shandong Provincial Hospital Affiliated to Shandong University. The tumor stage of HCC cases was evaluated on the basis of the tumor-nodule-metastasis (TNM) classification system. The HBsAg and anti-hepatitis C virus (HCV) antibody were tested by microparticle enzyme immunoassays using commercial assay kits, which were used to determine the infection status of hepatitis B or hepatitis C. Clinical characteristics data as well as related risk factors, including gender, age, smoking status, drinking status, serum a-FP levels, family history of HCC and HBV, HCV serological markers, were summarized (Table 1). The present study was approved by the Medical Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University, and informed consent was obtained from all participants.

DNA extraction
A 5 mL sample of venous blood was collected from each subject into a test tube containing EDTA as anticoagulant. Genomic deoxyribonucleic acid (DNA) was extracted from 2 mL of peripheral blood by the standard method with proteinase K digestion followed by phenol chloroform extraction. After ethanol precipitation, the DNA was dissolved in double distilled water and frozen at −20°C until use.

Table 1 Clinical characteristics of hepatocellular carcinoma cases and healthy controls

| Characteristics          | Cases (n) | Controls (n) | P-value |
|-------------------------|-----------|--------------|---------|
| Number                  | 753       | 760          | 50.2    |
| Gender                  |           |              |         |
| Male                    | 467       | 450          | 0.59    |
| Female                  | 286       | 310          | 0.40    |
| Age (years)             |           |              |         |
| <55                     | 307       | 303          | 0.45    |
| ≥55                     | 446       | 457          | 0.06    |
| Alcohol drinking        |           |              |         |
| Yes                     | 713       | 711          | 0.33    |
| No                      | 40        | 49           | 0.40    |
| Tobacco smoking         |           |              |         |
| Yes                     | 719       | 701          | 0.31    |
| No                      | 34        | 59           | 0.78    |
| HBsAg                   |           |              |         |
| +                       | 311       | 41.3 -       |         |
| -                       | 442       | 58.7 -       |         |
| Anti-HCV                |           |              |         |
| +                       | 241       | 32.0 -       |         |
| -                       | 512       | 68.0 -       |         |
| Serum a-FP levels       |           |              |         |
| <400 ng/ml              | 275       | 36.5 -       |         |
| >400 ng/ml              | 478       | 63.5 -       |         |
| Family history of HCC   |           |              |         |
| Yes                     | 119       | 15.8 -       |         |
| No                      | 634       | 84.2 -       |         |

Polymorphism genotyping
The −308 TNF-α promoter polymorphisms were determined by method previously described [16]. Amplification was performed in GeneAmp PCR System 9700 termocycler (Applied Biosystems, Singapore) with 100 ng of genomic DNA, 25 pmol of each primer, 200 μM total dNTP, 1.5 mM MgCl₂, 1 PCR buffer and 2.5 U Taq DNA polymerase (Promega, Madison, WI, USA). The following cycling conditions were used: 95°C for 5 min, followed by 35 cycles of 94°C for 60 s, 58°C for 30 s and 72°C for 60 s, with a final extension at 72°C for 10 min. Restriction enzyme digestion with NcoI (Promega, Madison, WI, USA) of the PCR product was carried out overnight and analyzed on a 3% agarose gel. DNA products were visualized by ethidium bromide staining. The -308G showed two fragments (homozygous for the allele -308G), while its homologue -308A was undigested and resulted in a single band (homozygous for allele -308A, lacking NcoI site). The presence of all three fragments defined heterozygotic individuals.
Statistical analysis
All statistical analyses were performed using the Statistical Package for Social Science software version 18.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were reported as means standard deviation (SD), and categorical variables were reported as frequencies (N) and percentages (%). The χ² test was used to assess differences between cases and controls with regard to clinical characteristics. A goodness-of-fit χ² test was used to evaluate the Hardy-Weinberg equilibrium in controls. Conditional logistic regression was performed to analyze the association between TNF-α −308 G > A polymorphism and the risk of HCC, which were estimated by odds ratios (ORs) and their 95% confidence intervals (95% CIs). The significance levels of all tests were set at P < 0.05.

Results
Clinical characteristics of cases and controls
In the present case control study, a total of 1,513 Chinese Han subjects were enrolled, which consisting of 753 HCC patients and 760 cancer-free controls. Table 1 showed the general characteristics of the studied subjects. There were 467 males and 286 females in the HCC group, and 450 males and 310 females in the control group. The mean age of HCC patients was 53.3 years, and mean age of cancer-free controls was 52.9 years. There were no significant differences between HCC cases and cancer-free controls (all P >0.05, shown in Table 1).

Genotypic frequencies of TNF-α −308 G > A in cases and controls
The observed genotype distribution in the controls did not differ from those expected from Hardy Weinberg equilibrium (P >0.05). Compared to healthy controls, patients with HCC had lower frequency of GG genotype (81.1% vs. 96.4%) and a higher frequency of AG (13.0% vs. 3.2%). Homozygous AA genotype was found in 44 HCC patients and in 2 of controls. Thus, genotypic frequencies in the cases were not similar to that of the controls, differences being statistically significant (P = 0.002, shown in Table 2).

The association of TNF-α −308G > A polymorphism with HCC risk
We further analyzed the effects of the tested genotypes under different genetic models (shown in Table 3). Using the GG genotype as the reference genotype, AA was significantly associated with increased risk of HCC (adjusted OR = 5.12, 95% CI = 2.31 7.82). Similarly, AG + AA genotype showed 5.59-fold increased HCC risk in a dominant model. Furthermore, we found A allele was significantly associated with increased risk of HCC, compared with G allele (OR = 4.18, 95% CI = 1.76 6.97).

Discussion
Advances in molecular and genetic epidemiology have increased our knowledge of the mechanisms underlying hepatocarcinogenesis and the relationships between susceptibility and individual genetic variations [17]. Based on the genetic information, we determine the disease etiology in terms of generic determinants to be used for identifying the high-risk individuals and perform targeting therapy to the individual’s genetic make-up.

TNF-α is a member of the TNF/TNFR cytokine superfamily, and is an intercellular communicating molecule involved in building transient or long-lasting multicellular structures [18]. It interacts with receptors TNFR1 and TNFR2, which participate in cellular signal transduction pathways [19]. TNF-α plays an important role in the regulation of cell differentiation, proliferation and death as well as in inflammation and the innate and adaptive immune response. It has also been implicated in a wide variety of human diseases. The presence of DNA sequence variations in the regulatory region might

### Table 2 Distribution of TNF-α −308 G > A genotypes in cases and controls

| TNF-α −308 G > A | Cases | % | Controls | % | P value |
|------------------|-------|---|----------|---|---------|
| GG               | 611   | 81.1 | 733      | 96.4 | 0.002   |
| AG               | 98    | 13.0 | 25       | 3.2  |
| AA               | 44    | 5.9  | 2        | 0.4  |

### Table 3 The association of TNF-α −308G > A polymorphism with hepatocellular carcinoma risk

| TNF-α −308G > A polymorphism | HCC patients | Controls | OR (95% CI) | P value |
|-------------------------------|--------------|----------|-------------|---------|
| General genotype              |              |          |             |         |
| GG                            | 611          | 733      | 1.00 (Reference) |          |
| AG                            | 98           | 25       | 2.12 (1.25 3.46) | 0.02    |
| AA                            | 44           | 2        | 5.12 (2.31 7.82) | 0.003   |
| Dominant genotype             |              |          |             |         |
| GG                            | 611          | 733      | 1.00 (Reference) |          |
| AG + AA                       | 142          | 27       | 5.59 (2.41 8.02) | <0.001  |
| Recessive genotype            |              |          |             |         |
| AG + GG                       | 709          | 758      | 1.00 (Reference) |          |
| AA                            | 44           | 2        | 1.98 (0.95 3.27) | 0.06    |
| Allele frequency              |              |          |             |         |
| G                             | 1,320        | 1,491    | 1.00 (Reference) | 0.006   |
| A                             | 186          | 29       | 4.18 (1.76 6.97) |         |

1 Adjusted for sex, age, smoking status, and drinking status.
interfere with transcription of the TNF gene, influencing the circulating level of TNF and thus increasing susceptibility to human diseases, such as cancer. The TNF enhancer polymorphism has been implicated in several diseases, and the TNF-α −308 polymorphism has been described as the most important TNF polymorphism in human disease susceptibility. The significance of these polymorphisms reflects their possible influence on the transcription of the TNF gene. TNF-α −308 G > A polymorphism involves the substitution of a guanine (G) by an adenine (A) and is associated with an increase in TNF-α expression levels [7]. TNF-α −308G > A polymorphism has been reported to alter the risk of several types of cancers, such as breast cancer, lung cancer, non-Hodgkin lymphomas, and prostate cancer [8-11]. For example, Jin et al. found that the TNF-α −308G > A polymorphism was not associated with breast cancer risk in the overall population but that the A allele might be a protective factor for breast cancer in postmenopausal women, and the AA genotype might be a breast cancer risk factor in premenopausal women [8]. In Ma et al. study, a significantly increased prostate cancer risk was found to be associated with the TNF-α-308 G > A polymorphism (AA + AG vs. GG: OR = 1.531, 95% CI = 1.093–2.145; P = 0.013; AG vs. GG: OR = 1.477, 95% CI = 1.047–2.085; P = 0.026). Their results suggested that the TNF-α-308 G > A polymorphism might significantly contribute to prostate cancer susceptibility [11]. However, the association between TNF-α −308G > A polymorphism and risk of HCC is controversial [12-15]. The present case control study was performed to assess the association of HCC risk and TNF-α −308G > A polymorphism in a Han Chinese population. We found that it is a genotypic frequency in the cases were not similar to that of the controls. We then analyzed the effects of the tested genotypes under different genetic models. Using the GG genotype as the reference genotype, AA was significantly associated with increased risk of HCC. Similarly, AG + AA genotype showed 5.59-fold increased HCC risk in a dominant model. Furthermore, we found A allele was significantly associated with increased risk of HCC, compared with G allele. Our results were consistent with the findings previously reported by Heneghan et al. and Ho et al. [20,21], but different from Chen et al’s study [22].

Conclusions
In conclusion, the present study showed that TNF-α −308 G > A polymorphism was associated with increased HCC risk in a Han Chinese population. Further prospective studies on large and different ethnic populations will be necessary to confirm our findings and elucidate the underlying molecular mechanism for the development of HCC.

Competing interests
The authors declare that they have no competing interests.

Authors contributions
HF and JHK designed the study and drafted the manuscript; HF, JHK, MYZ, GCW, YJS, and JYZ carried out the experiments and performed the data analysis. All authors read and approved the final manuscript.

Author details
1Digestive Disease Department, Shandong Provincial Hospital, Affiliated to Shandong University, Jinan, Shandong, China. 2Digestive Disease Department, Qi Lu Hospital of ShanDong University (QingDao), QingDao, Shandong, China. 3Digestive Disease Department, Shandong Provincial Hospital, Num 324, JingWu Wei Qi Road, Jinan, PR China.

Received: 4 September 2014 Accepted: 8 October 2014
Published online: 25 November 2014

References
1. Siegel R, Ma J, Zou Z, Jemal A: Cancer statistics, 2014. CA Cancer J Clin 2014, 64(1):9-29.
2. Parkish S, Hyman D: Hepatocellular cancer: a guide for the internist. Am J Med 2007, 120(3):194-202.
3. Schutte K, Bornschein J, Malfertheiner P: Hepatocellular carcinoma epidemiological trends and risk factors. Dig Dis 2009, 27(2):80-92.
4. El-Serag HB: Hepatocellular carcinoma. N Engl J Med 2011, 365(12):1119-1127.
5. Kerkhoffs A, Bidwell J, van den Brule AJ, Meijer CJ, Pavade J, Glev S: TNFalpha polymorphism frequencies in HPV-associated cervical dysplasia. Gynecol Oncol 2004, 92(3):675-679.
6. Kroeger KM, Steer JH, Joyce DA, Abraham LJ: Effects of stimulus and cell type on the expression of the −308 tumour necrosis factor promoter polymorphism. Cytokine 2000, 12(1):110-119.
7. Louis E, Franchimont D, Piron A, Gevaert Y, Schaaf-Lafontaine N, Roland S, Mahieu P, Malaise M, De Groote D, Louis R, Belaiche J: Tumour necrosis factor (TNF) gene polymorphism influences TNF-alpha production in lipopolysaccharide (LPS)-stimulated whole blood cell culture in healthy humans. Clin Exp Immunol 1998, 113(3):401-406.
8. Jin G, Zhao Y, Sun S, Kang H: Association between the tumour necrosis factor alpha gene −308G>A polymorphism and the risk of breast cancer: a meta-analysis. Tumour Biol 2014. [Epub ahead of print].
9. Xie H, Yao H, Huo Y, Li N, Cheng Y: Association between TNF-alpha gene 308G>A polymorphism and lung cancer risk: a meta-analysis. Tumour Biol 2014, 35(10):9693-9699.
10. Zhai K, Ding J, Zhou Y: Different role of tumor necrosis factor-alpha polymorphism in non-Hodgkin lymphomas among Caucasian and Asian populations: a meta-analysis. Int J Mol Sci 2014, 15(5):7684-7698.
11. Ma L, Zhao J, Li T, He Y, Wang J, Xie L, Qin X, Li S: Association between tumor necrosis factor-alpha gene polymorphisms and prostate cancer risk: a meta-analysis. Diagn Pathol 2014, 9(74).
12. Talaat RM, Esmail AA, Elwaili R, Gurgis AA, Nasr MI: Tumor necrosis factor-alpha −308G/A polymorphism and risk of hepatocellular carcinoma in hepatitis C virus-infected patients. Chin J Cancer 2012, 31(1):29-35.
13. Chen X, Zhang L, Chang Y, Shen T, Wang L, Zhuang H, Lu F: Association of TNF-alpha genetic polymorphisms with hepatocellular carcinoma susceptibility: a case control study in a Han Chinese population. Int J Biol Markers 2011, 26(3):181-189.
14. Shi Z, Du C: Tumor necrosis factor alpha 308 G/A polymorphism and hepatocellular carcinoma risk in a Chinese population. Genet Test Mol Biomarkers 2011, 15(7-8):659-672.
15. Akkiz H, Bayram S, Beker A, Ozdil B, Akgollu E, Sunbul AT, Demiryurek H, Doran F: G-308A TNF-alpha polymorphism is associated with an increased risk of hepatocellular carcinoma in the Turkish population: a case control study. Cancer Epidemiol 2009, 33(3):4261-264.
16. Wilson AG, di Giovine FS, Blakemore AI, Duch GF: Single base polymorphism in the human tumour necrosis factor alpha (TNF alpha) gene detectable by Ncol restriction of PCR product. Hum Mol Genet 1992, 1(5):353.
17. Akkiz H, Bayram S, Beker A, Akgollu E, Uuskurad O: Genetic variation in the microRNA-499 gene and hepatocellular carcinoma risk in a Turkish
population: lack of any association in a case control study. Asian Pac J Cancer Prev 2011, 12(11):3107-3112.

18. Locksley RM, Killeen N, Lenardo MJ: The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001, 104(4):487-501.

19. Leek RD, Landers R, Fox SB, Ng F, Harris AL, Lewis CE: Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. Br J Cancer 1998, 77(12):2246-2251.

20. Ho SY, Wang YJ, Chen HL, Chen CH, Chang CJ, Wang PJ, Chen HH, Guo HR: Increased risk of developing hepatocellular carcinoma associated with carriage of the TNF2 allele of the −308 tumor necrosis factor-alpha promoter gene. Cancer Causes Control 2004, 15(7):657-663.

21. Heneghan MA, Johnson PJ, Clare M, Ho S, Harrison PM, Donaldson PT: Frequency and nature of cytokine gene polymorphisms in hepatocellular carcinoma in Hong Kong Chinese. Int J Gastrointest Cancer 2003, 34(1):19-26.

22. Chen CC, Yang SY, Liu CJ, Lin CL, Liaw YF, Lin SM, Lee SD, Chen PJ, Chen CJ, Yu MW: Association of cytokine and DNA repair gene polymorphisms with hepatitis B-related hepatocellular carcinoma. Int J Epidemiol 2005, 34(6):1310-1318.

doi:10.1186/s13000-014-0199-3
Cite this article as: Feng et al.: Tumor necrosis factor-alpha gene −308G > A polymorphism alters the risk of hepatocellular carcinoma in a Han Chinese population. Diagnostic Pathology 2014 9:199.