# Correlation of *TP53* mutations with HCV positivity in hepatocarcinogenesis: Identification of a novel *TP53* microindel in hepatocellular carcinoma with HCV infection

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**Abstract.** Although it is known that chronic hepatitis C virus (HCV) infection may contribute to tumor initiation and development, the molecular processes causing hepatocellular carcinoma (HCC) remain unclear. Microindels are unique, infrequent mutations that result in inserted and deleted sequences at the same nucleotide position, and are important contributors to cancer. To date, microindels in the p53 tumor suppressor gene (*TP53*) have not been fully examined in tumors. In the present study, 116 cases of HCC were screened for mutations in the *TP53* gene (exon 5-8) by single-stranded conformational polymorphism analysis followed by direct sequencing. A special type of complex *TP53* mutation, 616ins14del1 (14-1 microindel), was identified in a case of HCC with HCV infection. This rare *TP53* microindel led to the generation of a truncated protein of 211 amino acids that lacked the DNA-binding domain and tetramerization domain. Immunohistochemistry showed loss of p53 protein expression and downregulation of $p21^{WAF/CIP}$, Mdm2 and Bax in the tumor cells, indicating an impaired p53 signaling pathway. Nineteen of the 116 (16.4%) HCCs carried a total of 19 *TP53* mutations. Notably, 5 of the 13 HCV-positive (38.5%) cases contained a *TP53* mutation, and there was a significant association between *TP53* mutations and HCV positivity (P=0.0379). No correlation of *TP53* mutations with hepatitis B virus (HBV) positivity was observed. In summary, we identified a novel *TP53* microindel in HCC, and provided evidence of HCC characterized by HCV infections typically associated with mutational inactivation of the *TP53* gene.

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## Introduction

Hepatocellular carcinoma (HCC) is a common malignancy worldwide. Major risk factors include chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and exposure to dietary aflatoxin B1 (1). It is estimated that HBV is responsible for 50-80%, whereas HCV is associated with 10-25% of HCC cases (2). HBV is the predominant cause of HCC in most Asian, African and Latin American countries. By contrast, HCV is more common than HBV in Europe, Japan and the USA (3). HCV-associated HCC typically develops 20-30 years after infection and is usually, but not always, preceded by cirrhosis (4).

Previous studies have demonstrated that multiple genetic and epigenetic changes are involved in the molecular pathogenesis of HCC, including somatic mutations in the p53 tumor suppressor gene (*TP53*), which has been reported with a rate of 14-35% worldwide, depending on the level of aflatoxin exposure (5,6). *TP53* mutations are frequently observed in HCC cases with HBV or HCV infection (4,7,8). The identification of the interactions between p53 and virus proteins is highly significant for therapeutic strategies aimed at reducing the chronicity and/or carcinogenicity of the virus. However, the association between *TP53* mutations and virus carried state in the pathogenesis of HCC has yet to be fully elucidated.

The most common missense mutations in human cancer are known as hotspot mutations. More complex mutations such as insertion/deletion/nonsense are less frequently described. Microindels are unique, very rare mutations that result in inserted and deleted sequences of different sizes (between one and 50 nucleotides) at the same nucleotide position, with relevance to evolution and the onset of cancer (9). Little is known about the mechanisms responsible for these mutations (10). As a tumor suppressor gene frequently mutated in almost any tumor type, *TP53* microindels have not been reported as much in tumor.

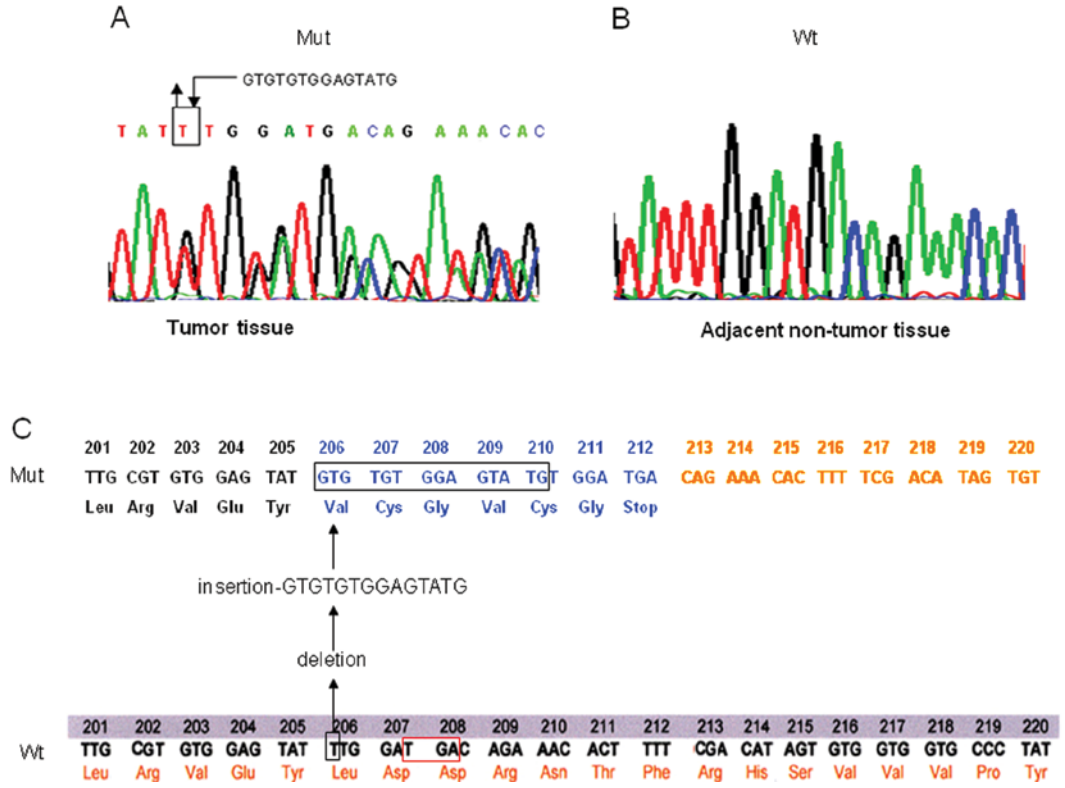


Figure 1. Identification and analysis of a rare *TP53* 14-1 microindel in HCC. (A) DNA sequencing of the *TP53* mutation 616ins14del1 (14-1 microindel) in exon 6 of the *TP53* gene in a case of HCC. (B) The *TP53* 14-1 microindel was not identified in the adjacent non-tumorous tissue, indicating that the mutation was somatic. (C) The mutation led to a frameshift of *TP53* mRNA starting from locus 616 (coden 206), generating a stop codon (TGA) at locus 621 (coden 207-208) and a truncated p53 protein with 211 amino acids. Mut, mutant type; Wt, wild-type.

In the present study, we identified and analyzed a novel somatic *TP53* microindel in a case of early stage HCC with HCV but not HBV infection; we also examined the association between *TP53* mutations and HCV or HBV positivity in the development of HCC with a panel of HCC cases from North China.

## Materials and methods

*Tissue samples*. One hundred and sixteen cases (102 males, 14 females) of HCC excised surgically at Beijing Friendship Hospital, Capital Medical University and Beijing Youan Hospital, Capital Medical University between January 2005 and December 2010 were examined in this study, including 13 HCV-positive cases, 93 HBV-positive cases, and 10 cases that were both HBV and HCV negative. Three cases were both HBV and HCV positive. The mean age of the patients was 52.8±9.1 years (range, 22-81 years).

The tumors were immediately frozen and stored at -80˚C or fixed in buffered formalin and embedded in paraffin. Written informed consent was obtained from all patients for use of their clinical materials in research. The study protocol was approved by the Clinical Research Ethics Committee of the Beijing Friendship Hospital, Capital Medical University.

*PCR-SSCP analysis followed by direct sequencing for TP53 mutations*. Genomic DNA was extracted using an EZ DNA FFPE kit™ (Omega) or tissue DNA kit™ (DP304-02, Tiangen, Beijing) according to the manufacturer's instructions. Prescreening for mutations in exon 5 to 8 of the *TP53* gene by single-strand conformational polymorphism (SSCP) analysis followed by direct sequencing was conducted as previously described (11). Samples exhibiting mobility shifts on SSCP analysis were subsequently re-amplified using the same primers as for SSCP and sequenced using a BigDye Terminator Cycle Sequencing kit (ABI PRISM; Applied Biosystems) in an ABI PRISM 3100 DNA sequencer (Applied Biosystems). The identified mutations were verified by sequencing a second product of amplification on both strands.

*Immunohistochemical assay for expression of p53, $p21^{WAF/CIP}$, Bax and Mdm2*. Sections (4 $\mu$m) were cut for immunohistochemistry (IHC). Immunohistochemical staining was performed using antibodies against p53 (monoclonal, sc-126; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200), $p21^{WAF/CIP}$ (monoclonal, BD556431; BD Biosciences; 1:200), Mdm2 (monoclonal, Batch 3012; Novocastra; 1:100) and Bax (polyclonal, 06-499; Upstate Biotechnology, Inc.; 1:200) at 4˚C overnight. Secondary antibody (anti-mouse IgG, cat. no. MP-7402; Vector Laboratories) was used at 37˚C for 1 h and antigen-antibody reactions were visualized with 3,3'-diaminobenzidine (SK-4100; Vector Laboratories). Tissue structures were visualized by counterstaining with hematoxylin.

*Statistical analysis*. Fisher's exact test (for expected value, ≤5) was used to identify molecular associations using SAS v9.2 software (SAS Institute, Inc., Cary, NC, USA). A P-value of <0.05 was considered to indicate a statistically significant difference in all tests.

Table I. Mutations in the *TP53* gene identified in HCC cases.

| Case | Exon | Codon | Nucleotide changes | Amino acid substitution |
|---|---|---|---|---|
| 1 | 5 | 157 | GTC→TTC | Val→Phe |
| 2 | 5 | 157 | GTC→TTC | Val→Phe |
| 3 | 5 | 175 | CGC→CAC | Arg→His |
| 4 | 6 | 220 | TAT→TGT | Tyr→Cys |
| 5 | 6 | 192 | CAG→CAT | Gln→His |
| **6** | **6** | **206** | **616ins14del1** | **Frameshift** |
| 7 | | | IVS6+19G→C | Splicing mutation |
| 8 | 7 | 245 | GGC→GAC | Gly→Asp |
| 9 | 7 | 248 | CGG→GGG | Arg→Gly |
| 10 | 7 | 248 | CGG→GGG | Arg→Gly |
| 11 | 7 | 248 | CGG→GGG | Arg→Gly |
| 12 | 7 | 249 | AGG→AGT | Arg→Ser |
| 13 | 8 | 301 | CCA→CTA | Pro→Leu |
| 14 | 8 | 298 | CAG→TAG | Gln→Stop |
| 15 | 8 | 285 | GAG→AAG | Glu→Lys |
| 16 | 8 | 285 | GAG→AAG | Glu→Lys |
| 17 | 8 | 273 | CGT→TGT | Arg→Cys |
| 18 | 8 | 273 | CGT→TGT | Arg→Cys |
| 19 | 8 | 286 | GAA→GTA | Arg→Val |

## Results

*Identification and analysis of TP53 mutations in HCC*. One hundred and sixteen cases of HCC were examined for *TP53* mutations. In a case with HCV infection (case 360), a novel heterozygous mutation, 616ins14del1 (14-1 microindel), was identified in exon 6 of the *TP53* gene; in this mutation, one base (T) was deleted followed by insertion of a 14 base nucleotide, GTGTGTGGAGTATG, at locus 616 (Fig. 1A). This *TP53* microindel was not observed in DNA from adjacent non-tumor tissue, indicating that it was a tumor-specific somatic mutation (Fig. 1B). The mutation led to a frameshift of *TP53* mRNA starting from locus 616 (codon 206) and generated a stop codon (TGA) at locus 621 (codons 207-208, corresponding to codon 212 in the mutant sequence), resulting in the generation of a truncated p53 protein with 211 amino acids (Fig. 1C).

Of the 116 HCC cases analyzed, 19 (16.4%) contained a *TP53* mutation (Table I). Aside from the *TP53* 14-1 microindel, 18 transition mutations in the *TP53* gene which have been reported in the International Agency for Research on Cancer (IARC) database (R15 release, http://www-p53.iarc.fr) were also identified, including 16 missense point mutations, one nonsense mutation and one splicing mutation. The pattern and effect of *TP53* mutations in HCC identified in the present study are similar as those reported in the IARC database (Fig. 2).

All *TP53* mutations were identified in the HCC cases with HBV or HCV infection. Of the 13 HCV-positive HCC cases, 5 (38.5%) contained a *TP53* mutation, i.e. the 14-1 microindel in case 360, R249S in case 212, E285K in case 421, R248G in case 522 and P301L in case 568, and there was a significant association between *TP53* mutations and HCV positivity (Table II). By contrast, 15 of the 93 HBV-positive HCC cases contained a *TP53* mutation (16.1%), and there was no significant association between *TP53* mutations and HBV positivity (Table II). In one case with both HCV and HBV positivity, a *TP53* mutation E285K was identified.

Table II. Correlation of *TP53* mutations with HBV and/or HCV positivity in hepatocellular carcinoma.[a]

| | *TP53* mutation | | |
|---|---|---|---|
| Virus carried state | Yes | No | Significance (P-value) |
| HBV | | | |
| Positive | 15 | 78 | 1.0000 |
| Negative | 4 | 19 | |
| HCV | | | |
| Positive | 5 | 8 | 0.0379 |
| Negative | 14 | 89 | |
| HBV or HCV | | | |
| Positive | 19 | 87 | 0.2116 |
| Negative | 0 | 10 | |
| HBV and HCV | | | |
| Positive | 1 | 2 | 0.4183 |
| Negative | 18 | 95 | |

[a]Estimated by Fisher's exact test.

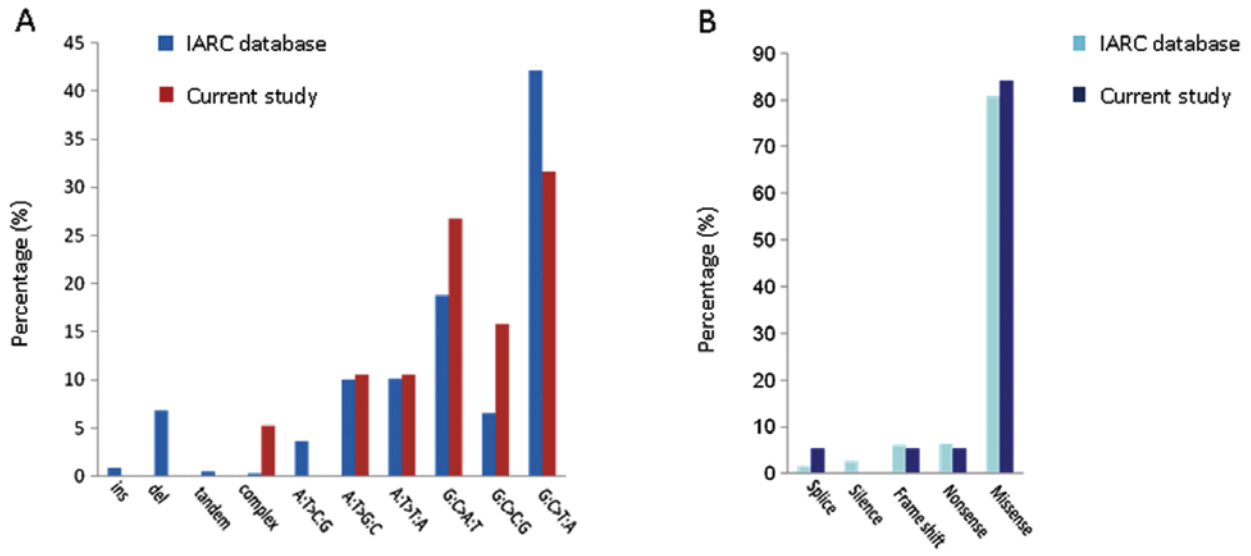


Figure 2. Comparison of *TP53* mutations in HCC identified in the current study and those reported in the IARC database. (A) Mutation pattern; (B) mutation effect. The pattern and effect of *TP53* mutations in HCC identified in the current study are similar to those reported in the IARC database.

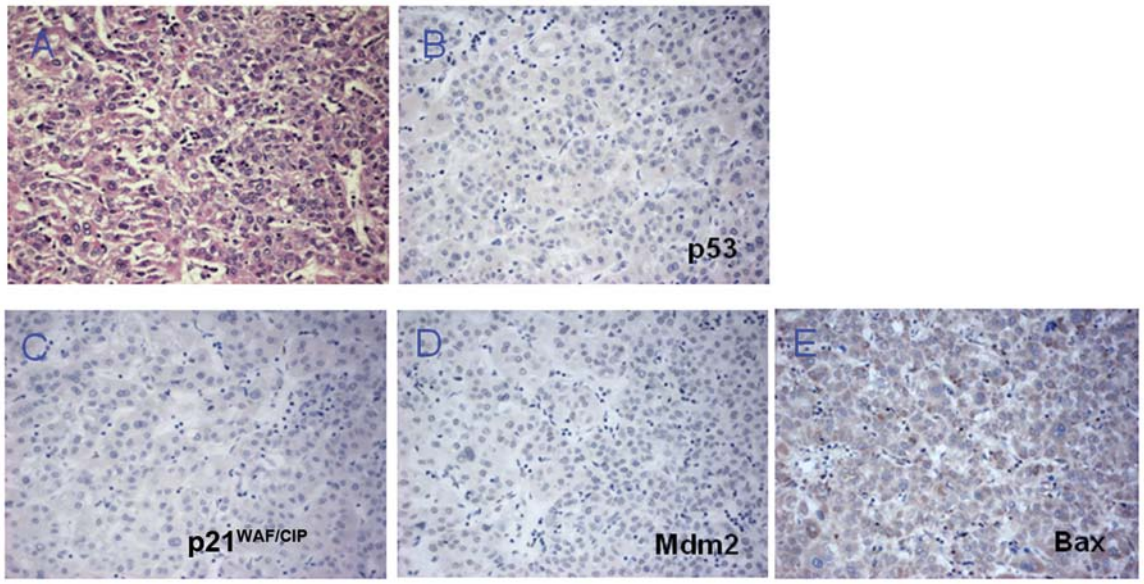


Figure 3. Immunohistochemistry (IHC) for expression of p53 protein and its downstream target proteins in the HCC case with the *TP53* 14-1 microindel. (A) Hematoxylin and eosin staining showing tumor cells; (B) expression of p53 protein; (C) expression of $p21^{WAF/CIP}$; (D) expression of Mdm2; (E) expression of Bax (original magnification, x40). IHC indicates that the loss of p53 expression caused by the *TP53* 14-1 microindel may result in downregulation of $p21^{WAF/CIP}$, Mdm2 and Bax.

*Functional consequences of the novel TP53 microindel in HCC*. To evaluate the functional consequences of the *TP53* microindel, IHC was conducted to determine the expression of the p53 protein, as well as its downstream targets $p21^{WAF/CIP}$, Bax and Mdm2 in the HCC case. The results showed no positive staining for p53 (Fig. 3A and B) and negative staining for $p21^{WAF/CIP}$, Mdm2 and Bax in tumor cells (Fig. 3C-E). These IHC results suggest that loss of p53 activity may result in the downregulation of $p21^{WAF/CIP}$, Mdm2 and Bax, which are crucial for inhibition of the cell cycle and for inducing apoptosis. Thus, the truncated p53 protein caused by the *TP53* microindel may result in loss function of normal p53, leading to loss of control of the cell cycle and apoptosis.

## Discussion

Germline mutations in the *TP53* gene have been identified in patients with Li-Fraumeni syndrome (LFS), which is an inherited cancer predisposition syndrome characterized by a wide spectrum of neoplasms (12). Somatic *TP53* mutations were identified in almost all tumor types including HCC, particularly following exposure to aflatoxin (7,13). According to the IARC database, 1020 *TP53* mutations have been identified in 31.3% of HCC cases. These mutations comprise 933 base transitions (91.5%), nine insertions (0.88%), 70 deletions (6.86%), five tandems (0.49%), and three complex mutations [0.29%; an 8.5-kb DNA rearrangement in a human hepatoma

cell line (Hep3B) identified by restriction fragment analysis (14), a 248-289dup (A83-S96dup) in an HCC case from France (15), and a TGAAAAC-AG replacement (exon 3) in a case from Hong Kong (8); no detailed clinical data or information on the consequences of the mutations is provided by any of these studies]. In the present study, all patients came from North China (regions with low aflatoxin exposure), and *TP53* mutation was identified in 16.4% of total HCC cases, lower than the average rate (31.3%) reported in the IARC *TP53* database. Notably, a special type of complex *TP53* mutation, 616ins14del1 (14-1 microindel), was identified in HCC with HCV infection in the population of North China.

Microindels in coding regions often lead to a frame-shift, with devastating consequences for protein function. Meanwhile, microindels can also be in-frame and thereby alter the properties of a protein by adding or subtracting a small number of amino acids (protein tinkering). Protein tinkering can sometimes be a critical step in carcinogenesis (such as EGFR microindels in lung cancer, KIT mutation in gastrointestinal stromal tumors) (9,16). To date, a total of 66 *TP53* somatic microindels have been reported in the context of other mutations in the IARC database. The majority (79%) of these microindels result in a reading frame shift; the others are in-frame, resulting in protein tinkering (17). The novel *TP53* microindel identified in the present study brings into frame a termination codon at the equivalent of codon 207-208, with an altered C-terminus from codon 206-ter. A truncated and presumably inactivated product is predicted, lacking a number of key domains including part of the DNA-binding domain and the tetramerization domain. We did not examine the loss of heterozygosity (LOH) of *TP53* in the specific HCC case. However, IHC confirmed that the truncated p53 protein caused by the *TP53* microindel may result in loss function of normal p53, leading to loss of control of the cell cycle and apoptosis. The loss of p53 protein expression could be explained by the truncated protein or by damage to the *TP53* mRNA through a nonsense-mediated digestion pathway due to premature termination of transcription (18).

The patient with the *TP53* microindel was a 67-year-old male diagnosed with HCC (T1N0M0, Union for International Cancer Control standard) following physical examination. The patient underwent surgical resection two weeks after diagnosis. The characteristics of the HCC were as follows: i) location, left lobe of liver, baseline computed tomography scan revealed no distant dissemination of cancer; ii) size, 4x4×3.8 cm, with an intact capsule; iii) serology, HCV(+), hepatitis B surface antigen (-), HBV DNA (-); and iv) histology, moderate differentiation. The patient had no specific exposure to dietary aflatoxin and did not consume alcohol excessively. There was no family history of tumor, inherited disease, or infectious diseases. According to the above clinical data, the HCC case with the *TP53* microindel was at an early stage, and HCV but not HBV infection was present; there were no other specific risk factors for HCC. Since the novel *TP53* microindel resulted in the disruption of the *TP53* signaling pathway, it may act as a driver mutation and contribute to the development of HCC.

Although it is evident that HCV infection may contribute to tumor initiation and development, the direct molecular role for HCV in the pathogenesis of HCC and the molecular processes causing HCC remain unclear (19). To gain insight into HCV-related hepatocarcinogenesis, microarray analysis has been applied in several studies (7,20,21). Analysis of HCV-associated cirrhosis revealed an upregulation of pro-inflammatory, pro-apoptotic and pro-proliferative genes, which might reflect groups of genes involved in HCV-related cirrhosis progressing to HCC (20). Notably, microarray analysis of numerous *TP53* mutant and *TP53* wild-type HCC cases showed significant differences in their gene expression patterns. Cell cycle-related genes (*CCNG2* and *BZAP45*) and cell proliferation-related genes (*SSR1*, *ANXA2*, *S100A10* and *PTMA*) were overexpressed in mutant *TP53* tumors compared with wild-type *TP53* tumors (21). This observation indicates a higher potential for malignancy in HCV-related HCC with a *TP53* mutation. In addition, it has been postulated that the HCV protein might cause mutation of the *TP53* gene, but this is controversial (22,23). The above observation suggested a significant role of co-presence of *TP53* mutation and HCV infection in the pathogenesis of HCC, consistent with the findings in the present study.

As previously described, HBV is the predominant cause of HCC in most Asian countries (3), and the majority of the cases analyzed in the present study were HBV positive (80.2%). Some evidence supports a direct oncogenic role for HBV in the development of HCC, i.e. the integration of HBV DNA into the chromosomal DNA of HCC; the role of the HBV X gene in the pathogenesis of HBV-associated HCC, in particular, binding to and inactivation of p53 (24), and a recent whole-genome study showed the role of interferon regulatory factor 2 (IRF2) as a tumor suppressor in HBV-associated HCC and its function as a regulator of the p53 pathway (25). However, the present study did not reveal any relationship between *TP53* mutations and HBV infection in HCC in the Chinese population, consistent with a recent study which showed no correlation between mutational change in *TP53* and the number of HBV integration events in a Chinese population (26).

Collectively, the present study shows the co-presence of *TP53* mutations and HCV infection but not HBV infection in HCC in the small subset of HCCs from North China, suggesting different carcinogenetic pathways between HBV- and HCV-related hepatocarcinogenesis in relation to disruption of p53. A large scale deep study is required to fully understand the molecular role of p53 in virus infection-related hepatocarcinogenesis.

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