Null Results In Brief

No Association of MMP-7, MMP-8, and MMP-21 Polymorphisms with the Risk of Hepatocellular Carcinoma in a Chinese Population

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Abstract

Previous studies have suggested that the functional polymorphisms in the promoters of matrix metalloproteinases (MMP) genes were associated with the risk of cancers, but no study has ever explored these polymorphisms as risk factors for hepatocellular carcinoma. Recently, we firstly examined whether seven functional polymorphisms in the promoters of MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, and MMP-13 have any bearing on the risk of hepatocellular carcinoma, but we found none. In this study, we focused on an additional six MMP polymorphisms, including four functional polymorphisms in the promoters of MMP-7 (A-181G and C-153T) and MMP-8 (C-799T and A-381G), and two nonsynonymous polymorphisms in MMP-10 (A180G) and MMP-21 (C572T). With the polymorphism validation, we found that only MMP-7 A-181G, MMP-8 C-799T, and MMP-21 C572T were polymorphic. These three polymorphisms were then genotyped in 434 patients with hepatocellular carcinoma and 480 controls by PRC-RFLP analysis. The associations between the polymorphisms and hepatocellular carcinoma risk were evaluated while controlling for confounding factors. No significant association with the risk of hepatocellular carcinoma was observed with the three polymorphisms in the overall sample, hepatitis B virus carriers, and non–hepatitis B virus carriers after correction for multiple comparisons. Furthermore, when the analyses were stratified by age, sex, status of smoking and drinking, pack-years of smoking, and family history of hepatocellular carcinoma, there was also no significant association between these polymorphisms and hepatocellular carcinoma risk. Our findings suggest that the polymorphisms MMP-7 A-181G, MMP-8 C-799T, and MMP-21 C572T may not play a major role in mediating susceptibility to hepatocellular carcinoma. (Cancer Epidemiol Biomarkers Prev 2008;17(9):2514–8)

Introduction

The matrix metalloproteinases (MMP) are a family of enzymes that proteolytically degrade a wide spectrum of both extracellular matrix and nonmatrix proteins (1). The broad range of substrates conveys a pivotal role for MMP involvement during both normal physiologic processes and pathologic states (2). With regard to the cancers, MMPs can regulate the tumor microenvironment and were considered to be involved in several steps of cancer development by regulating cancer cell growth, differentiation, apoptosis, invasion, migration, metastasis, angiogenesis, and immune surveillance (3). Several polymorphisms in the promoters of a number of MMP genes, which are thought to affect the respective MMP production in an allele-specific manner, have been well characterized (4-6). Furthermore, there is increasing evidence indicating that these functional polymorphisms may contribute to interindividual differences in susceptibility to a wide spectrum of cancers (4-9). Recently, we examined whether seven functional polymorphisms in the promoters of MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, and MMP-13 have any bearing on the risk of hepatocellular carcinoma in a Chinese population. We found no evidence, however, of an association between these polymorphisms and hepatocellular carcinoma risk (10). The additional studies for the polymorphisms in other members of MMP family and their associations with hepatocellular carcinoma risk are warranted before the importance of MMP polymorphisms in hepatocellular carcinoma risk can be fully ascertained.

In this study, we focused on six other MMP polymorphisms. The first and second polymorphisms, A-181G and C-153T, are located in the promoter of MMP-7. The electrophoretic mobility shift assays showed that these two polymorphisms influenced the binding of nuclear protein(s). Furthermore, the basal promoter activity was
higher in promoter constructs harboring the combination of the two rare alleles in transient transfection assays (11). Several studies have shown the A-181G polymorphism to be associated with ovarian, oral, colorectal, esophageal, gastric, and lung cancer (8, 12–14). The third and fourth polymorphisms, C-799T and A-381G, are located in the promoter of the MMP-8 gene. The electrophoretic mobility shift assays revealed differences in nuclear protein binding to oligonucleotides representing the C-799T and A-381G. The promoter constructs containing the minor alleles of the two polymorphisms had a 3-fold greater activity in choriocarcinoma-like trophoblast cells compared with the constructs containing the major alleles (15). Moreover, the C-799T has been shown to be associated with the prognosis of breast cancer (16). The fifth polymorphism, A180G, is caused by a C→A transition at codon 53 in exon 2 of the MMP-10. The sixth polymorphism, C572T, is caused by a T→C transition at codon 191 in exon 2 of the MMP-21. These two polymorphisms can lead to the substitution of specific amino acids (17, 18), and may have potential functional effects. The role of the six polymorphisms in hepatocellular carcinoma has never been specifically investigated previously. We therefore examined whether these polymorphisms have any bearing on the risk of hepatocellular carcinoma in a Chinese population.

Materials and Methods

Patients and Controls. This case-control study included 434 incident patients with hepatocellular carcinoma and 480 control subjects. All the subjects were enrolled at Fushui County and its surrounding regions at Guangxi province located in southern China, a well-known high-risk region for hepatocellular carcinoma. The diagnosis of cases, the inclusion and exclusion criteria for cases and controls, and the definition of hepatitis B virus (HBV) carriers, smokers, and drinkers were described previously (10, 19). At recruitment, informed consent was obtained from each subject, and personal information on demographic factors, medical history, tobacco and alcohol use, and family history of hepatocellular carcinoma was collected via structured questionnaire. This study was done with the approval of the Medical Ethical Committee of the Chinese National Human Genome Center.

Validation of Polymorphisms. The six polymorphisms were validated by PCR direct sequencing. The screening panel used for polymorphism validation included DNAs from 96 individuals randomly chosen from the total control population of 480 subjects. The primers and conditions used for amplifying and sequencing the target region containing these six polymorphisms are available on request. The PCR and DNA sequencing conditions were identical to those for the polymorphism discovery described previously (20, 21), except for a PCR annealing temperature of 60°C (MMP-7 A-181G and C-153T), 59°C (MMP-8 C-799T), 60°C (MMP-8 A-381G), 60°C (MMP-10 A180G), and 58°C (MMP-21 C572T), respectively. Polymorphism candidates were identified by the PolyPhred program4 and inspected by two observers. Polymorphism positions and individual genotypes were confirmed by reamplifying and resequencing the polymorphism sites from the opposite strand.

Genotyping of Polymorphisms. The polymorphisms MMP-7 A-181G (rs11568818), MMP-8 C-799T (rs11225395), and MMP-21 C572T (rs10901425) were genotyped by PCR-based RFLP analysis. For the MMP-7 A-181G polymorphism, an amplification using forward primer TCAATTTATGCACAGACAGAAAGAAAAATCCCTTGGAAAGT and reverse primer CTCCACCTGGAATGTGGAAAT was done. A Taq I recognition site was introduced by a one base mismatch (underlined) in the forward primer. PCR conditions were identical to those for polymorphism discovery except for an annealing temperature of 59°C and a total reaction volume of 25 μL (20, 21). The reaction yielded a 180-bp amplicon. An aliquot (5 μL) of PCR product was digested with 1 unit of Taq I (TaKaRa; Otsu) and separated on a 3% agarose gel. The presence of the −181G allele creates an Taq I restriction site; digested amplicons from −181G homozygotes appear as a 138-bp and a 42-bp band, homozygotes for the −181A allele appear as a 180-bp band, and heterozygotes have all three of these bands.

For the MMP-8 C-799T polymorphism, an amplification of a 255-bp fragment using forward primer GCCGAGAGACTCTAGTGGAAGCTACCATGCA- GATC and reverse primer CTCAGCCTCCAGCACATTTG was done. A Bgl II recognition site was introduced by a one base mismatch (underlined) in the forward primer. PCR conditions were identical to those for MMP-7 A-181G, except for an annealing temperature of 56°C. The 255-bp amplicon was digested with 1 unit of Bgl II (TaKaRa; Otsu) and separated on a 3% agarose gel. Homozygotes for the −799T allele yielded two restriction fragments of 224 and 31 bp after Bgl II digestion, homozygotes for −799C remained uncut (255-bp band), and heterozygotes yielded all three of these bands.

For the MMP-21 C572T polymorphism, an amplification of a 197-bp fragment using forward primer TTATGATGTTGCCCAGATCTCC and reverse primer TGCTTTATGCCTCTCCCAAGG was done. PCR conditions were identical to those for MMP-7 A-181G, except for an annealing temperature of 58°C. The 197-bp amplicon was digested with 1 unit of Eco R I (TaKaRa; Otsu) and separated on a 3% agarose gel. Homozygotes for the 572C allele yielded two bands of 134 and 63 bp after Eco R I digestion, homozygotes for the 572T appeared as a 197-bp band, and heterozygotes yielded all three of these bands.

Genotyping was done by staff blinded to the subjects’ case/control status. The accuracy of genotyping data for each polymorphism obtained from PCR-RFLP analyses was validated by direct sequencing of a 15% masked, random sample of cases and controls.

Statistical Analysis. Genotype and allele frequencies for the MMP polymorphisms were determined by gene counting. The fitness to the Hardy-Weinberg equilibrium was tested using the χ² test. The association between the genotypes and hepatocellular carcinoma risk was evaluated by multiple logistic regression analyses while controlling for confounding factors (including age, sex, status of smoking and drinking, pack-years of smoking, and family history), and the P values, odds ratios (OR), and 95% confidence intervals (CI) were calculated. The potential modification effect of the polymorphisms on hepatocellular carcinoma risk was...
assessed for the above confounding factors by the addition of interaction terms in the logistic model and by separate analyses of subgroups of subjects stratified by these factors. In view of the multiple comparisons in our study, the correction factor \( n(n-1); n \) loci with \( m \) alleles each) was applied to correct the significance level. An association was considered significant at a \( P \) value of \(<0.017 \) (i.e., \( 0.05/m \)), and all statistical tests were two-sided. These analyses were done using SPSS software (version 9.0; SPSS Inc.).

### Results

The results of the polymorphism validation show that the MMP-7 A-181G, MMP-8 A-381G, and MMP-10 A180G are not polymorphic in our study population. The minor allele frequencies of the polymorphisms MMP-7 A-181G, MMP-8 C-799T, and MMP-21 C572T are 0.047, 0.35, and 0.30, respectively. We therefore selected these three polymorphisms for the subsequent genotyping analyses.

The genotyping results of the three polymorphisms are presented in Table 1. The observed genotype frequencies of the MMP-21 C572T polymorphism conformed to the Hardy-Weinberg equilibrium (\( P > 0.05 \)) in patients but not in controls (\( P = 0.008 \)). However, the other two polymorphisms conformed to the Hardy-Weinberg equilibrium in both patients and controls (\( P > 0.05 \)). On the basis of logistic regression analysis with adjustment for age, sex, smoking and drinking status, and family history, significant association with the risk of hepatocellular carcinoma was observed with the MMP-8 C-799T in non-HBV carriers (Table 1). An increased risk of hepatocellular carcinoma was found to be associated with the −799CT genotype, with the OR being 1.81 (95% CI, 1.05-3.12; \( P = 0.03 \)) compared with the −799CC genotype. After correction for multiple comparisons, however, the association was never again significant. For the other two polymorphisms, that is, MMP-8 C-799T and MMP-21 C572T, we found no association with the risk of hepatocellular carcinoma in the overall sample, HBV carriers, and non-HBV carriers (Table 1). The associations between these polymorphisms and the risk of hepatocellular carcinoma were further examined with stratification by age, sex, family history, status of smoking and drinking, and pack-years of smoking. Again, no significant association was found in the overall sample set, HBV carriers, and non-HBV carriers (data not shown).

### Discussion

Previous studies suggested that the functional polymorphisms in the promoters of MMP genes were strongly associated with the risk of a wide spectrum of cancers (4-9), but no study has ever explored these polymorphisms as risk factors for hepatocellular carcinoma. Recently, we first examined whether seven functional polymorphisms in the promoters of MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, and MMP-13 have any bearing on the risk of hepatocellular carcinoma in a Chinese population, but we found none (10). In the present study, we assessed whether there was an association between an additional three MMP polymorphisms (i.e., MMP-7 A-181G, MMP-8 C-799T, and MMP-21 C572T) and hepatocellular carcinoma risk. Again, no significant association was observed in our case-control population. Our findings suggest that these MMP polymorphisms might not play a major role in mediating susceptibility to hepatocellular carcinoma.

It would be expected that the MMP genotypes would alter the risk of hepatocellular carcinoma under specific conditions such as exposure to cigarette smoke and alcoholic consumption. Indeed, many factors such as chronic infection with HBV, male gender, family history, smoking, and alcoholic consumption have been shown as independent risk factors for hepatocellular

### Table 1. Genotype and allele frequencies of MMP polymorphisms in patients with hepatocellular carcinoma and controls and adjusted OR for hepatocellular carcinoma risk

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Overall</th>
<th>HBV carriers</th>
<th>Non-HBV carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases/controls</td>
<td>OR* (95% CI)</td>
<td>Cases/controls</td>
</tr>
<tr>
<td>MMP-7 A-181G</td>
<td>AA 374/435</td>
<td>1.00 (reference)</td>
<td>286/167</td>
</tr>
<tr>
<td></td>
<td>AG 50/40</td>
<td>1.45 (0.93-2.26)</td>
<td>35/16</td>
</tr>
<tr>
<td></td>
<td>GG 1/0</td>
<td>NA</td>
<td>1/0</td>
</tr>
<tr>
<td></td>
<td>G allele 0.06/0.04</td>
<td>NA</td>
<td>0.06/0.04</td>
</tr>
<tr>
<td>MMP-8 C-799T</td>
<td>CC 140/184</td>
<td>1.00 (reference)</td>
<td>114/64</td>
</tr>
<tr>
<td></td>
<td>CT 196/216</td>
<td>1.16 (0.86-1.57)</td>
<td>141/88</td>
</tr>
<tr>
<td></td>
<td>TT 81/80</td>
<td>1.31 (0.89-1.93)</td>
<td>61/34</td>
</tr>
<tr>
<td></td>
<td>T allele 0.43/0.39</td>
<td>NA</td>
<td>0.42/0.42</td>
</tr>
<tr>
<td>MMP-21 C572T</td>
<td>CC 160/182</td>
<td>1.00 (reference)</td>
<td>123/63</td>
</tr>
<tr>
<td></td>
<td>CT 201/238</td>
<td>0.96 (0.72-1.27)</td>
<td>151/98</td>
</tr>
<tr>
<td></td>
<td>TT 44/60</td>
<td>0.84 (0.53-1.31)</td>
<td>34/25</td>
</tr>
<tr>
<td></td>
<td>T allele 0.36/0.37</td>
<td>NA</td>
<td>0.36/0.40</td>
</tr>
</tbody>
</table>

**NOTE:** The number of genotyped samples varies because of genotyping failure for some individuals.

Abbreviation: NA, not applicable.

*OR and 95% CI were calculated by logistic regression and adjusted for age, sex, smoking status, smoking level, and drinking status.
cancer (22, 23). However, we did not find a statistically significant interaction between the MMP polymorphisms and these risk factors, suggesting that these factors may not have a modification effect on the susceptibility to hepatocellular carcinoma related to MMP genotypes.

There are several possible reasons for our negative results. First, inadequate power may be an explanation of our results. For the MMP-7 A-181G polymorphism, this study had 80% power (two-sided test of significance, \( \alpha = 0.05 \)) to detect an OR of >1.86 (assuming a risk effect) or <0.42 (assuming a protective effect) for carriers of the −181G allele (AG + GG genotype) relative to the carriers of the AA genotype in the overall sample set. For the MMP-8 C-799T polymorphism, this study had 80% power to detect an OR of >1.53 or <0.65 for carriers of the CT genotype, and an OR of >1.91 or <0.43 for carriers of the TT genotype relative to the carriers of the CC genotype. For the MMP-21 C572T polymorphism, this study had 80% power to detect an OR of >1.52 or <0.66 for carriers of the CT genotype, and an OR of >2.10 or <0.34 for carriers of the TT genotype relative to the carriers of the CC genotype. Additional larger population-based case-control studies are warranted to understand the roles of these MMP polymorphisms in the etiology of hepatocellular carcinoma.

Alternatively, differences in the genetic effect among ethnic groups may be another explanation. There may be a small, population-specific effect of MMP polymorphisms on the development of hepatocellular carcinoma. This might occur if there were population differences in linkage disequilibrium pattern or allele frequencies of MMP genes. Indeed, the allele and genotype frequencies of the MMP polymorphisms vary with ethnicity. For instance, in this study with 480 control subjects, we found that the frequencies of the MMP-7-181G allele and GG genotype were 0.04 and 0.00, compared with around 0.40 and 0.13, respectively, among Caucasians from Italy (13). Thus, ethnic variation in the MMP genotype distribution warrants additional comparative studies in other populations of different ancestry, such as Caucasians and Africans, to confirm our results.

Lastly, our negative results may be due to an inherent selection bias. As a hospital-based study, our hepatocellular carcinoma cases were enrolled from the hospitals and the control subjects were selected from the community population. Thus, inherent selection bias cannot be completely excluded. By matching on age and residential area, however, potential confounding factors might have been minimized and any inadequacy in matching might have been controlled in data analyses with further adjustment and stratification.

In summary, to our best knowledge, this is the first case-control study of polymorphisms in the MMP-7, MMP-8 and MMP-21 genes in relation to hepatocellular carcinoma. Our results suggest that the functional polymorphisms in the promoters of MMP-7 and MMP-8, and a non-synonymous polymorphism in the MMP-21 do not significantly confer susceptibility to hepatocellular carcinoma in a southern Chinese population. However, additional studies are warranted before the importance of MMP polymorphisms in hepatocellular carcinoma risk can be fully ascertained. First, data from larger population-based case-control studies among Chinese, and from diverse ethnic populations, are required to confirm our initial observation. Second, the polymorphisms in other members of MMP family and their association with hepatocellular carcinoma risk should be systematically investigated.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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Cancer Epidemiol Biomarkers Prev 2008;17(9). September 2008


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