Polymorphisms of a disintegrin and metalloproteinase with thrombospondin motifs 5 and aflatoxin B1 related-hepatocellular carcinoma

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ABSTRACT

Background: Altered expression of a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) is observed in hepatocellular carcinoma (HCC). The genetic polymorphisms of this gene in aflatoxin B1 (AFB1)-related HCC have not yet been elucidated.

Methods: We conducted a hospital-based case-controlled study, including 1706 HCC cases and 2270 controls without any liver diseases or tumors, to assess the association between 74 polymorphisms in ADAMTS5 and AFB1-related HCC risk and prognosis. Genotype, mRNA levels, and TP53 gene mutation (TP53M) related to AFB1 exposure were tested using TaqMan-PCR or sequencing technique. ADAMTS5 protein level and micro-vessel density were analyzed by immunohistochemistry.

Results: Among these 74 polymorphisms, only rs2830581 affected HCC risk. Compared with the homozygote of rs2830581 G alleles (rs2830581-GG), the genotypes of rs2830581 A alleles (rs2830581-GA or -AA) increased HCC risk (odds ratio: 1.85 and 4.40, 95% confidence interval: 1.57-2.19 and 3.43-5.64, respectively). Significant interactive effects between risk genotypes and AFB1 exposure status were also observed in the joint effects analysis. Furthermore, the rs2830581 polymorphism modified the tumor recurrence-free survival and overall survival of patients. This polymorphism not only affected pathological features of HCC such as tumor dedifferentiation and micro-vessel density, but also modified ADAMTS5 expression and the effects of transarterial chemoembolization treatment on HCC.

Conclusions: These results suggest ADAMTS5 polymorphisms may be risk and prognostic biomarkers of AFB1-related HCC, and rs2830581 is a potential candidate.

Impact: Our findings support the hypothesis that ADAMTS5 rs2830581 polymorphism modifies AFB1-related HCC risk and prognosis.
Introduction

Hepatocellular carcinoma (HCC), in addition to being the most common cancer worldwide in 2008, is the most common histopathological type of liver cancer (1). Approximately 50% all HCC cases and deaths were estimated to occur in the People’s Republic of China (especially in the southeast areas such as Guangxi), mainly because of high aflatoxin B1 (AFB1) exposure and/or chronic infection of hepatitis B and C viruses (HBV and HCV, respectively) (2). Therefore, a better understanding of the molecular characteristics and underlying mechanism are of the utmost importance and may prove useful in developing novel strategies for the prevention, early detection, treatment, and prognosis of HCC. The hot-spot mutation of the TP53 gene (TP53M) has been shown to be positively associated with AFB1 exposure levels and HCC risk factor (2, 3). Until now, it has been regarded as an important biomarker of AFB1-related HCC as well as the serum AFB1 albumin adducts (AAA) (2, 3).

Recently, increasing evidence has shown potential associations of common genetic variants with AFB1-related HCC risk in biologically plausible pathways including DNA repair and detoxication pathways (4). These genetic factors could feasibly be utilized as biomarkers of HCC induced by AFB1 exposure and prognostic indicators.

The disintegrin-like and metalloproteinase (ADAMTS) family member ADAMTS5 acts as a proteoglycanase and has been shown to be a major aggrecanase in cartilage destruction (5, 6). Recently, a preponderance of evidence has implicated aberrant ADAMTS5 expression in the development and progression of some tumors including breast cancer, colorectal cancer, glioblastoma, HCC, and T-cell acute lymphoblastic leukemia (7-18). Despite the collected data, the association of altered ADAMTS5 expression and genetic variants of AFB1-related HCC remains to be elucidated. Here, we evaluated whether 74 single-nucleotide polymorphisms (SNPs) in this gene modify AFB1-related HCC risk and prognosis.

Materials and Methods
Study subjects

We utilized a hospital based case-controlled study of AFB1-related HCC in the Guangxi area according to a previously described protocol (19, 20). Briefly, patients diagnosed with histopathologically confirmed HCC in hospitals affiliated with Guangxi Medical University and Youjiang Medical College for Nationalities in the Southwestern Guangxi from January 2004 to July 2014 were utilized. Individuals without clinical evidence of hepatic disease or tumors were recruited from the general health check-up center at the same hospitals during the same period for comparison. To control the effects of confounders, cases were individually matched (1:1 or 1:2) to controls based on gender, ethnicity (Han, Zhuang), age (± 5 years), and hepatitis B virus (HBV and HCV) infection. All controls were surveyed in order to ascertain their willingness to participate in the study and to provide preliminary demographic data.

In this study, a total of 1706 cases and 2,076 controls, representing 95% of eligible cases and 98% of eligible controls were interviewed and included the final analysis. From these subjects, 200 matched cases and controls were randomly selected for inclusion in the screening set while the remainder was utilized for the validation group. Once informed, written consent was obtained for all participants and 4 mL of peripheral blood was collected along with demographic data. Surgically removed tumor samples and 207 fresh cancer tissue specimens were collected for ADAMTS5 expression analysis. All protocols were approved by the ethic committees of the participating hospitals and more detailed data collection can be found in the Supplementary Methods.

AFB1 exposure analysis

In this study, subject AFB1 exposure level was evaluated via the serum AAA levels using a comparative enzyme-linked immunosorbent assay (21). For statistical analysis, AAA values were logarithmically transformed and then were divided into three subgroups: low (<2.18 ln fmol/mg), medium (2.18-2.98 ln fmol/mg), and high
(>2.98 ln fmol/mg), according to the mean logit value of serum AAA among controls and cases. These three subgroups represented low, medium, and high AFB1 exposure, respectively.

Mutation of TP53M at codon 249 of the TP53 gene was examined using the TaqMan-PCR methods (21) in order to assess the relationship between AFB1 exposure and HCC diagnosis. Briefly, Genomic DNA was isolated from cancerous tissues using FFPE DNA Kit (catalog # CW0547) by Cowin Biotech Co., Ltd (Beijing, China). Each PCR was carried out in a total volume of 25 μL consisting of 1 × Premix Ex Taq™ (catalog # DRR039A, TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China), 0.2 μM of each probe, 0.2 μM of each primer, and 50 – 100 ng of genomic DNA. The PCR program had an initial denaturation step of 2 min at 95 °C followed by 50 cycles of 10 sec at 95 °C and 1 min at 60 °C. For quality control, controls were included in each run, and repeated genotyping and sequencing of a random 10% subset yielded 100% identical genotypes. In this study, TP53M positive was defined as the TP53 genotypes with mutant codon 249 alleles.

**ADAMTS5 SNPs selection and analysis**

Utilizing the application programming interface tools available from the ENSEMBL database, 1143 SNPs in the ADAMTS5 gene were obtained. According to the criteria of minor allele frequency of more than 0.10 in Asians, 74 SNPs were selected for further analysis in the first stage of the study (Supplementary Table S1). Genotyping was performed by using the SNaPshot method. In the second stage of the study, four SNPs (rs2830581, rs162499, rs233895, and rs62215997) were further analyzed using the TaqMan-PCR method. More detailed information for SNPs selection and analysis is available in the Supplementary Materials and Methods.

**ADAMTS5 expression analysis**
ADAMTS5 expression levels were evaluated using both ADAMTS5 mRNA-expressing levels and protein-expressing levels. Detailed information about ADAMTS5 expression analysis is described in the Supplementary Materials and Methods.

**Statistical analysis**

We used the Student’s t test, the Mann-Whitney U test, or the $\chi^2$ test to evaluate the differences between groups, demographic characteristics, AFB1-exposure information, and SNP genotypes. From the individually matched design, conditional logistical regression was conducted (with multivariate factors including known causes of HCC among Guangxi population) to estimate odds ratios (ORs) for HCC along with the 95% confidence intervals (CIs). Screening the main effects of the 74 SNPs utilized was based on the additive model with the genotypes treated as ordinal variables. For the correction of multiple testing in the screen stage, the Bonferroni correction treats each SNP test as an independent test. This correction is overly conservative for SNPs that are in the linkage disequilibrium (LD), because it ignores the correlation among SNPs. To address this limitation, the correlation matrix-based method was used to takes into account LD between SNPs (22). Based on this method, 2-sided $P$ values smaller than $6.74 \times 10^{-4}$ were considered significant for the main effects of SNPs in the screen stage. In the joint analysis stage of SNPs and AFB1 exposure, genotype frequencies in these groups were further adjusted for multiple comparisons using Bonferroni’s method, and the significance threshold was lowered to $\alpha_{\text{correct}} = 3.37 \times 10^{-4}$.

Kaplan-Meier survival analysis with the log-rank test was used to evaluate the effects of rs2830581 polymorphism on HCC prognosis. Risk factors for HCC prognosis were selected using the Cox multivariate regression model (including all possible multiplicatively interactive variables) with stepwise forward selection based on a likelihood ratio test. Hazard ratios (HRs) and 95% CIs for risk factors were then
calculated from a multivariate Cox regression model. All statistical analyses were carried out utilizing SPSS version 18 (SPSS Institute, Chicago, IL).

RESULTS

Characteristics of subjects

The distributions of demographic characteristics (including sex, age, race, and HBV and HCV status) between HCC cases and controls were not significantly different. However, HCC cases exhibited a higher level of AFB1-album adducts in the peripheral serum than controls (2.98 vs. 2.18 ln fmol/mg, $P = 1.81 \times 10^{-177}$). Logistic regression analysis revealed that HCC risk increased with an increasing exposure level (OR, 1.89 to 5.88; $P < 0.01$; Supplementary Table S2). However, there was not a significant difference of AFB1 levels between the early stage and the advanced stage patients ($2.99 \pm 0.98$ vs. $2.97 \pm 0.75$ ln fmol/mg, $P = 0.61$).

Effects of ADAMTS5 polymorphisms on HCC risk

Analysis of the selected SNPs revealed the genotype distribution of control individuals was consistent with predictions from the Hardy-Weinberg equilibrium (Supplementary Table S3; $P > 0.05$). Logistic regression analysis indicated that four SNPs (rs162499, rs233895, rs62215997, and rs2830581) were significant upon initial screening (Table 1, Supplementary Table S4). From the validation group, it was found that only rs2830581 (located on the 3 prime UTR of the ADAMTS5 gene) was significantly associated with HCC risk (OR = 2.35; 95% CI: 2.03 - 2.72; $P = 3.38 \times 10^{-31}$). In the combined analysis utilizing all subjects (Merged set, Table 1), the adjusted OR for HCC among those heterozygous for rs2830581 G and A alleles (rs2830581-GA) versus those homozygous for rs2830581 G alleles (rs2830581-GG) was 1.85 (1.57–2.19). The corresponding OR for those homozygous for rs2830581 A
alleles (rs2830581-AA) was 4.40 (3.43–5.64). Thus, HCC risk was associated with the number of rs2830581 A alleles.

To evaluate possible interactive effects of individually matching variables (including age, race, gender, HBV infection, and HCV infection) and ADAMTS5 rs2830581 polymorphism on HCC risk, the polymorphism analysis was stratified by matching factors. Results showed that these factors did not substantially modulate the effect of ADAMTS5 rs2830581 on cancer risk ($P_{\text{interaction}} > 0.05$; Supplementary Table S5).

**Joint effects of ADAMTS5 rs2830581 polymorphism and AFB1 exposure on HCC risk**

The joint effects of ADAMTS5 rs2830581 polymorphism and AFB1 exposure levels on HCC risk was carried out with the lowest risk group (those with rs2830581-GG and low level of AFB1 exposure) as a reference (Table 2). It was found that higher levels of AFB1 exposure consistently increased HCC risk; the risk was more pronounced among subjects with ADAMTS5 risk genotypes. There was evidence of multiplicatively interactive effects of genotypes and exposure levels on HCC risk ($16.67 > [1.88 \times 3.97]$) according to the previously published formula ($\text{OR}_{eg} > [\text{OR}_{eg} \times \text{OR}_{eg}]$ (23)). To determine more detailed interactive value, we conducted multiplicatively interactive analysis. In this analysis model, risk factors for HCC risk were selected using a multivariate logistic regression model (including all possible multiplicatively interactive variables) with stepwise forward selection based on a likelihood ratio test. Next, the OR$_{\text{interaction}}$ and 95% CIs for AFB1-genotypes interactive variables were calculated in the same multivariate model (simultaneously including all risk variables and multiplicatively interactive variables). We found significantly interactive values [OR$_{\text{interaction}}$ (95% CI/P), 1.63 (1.08 - 2.46/0.02)] for high AFB1 exposure level $\times$ rs2830581-GA; 4.37 (1.76 - 10.86/1.48 $\times 10^{-3}$) for high AFB1 exposure level $\times$ rs2830581-AA, respectively]. Together, the data indicated
that AFB1 exposure multiplicatively interacted with ADAMTS5 rs2830581 polymorphism.

**ADAMTS5 rs2830581 polymorphism modified ADAMTS5 expression**

To characterize the functional relevance of the ADAMTS5 rs2830581 polymorphism, we conducted a correlation analysis between the rs2830581 genotypes and the expression of ADAMTS5 protein assessed using immunohistochemistry on the cancerous tissues collected in the study. The results showed that the genotypes with rs2830581 A alleles were significantly related to decreased ADAMTS5 expression in hepatocellular tumor tissues, compared with rs2830581-GG (Fig. 1A; \( P < 0.01 \)). To analyze further this correlation, subjects were divided into three groups based on ADAMTS5 expression scores in the tumors, representing low, medium, and high expression of ADAMTS5. ADAMTS5 expression was found to be negatively related to the expression of the polymorphism (\( r = -0.313 \); Supplementary Table S6; Fig. 1B). Moreover, mRNA levels of ADAMTS5 in cancerous tissues with rs2830581-GA or -AA were significantly lower than in those with rs2830581-GG (Fig. 1C; \( P < 0.01 \)). Together, these results suggest that this polymorphism modulates the expression of ADAMTS5.

**ADAMTS5 rs2830581 polymorphism correlated with micro-vessel density (MVD)**

From previous reports, it is known that ADAMTS5 expression is capable of regulating HCC angiogenesis (14). Thus, we explored how expression of ADAMTS5 rs2830581 polymorphism affected MVD (Fig. 1D). The data showed that rs2830581-GA or -AA had a higher frequency of MVD, compared with rs2830581-GG [OR (95% CI/\( P \)), 2.50 (1.98 - 3.16/1.65 \times 10^{-14}) for rs2830581-GA and 7.29 (5.09 - 10.43/2.33 \times 10^{-27}) for rs2830581-AA, respectively]. Moreover, these risk-associated genotypes increased the risk of portal vein tumor (PVT) [OR = 2.14 (1.65 - 2.79) and 5.74 (3.64 - 9.06) for rs2830581-GA and -AA, respectively] (Fig. 1E).
**ADAMTS5 rs2830581 polymorphism modified HCC prognosis**

To study the effects of ADAMTS5 rs2830581 polymorphism on the outcome of HCC patients, survival follow-up information of all HCC patients was analyzed. The association analysis between high-risk genotypes (namely, genotypes with rs2830581 A alleles; rs2830581-GA/AA) or low/no-risk genotypes (rs2830581-GG) and the pathological characteristics of HCC were performed separately (Supplementary Table S7). A significant difference between genotypes among different AFB1 exposure levels, tumor size, tumor grade and TNM stage, and PVT and TP53M status, but not in age, gender, race, HBsAg, anti-HCV, or cirrhosis was observed. Results of the Kaplan-Meier survival analysis showed that ADAMTS5 polymorphism significantly correlated with shorter overall and recurrence-free survival of HCC cases (Fig. 2A and 2B), particularly under conditions of high aflatoxin exposure (Fig. 2C – 2H). From Cox regression analysis (Table 3) we showed that the ADAMTS5 polymorphism is capable of affecting HCC prognosis (rs2830581-AA risk value, HR = 3.57 for RFS and 2.18 for OS, respectively). Furthermore, some evidence of multiplicative interaction was found for rs2830581 polymorphism and AFB1 exposure ($P_{interaction} < 0.05$). Taken together, these results indicated that this polymorphism is independent of other clinical covariates and suggested its potential as an independent prognostic factor for HCC.

**ADAMTS5 rs2830581 polymorphism modified the effects of TACE treatment on HCC**

The effect on patient survival in ADAMTS5 rs2830581 polymorphism was investigated to determine if there was a correlation with effectiveness of a chosen therapy. Information from TACE-treated HCC patients who received curative resection treatment (Supplementary Table S8) was collected in accordance with the Chinese Manage Criteria of HCC (24). Although TACE treatment improved HCC
prognosis as a whole (Fig. 3A-3B, Supplementary Fig. S1A-S1B), stratified analysis based on the different genotypes of ADAMTS5 rs2830581 revealed that TACE treatment had no effect on the RFS and OS of HCC patients with rs2830581-AA genotype (Fig. 3C-3D, Supplementary Fig. S1C-S1D). Interestingly, patients with rs2830581-GA or -GG polymorphisms that received TACE treatment had a prolonged OS and RFS (Fig. 3E-3H, Supplementary Fig. S1E-S1H). By using these with rs2830581-AA but not receiving TACE treatment as a reference, a joint statistical analysis between ADAMTS5 genotypes and TACE treatment on HCC prognosis was performed (Supplementary Fig. S2A-S2D). The results indicated that HCC cases with rs2830581-GG would face a noticeably decreased risk of death and tumor reoccurrence under the conditions of TACE treatment (OS: HR = 0.28, 95% CI, 0.22 - 0.36; RFS: HR = 0.17, 95% CI, 0.12 - 0.24). Taken together, the data suggest that ADAMTS5 rs2830581 polymorphism could potentially modify the effects of TACE treatment and improve the prognosis of HCC patients.

DISCUSSION

Based a large-scale case-control study, we investigated the association between ADAMTS5 polymorphisms and the risk of HCC in a high AFB1 exposure area of Guangxi province. It was found that the genotypes of ADAMTS5 with rs2830581 A alleles had a substantial association with the increasing risk of HCC (OR, 2.37; 95% CI, 2.05-2.74). Interestingly, an interaction between genotype and environment was found for populations having the rs2830581 A alleles, and exposed to different levels of AFB1. This study represents the first report indicating the potential of ADAMTS5 polymorphisms as biomarkers and prognostic indicators of HCC. Additionally, we report that these polymorphisms alter the effects of TACE treatment as well.

ADAMTS5 is well known for its role in cartilage biology (1, 25), and that it is the major aggrecanase in mouse cartilage in an inflammation model of arthritis (26). Previously, studies have suggested that ADAMTS5 may be involved in the
development and progression of cancers affecting the breast, prostate, brain, colorectal tissue, and liver (10, 14, 16, 27-29). Porter et al. (27) found that ADAMTS5 expression in breast cancer tissues was downregulated in cancerous tissues with respect to nonneoplastic mammary tissue, irrespective of the heterogeneity of the samples and the tumor type or grade. Kim and Roman-Gomez et al. (10, 16) observed that the ADAMTS5 gene exhibited hyper-methylation in colorectal cancer and T-cell acute lymphoblastic leukemia. Additional studies have indicated that it may be possible for this gene to suppress tumor growth and progress (9, 14, 16). In contrast, other reports indicated that ADAMTS5 expression was upregulated in human glioblastoma compared with normal brain tissues and promoted glioma cell invasiveness in culture (28). Together, the collected data indicate that ADAMTS5 may serve different functions in different cancer types. Thus, the role of ADAMTS5 in cancer, including HCC, needs to be further evaluated.

More than 1000 SNPs have been reported in the ADAMTS5 gene, some of which were found in correlation with human disease (11, 13, 17, 18). In the present study, 74 known SNPs were analyzed because of their indicated involvement with the structure and expression of ADAMTS5, and potential involvement in HCC carcinogenesis (13). Of the polymorphisms analyzed, only rs2830581 polymorphism (located in the 3 prime UTR of ADAMTS5) was found to be associated with increased HCC risk from first stage screening and second stage validation. Furthermore, this polymorphism was found in correlation with AFB1 exposure status in HCC patients. These data suggest this polymorphism may be an important genetic susceptibility factor in cancer risk. Coincidentally, a previous case-control study from Chongqing Region, a non-AFB1 exposure area in China, has shown that rs2830585 modifies HCC risk. Supplementary the aforementioned modifying role, the data indicated that the polymorphism affected HCC risk in the first-stage screening analysis, despite a lack of statistical significance. Therefore, the possibility that the genetic polymorphisms in ADAMTS5 influence HCC risk through an alternative pathway should not be ignored.
To explore the effect of ADAMTS5 rs2830581 polymorphism on AFB1-related HCC risk, expression of ADAMTS5 and the pathological features of HCC were examined. It was found that this polymorphism was associated with the downregulation of ADAMTS5 expression, increasing MVD, tumor progression, dedifferentiation, and metastasis. Supplementary the data, a recent in vivo and in vitro study has shown that downregulation of this gene accelerates HCC angiogenesis and carcinogenesis by modifying VEGF expression (14). The survival analysis further indicated that the rs2830581 polymorphism was associated with poor outcome of HCC patients, highlighting the potential roles of this polymorphism in predicting the prognosis of HCC induced by AFB1 exposure. Collectively, these results suggest that ADAMTS5 rs2830581 polymorphism could modulate the anti-tumorigenesis role of ADAMTS5, increasing AFB1-related HCC risk and shortening prognosis of this tumor.

Another important finding in this study was that ADAMTS5 rs2830581 polymorphism was found to modify the effects of TACE treatment on HCC patients. This is likely due to the downregulation of ADAMTS5 expression and increased MVD, an important control factor for TACE treatment.

In the present study, major strengths were its prospective two-stage design, large sample size, and the panel of a relatively large number of systematically identified SNPs in ADAMTS5. The effects of possible confounders (including age, gender, nationality, and HBV and HCV infection status) were controlled with an individually matched design. Additionally, AAA and TP53M was used to test this exposure and correlation because AAA is a stable biomarker reflecting long-term AFB1 exposure status, whereas TP53M is the characteristic genetic change resulting from AFB1 exposure, and higher frequency of this mutation predicts higher AFB1 exposure and higher HCC risk. In the present study, we found HCC cases had a higher serum AAA
and more than 70% of them featured TP53M, which was similar to the data from other countries of South East Asia (30).

However, there were several limitations to our study. First, because 74 SNPs were evaluated in the screening set, whereas only 4 were evaluated in the validation set, false-negative results were a considerable issue. In this study, the correction for multiple testing was done using the correlation matrix-based method, which takes into account the linkage disequilibrium between SNPs (22). Second, potential selection bias might have occurred through the selection of hospital-based control subjects. Third, the increased risk with AFB1 exposure seen in this study was probably underestimated because liver disease itself may affect the metabolism of AFB1 and modify the levels of AFB1 adducts (2, 31). Finally, although we analyzed the effects of the ADAMTS5 rs2830581 polymorphisms on the ADAMTS5 expression and MVD, we failed to accomplish more functional analysis. Thus, more functional analyses deserve elucidation based on large samples and a combination of genes and AFB1 exposure.

This study represents the first report describing genetic polymorphisms of ADAMTS5 and their association with AFB1-related HCC risk and prognosis. The findings provide an additional insight into the ADAMTS5 rs2830581 polymorphism as biological determinants of HCC risk and prognosis related to AFB1 exposure. Interestingly, the effects of ADAMTS5 polymorphism on TACE therapy for HCC treatment indicated a positive association between the two. Understanding the different value in individual patients will allow more information counseling with regard to screening, prevention, treatment options, follow-up plans, and approaches for secondary prevention. Therefore, these findings, in combination with clinicopathologic information of HCC, could improve the identification of high-risk populations, especially from high AFB1 exposure areas.
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REFERENCES


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<td>AG/GG</td>
<td>47</td>
<td>79</td>
<td>2.14(1.39-3.30/5.86×10^{-4})</td>
<td>488</td>
<td>353</td>
<td>0.99(0.85-1.16/0.92)</td>
<td>535</td>
<td>432</td>
<td>1.10(0.95-1.27/0.20)</td>
</tr>
<tr>
<td>Rs2030581-GG</td>
<td></td>
<td></td>
<td></td>
<td>1,584</td>
<td>875</td>
<td>Reference</td>
<td>1,738</td>
<td>990</td>
<td>Reference</td>
</tr>
<tr>
<td>GA</td>
<td>36</td>
<td>52</td>
<td>1.92(1.18-3.13/8.93×10^{-3})</td>
<td>383</td>
<td>406</td>
<td>1.92(1.63-2.56/3.24×10^{-15})</td>
<td>419</td>
<td>458</td>
<td>1.85(1.57-2.19/1.88×10^{-13})</td>
</tr>
<tr>
<td>AA</td>
<td>10</td>
<td>33</td>
<td>4.40(2.08-9.30/1.04×10^{-6})</td>
<td>103</td>
<td>225</td>
<td>3.96(3.09-5.07/1.17×10^{-27})</td>
<td>113</td>
<td>258</td>
<td>4.40(3.43-5.64/1.13×10^{-31})</td>
</tr>
<tr>
<td>GA/AA</td>
<td>46</td>
<td>85</td>
<td>2.46(1.60-3.79/4.61×10^{-5})</td>
<td>486</td>
<td>631</td>
<td>2.35(2.03-2.72/3.38×10^{-31})</td>
<td>532</td>
<td>716</td>
<td>2.37(2.05-2.74/4.42×10^{-31})</td>
</tr>
</tbody>
</table>
Table 2 Joint Effects of AFB1 Exposure and ADAMTS5 rs2830581 polymorphism on HCC Risk

<table>
<thead>
<tr>
<th>AFB1 exposure level/ADAMTS5 genotype</th>
<th>Controls (n = 2270)</th>
<th>Cases (n = 1706)</th>
<th>OR (95%CI)</th>
<th>( P_{\text{trend}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low/GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>216 (9.5%)</td>
<td>98 (5.7%)</td>
<td>1.52 (1.16-2.00)</td>
<td>( 2.76 \times 10^{-3} )</td>
</tr>
<tr>
<td>AA(^b)</td>
<td>72 (3.2%)</td>
<td>63 (3.7%)</td>
<td>2.98 (2.07-4.30)</td>
<td>( 4.48 \times 10^{-9} )</td>
</tr>
<tr>
<td>GA/AA</td>
<td>288 (12.7%)</td>
<td>161 (9.4%)</td>
<td>1.88 (1.49-2.39)</td>
<td>( 1.47 \times 10^{-7} )</td>
</tr>
<tr>
<td>Medium/GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>137 (6.0%)</td>
<td>121 (7.1%)</td>
<td>3.01 (2.28-3.99)</td>
<td>( 1.28 \times 10^{-14} )</td>
</tr>
<tr>
<td>AA</td>
<td>35 (1.5%)</td>
<td>82 (4.8%)</td>
<td>7.91 (5.20-12.02)</td>
<td>( 3.82 \times 10^{-22} )</td>
</tr>
<tr>
<td>GA/AA</td>
<td>172 (7.6%)</td>
<td>203 (11.9%)</td>
<td>4.01 (3.14-5.12)</td>
<td>( 1.04 \times 10^{-28} )</td>
</tr>
<tr>
<td>High/GG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>66 (2.9%)</td>
<td>239 (14.0%)</td>
<td>12.33 (9.08-16.73)</td>
<td>( 1.73 \times 10^{-58} )</td>
</tr>
<tr>
<td>AA</td>
<td>6 (0.3%)</td>
<td>113 (6.6%)</td>
<td>64.56 (28.07-148.49)</td>
<td>( 1.05 \times 10^{-22} )</td>
</tr>
<tr>
<td>GA/AA</td>
<td>72 (3.2%)</td>
<td>352 (20.6%)</td>
<td>16.67 (12.50-22.25)</td>
<td>( 1.86 \times 10^{-81} )</td>
</tr>
</tbody>
</table>
Table 3 Cox proportional hazard model analysis for multivariate analysis of potential predictor factors for HCC cases

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI/P) for RFS</th>
<th>HR (95% CI/P) for OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3 cm</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>&gt;3 cm</td>
<td>1.41(1.20-1.66/2.19×10^{-5})</td>
<td>1.66(1.32-1.85/2.42×10^{-6})</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>III-IV</td>
<td>2.84(2.34-2.99/1.41×10^{-53})</td>
<td>2.32(1.73-3.10/1.49×10^{-8})</td>
</tr>
<tr>
<td>MVD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Positive</td>
<td>5.21(4.38-6.19/1.22×10^{-77})</td>
<td>4.31(3.83-4.86/8.03×10^{-126})</td>
</tr>
<tr>
<td>Curative treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>No</td>
<td>3.13(2.33-4.19/2.08×10^{-14})</td>
<td>2.11(1.86-2.39/1.90×10^{-31})</td>
</tr>
<tr>
<td>AFB1-exposure levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Median</td>
<td>1.31(1.00-1.71/0.04)</td>
<td>1.34(1.16-1.55/8.19×10^{-5})</td>
</tr>
<tr>
<td>High</td>
<td>3.18(2.53-3.99/2.49×10^{-21})</td>
<td>1.61(1.42-1.84/6.77×10^{-15})</td>
</tr>
<tr>
<td>ADAMTS5 rs2830581 genotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>GA</td>
<td>1.71(1.37-2.15/2.62×10^{-6})</td>
<td>1.40(1.24-1.58/8.78×10^{-8})</td>
</tr>
<tr>
<td>AA</td>
<td>3.57(2.83-4.49/2.59×10^{-27})</td>
<td>2.18(1.88-1.52/2.76×10^{-25})</td>
</tr>
<tr>
<td>Interaction of ADAMTS5 and AFB1 exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA × High exposure</td>
<td>2.20(1.27-3.83/4.91×10^{-3})</td>
<td>1.61(1.17-2.20/3.15×10^{-3})</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. ADMATS5 rs2830581 polymorphism correlated with ADAMTS5 expression and pathological features of HCC. (A) ADAMTS5 protein expression was evaluated using the IHC scores of IRS system according to the following formula: IRS = SI × PP, as described elsewhere. ADAMTS5 expression scores are shown as box plots, with horizontal lines representing the median, the bottom and the top of the boxes representing the 25th and 75th percentiles, respectively. We compared genotypes with rs2830581 A alleles (rs2830581-GA and rs2830581-AA) with rs2830581-GG using the Mann-Whitney U test. (B) Representative images show that different expression levels were observed in cancerous tissues from cases with different ADAMTS5 genotypes (scale bars: 50 μm). (C) Quantitative real-time PCR analysis of ADAMTS5 mRNA level in HCC tumor tissues from 207 subjects. The relative expression of ADAMTS5 mRNA is shown as box plots, with horizontal lines representing the median, the bottom and the top of the boxes representing the 25th and 75th percentiles, respectively, and vertical bars representing the range of data. We compared the difference among group using the Mann-Whitney U test. Any outliers are marked with a circle and extreme cases are marked with an asterisk. (D-E) The micro-vessel density (MVD) and percentage of portal vein tumor (PVT)-positive tumors in three groups with different genotypes of rs2830581 from 1706 subjects. Data were analyzed using Pearson’s chi-square test.

Figure 2. The association between HCC prognosis and both AFB1 exposure and ADMATS5 rs2830581 polymorphism in 1706 HCC patients. (A-B) The genotypes with rs2830581 A alleles (rs2830581-GA and -AA) were found to have a poorer overall survival (OS) or tumor recurrence-free survival (RFS) than rs2830581-GG. (C-H) The joint effects of AFB1 exposure and rs2830581 polymorphism further modified HCC prognosis. Cumulative hazard function was plotted by Kaplan-Meier’s methodology, and P value was calculated with two-sided log-rank tests. Abbreviations:
MST, the median overall survival time; MRT, the median tumor recurrence-free survival time.

**Figure 3.** The different therapeutic value of TACE on HCC cases in three groups with different genotypes of ADAMTS5 rs2830581 from 1299 subjects. (A-B) TACE treatment was found to improve overall survival (OS) or tumor recurrence-free survival (RFS). (C-H) The effects of TACE treatment on HCCs’ OS and RFS within strata of ADAMTS5 rs2830581 genotypes. Cumulative hazard function was plotted by Kaplan-Meier’s methodology, and *P* value was calculated with two-sided log-rank tests. *Abbreviations*: MST, the median overall survival time; MRT, the median tumor recurrence-free survival time.
Figure 1

**A**  
Box-Whisker plots showing the relative expression of ADAMTS5 mRNA in different genotypes: ADAMTS5-AA, ADAMTS5-GA, and ADAMTS5-GG. The relative expression is expressed as the ratio of mRNA levels in each genotype to the reference genotype. The x-axis represents the genotypes, and the y-axis represents the relative expression level. The box plots indicate the median, interquartile range, and outliers. The statistical significance was calculated using a two-sample t-test, with P-values of $P = 4.82 \times 10^{-41}$ for ADAMTS5-AA vs. ADAMTS5-GG and $P = 9.13 \times 10^{-41}$ for ADAMTS5-GA vs. ADAMTS5-GG.

**B**  
Immunohistochemical images of tumor sections stained for ADAMTS5 expression. The images show staining intensity and distribution, with different genotypes represented in the images. The images are labeled ADAMTS5-GG, ADAMTS5-GA, and ADAMTS5-AA.

**C**  
Histograms showing the relative scores of ADAMTS5 protein expression in different genotypes: ADAMTS5-GG, ADAMTS5-GA, and ADAMTS5-AA. The x-axis represents the genotypes, and the y-axis represents the protein score. The histograms indicate the distribution of protein scores across different genotypes. The statistical significance was calculated using a two-sample t-test, with P-values of $P = 3.58 \times 10^{-31}$ for ADAMTS5-GG vs. ADAMTS5-GA and $P = 1.48 \times 10^{-31}$ for ADAMTS5-GA vs. ADAMTS5-AA.

**D**  
Bar charts showing the tumor microvascular density (MVD) and the relative MVD risk in different genotypes: GG, GA, and AA. The x-axis represents the genotypes, and the y-axis represents the MVD percentage. The charts indicate the MVD distribution and the relative risk of MVD across different genotypes. The statistical significance was calculated using a chi-squared test, with P-values of $P = 1.46 \times 10^{-36}$ for GG vs. GA and $P = 2.33 \times 10^{-27}$ for GG vs. AA.

**E**  
Bar charts showing the vascularization (VT) and the relative VT risk in different genotypes: GG, GA, and AA. The x-axis represents the genotypes, and the y-axis represents the VT percentage. The charts indicate the VT distribution and the relative risk of VT across different genotypes. The statistical significance was calculated using a chi-squared test, with P-values of $P = 1.65 \times 10^{-34}$ for GG vs. GA and $P = 4.56 \times 10^{-10}$ for GG vs. AA.
Figure 2

All patients

Log-rank test, $P = 3.58 \times 10^{-26}$
- GG; MST 27.00 months; 95%CI 25.45-28.56 months
- GA; MST 20.00 months; 95%CI 18.76-21.24 months
- AA; MST 15.00 months; 95%CI 13.63-16.37 months

n = 990
n = 258
n = 458

Low AFB1 exposure

Log-rank test, $P = 4.49 \times 10^{-9}$
- GG; MST 32.00 months; 95%CI 29.52-34.48 months
- GA; MST 31.00 months; 95%CI 26.84-35.16 months
- AA; MST 18.00 months; 95%CI 15.82-20.18 months

n = 269
n = 98
n = 63

Medium AFB1 exposure

Log-rank test, $P = 4.04 \times 10^{-5}$
- GG; MST 27.00 months; 95%CI 24.04-29.96 months
- GA; MST 20.00 months; 95%CI 17.86-22.14 months
- AA; MST 17.00 months; 95%CI 14.89-19.11 months

n = 258
n = 82
n = 121

High AFB1 exposure

Log-rank test, $P = 1.28 \times 10^{-18}$
- GG; MST 25.00 months; 95%CI 23.09-26.92 months
- GA; MST 18.00 months; 95%CI 16.15-19.85 months
- AA; MST 13.00 months; 95%CI 11.63-14.32 months

n = 463
n = 113
n = 239

Recurrence-free survival

Log-rank test, $P = 1.90 \times 10^{-21}$
- GG; MRT 45.00 months; 95%CI 39.08-50.92 months
- GA; MRT 31.00 months; 95%CI 26.29-35.71 months
- AA; MRT 18.00 months; 95%CI 14.21-21.79 months

n = 990
n = 258
n = 458

Log-rank test, $P = 1.09 \times 10^{-12}$
- GA; MRT is not determined
- GG; MRT is not determined
- AA; MRT 24.00 (14.73-33.28) months

n = 269
n = 63
n = 98

Log-rank test, $P = 1.65 \times 10^{-8}$
- GA; MRT is not determined
- GG; MRT is not determined
- AA; MRT 24.00 (14.73-33.28) months

n = 258
n = 82
n = 121

Log-rank test, $P = 2.58 \times 10^{-11}$
- GG; MRT 30.00 months; 95%CI 27.77-32.23 months
- GA; MRT 20.00 months; 95%CI 16.38-23.62 months
- AA; MRT 13.00 months; 95%CI 11.26-14.74 months

n = 269
n = 63
n = 98

All patients in low, medium, and high AFB1 exposure groups are shown.
Survival time (months)

Overall survival

Recurrence-free survival

Log-rank test, $P = 1.68 \times 10^{-32}$

TACE(+); MST 34.00 months; 95%CI 32.50-35.50 months

TACE(-); MST 20.00 months; 95%CI 18.79-21.21 months

n = 597

n = 702

Overall survival

Recurrence-free survival

Log-rank test, $P = 3.64 \times 10^{-24}$

TACE(+); MST 25.00 months; 95%CI 22.81-27.19 months

TACE(-); MRT is not determined

n = 597

n = 702

Log-rank test, $P = 1.19 \times 10^{-3}$

TACE(+); MST 24.00 months; 95%CI 20.59-27.41 months

TACE(-); MST 19.00 months; 95%CI 17.81-20.19 months

n = 167

n = 180

Log-rank test, $P = 4.56 \times 10^{-4}$

TACE(+); MST 29.00 months; 95%CI 25.94-32.06 months

TACE(-); MST 18.00 months; 95%CI 14.48-21.52 months

n = 167

n = 180

Log-rank test, $P = 6.12 \times 10^{-38}$

TACE(+); MST 39.00 months; 95%CI 36.99-41.01 months

TACE(-); MST 22.00 months; 95%CI 20.09-23.91 months

n = 354

n = 435

Figure 3
Polymorphisms of a disintegrin and metalloproteinase with thrombospondin motifs 5 and Aflatoxin B1 related-hepatocellular carcinoma

Xiao-Ying Huang, Jin-Guang Yao, Bing-Chen Huang, et al.

Cancer Epidemiol Biomarkers Prev  Published OnlineFirst December 16, 2015.

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