A polymorphism at the miR-502 binding site in the 3'-untranslated region of the histone methyltransferase SET8 is associated with hepatocellular carcinoma outcome

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MicroRNAs (miRNAs) can bind to the 3'-untranslated regions (UTRs) of messenger RNAs, where they interfere with translation and thereby regulate cell differentiation, apoptosis and tumorigenesis. Genetic polymorphisms in the 3'-UTRs targeted by miRNAs alter the strength of miRNA binding in a manner that affects the behavior of individual miRNAs. The histone methyltransferase SET8 has been reported to methylate TP53 and regulate genomic stability. We analyzed a single-nucleotide polymorphism (rs16917496) within the miR-502 miRNA seed region for the 3'-UTR of SET8 in Chinese patients with hepatocellular carcinoma (HCC). The SET8 CC genotype was independently associated with longer postoperative survival in patients with HCC by multivariate analysis (relative risk, 0.175; 95% CI = 0.053–0.577; p = 0.004). The SET8 CC genotype was associated with reduced SET8 protein levels based on the immunostaining of 51 HCC tissue samples. We also found that the low SET8 levels were associated with longer HCC survival. Our data suggest that SET8 modifies HCC outcome by altering its expression, which depends, at least in part, on its binding affinity with miR-502. The analysis of genetic polymorphisms in miRNA binding sites can help to identify patient subgroups that are at high risk for poor disease outcomes.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and is responsible for more than half a million deaths each year, which makes it the third leading cause of cancer deaths worldwide.1 The severity of HCC and the lack of effective treatment strategies make the disease a major challenge faced by the cancer researchers. This disease is strongly associated with several risk factors, including chronic hepatitis B virus (HBV), chronic hepatitis C virus (HCV) and alcohol abuse.2 The incidence of HCC has increased steeply in Asia and Africa, where HBV and HCV are more prevalent than in other continents. HBV infection is a challenging health issue in China, where ~93 million people are HBV carriers and 30 million have chronic hepatitis B.2,3 Alcohol abuse is also increasing in China, where ~6.6% of males and 0.1% of females in the population have been diagnosed with alcohol dependence.4 Many of these people develop liver disease, such as alcoholic hepatitis and cirrhosis, which make them susceptible to HCC. Despite improved clinical detection methods and therapies, the prognosis of the patients with postoperative HCC is still poor because of a high recurrence rate. Although the molecular mechanism of HCC carcinogenesis is still not fully understood, there are many prognostic factors and predictors of recurrence associated with the disease, including tumor size, tumor quantity, cell differentiation, venous invasion and degree of inflammation.5–8

MicroRNAs (miRNAs) are RNA molecules with lengths of ~22 nucleotides that act as post-transcriptional regulators of mRNA expression by base pairing to the 3' untranslated region (UTR) or miRNAs and repressing translation.9,10 A growing body of evidence suggests that miRNA have important roles in a broad range of biological processes, such as embryonic development, cellular differentiation, proliferation, apoptosis, cancer development and insulin secretion.9,10 More than 700 miRNAs have been identified in humans, and these miRNAs are responsible for regulating the expression of at least 30% of protein-coding genes.11 Specifically, miRNAs target nucleotides 2–8 in the 5' end, which is known as the “seed region” of an mRNA's 3'-UTR. Perfect complementarity

Key words: HCC, SET8, microRNA, outcome, polymorphism

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between the miRNA and its target mRNA sequence results in reduced protein levels because of RNA silencing.\textsuperscript{12,13} There is increasing evidence suggesting that single-nucleotide polymorphisms (SNPs) in the 3'-UTR targeted by miRNAs alter a target genes' expression and thereby affect an individual's cancer risk.\textsuperscript{14,15} SNPs in miRNA binding sites were examined extensively in recent genotyping studies\textsuperscript{16–19}; however, few of these studies focused on SNPs in the miRNA binding sites and their association with cancer prognosis.

PR-Set7/SET8/KMT5a (SET8), which is regulated by miR-502 through the binding site in the 3'-UTR of SET8 mRNA, encodes a histone H4 lysine 20 monomethyltransferase that is implicated in normal cell cycle progression.\textsuperscript{20–22} Previous studies suggest that the SNP rs16917496, which is located within the SET8 miR-502 binding site in its 3'-UTR, modulates SET8 expression and, therefore, contributes to cancer risk and the early development of cancer.\textsuperscript{16,23} In our study, we genotyped this SNP in patients with HCC and normal controls to assess its relationships to cancer risk and outcome.

**Material and Methods**

**Tissue specimens and DNA extraction**

Blood samples were collected at the Fourth Hospital of Hebei University from 142 patients with HCC who underwent tumor resection in the Department of Hepatobiliary Surgery between 2003 and 2008. Blood samples were also collected from an equal number of age-matched healthy controls. Genomic DNA was extracted immediately with a Wizard Genomic DNA extraction kit (Promega, Madison, WI). All procedures were supervised and approved by the hospital’s Human Tissue Research Committee.

**PCR amplification and sequence analysis**

The forward and reverse primers, 5'-TCACGACGGTGC TACCTAAG-3' and 5'-CATGCTGGTG TGACACAGTC-3', respectively, were used to amplify DNA fragments flanking rs16917496 in the SET8 3'-UTR according to the NCBI database (http://www.ncbi.nlm.nih.gov/snp/). PCR was performed using a PCR Master Mix Kit according to the manufacturer’s instructions (Promega), and the PCR products were purified before sequencing. Cyclic sequencing was performed with a Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and the products were separated using an ABI PRISM Genetic Analyzer 3100 (Applied Biosystems). Polymorphisms were confirmed by repeating the analysis on both strands.

**Measurement of SET8 levels in hepatocarcinoma tissue**

SET8 immunochemistry was performed on hepatic tissue. The tissue sections were incubated with an anti-SET8 antibody (Abcam, Cambridge, UK) at a dilution of 1:100 overnight at 4°C followed by incubation with a biotinylated secondary anti-mouse IgG antibody for 1 hr at room temperature. The sections were subsequently incubated with HRP-conjugated streptavidin and developed using 3,3-diaminobenzidine.

The stained slides were scored by two investigators who were blinded to the sequencing results. The immunostaining results for all receptors were semiquantified using the HSCORE, as described previously.\textsuperscript{24,25} Briefly, we calculated the score based on the estimates of the percentage of positively stained hepatocyte cells in each of five intensity categories (0, 1+, 2+, 3+ and 4+). The HSCORE represents the sum of each of the percentages multiplied by the weighted intensity of staining as follows: HSCORE = (i + 1)\pi, where i = 1, 2, 3 and 4 and \pi varies from 0 to 100%. A score of >100% was defined as high expression and \leq 100 as low expression (Fig. 1).

**Statistical analysis**

Survival curves were calculated using the Kaplan–Meier method, and comparisons between the curves were made.
using the log-rank test. Multivariate survival analysis was performed using a Cox proportional hazards model. The distribution of expression grades for each SET8 genotype was compared using a $\chi^2$ test. All of the statistical analyses were performed using the SPSS 11.5 software package (SPSS Company, Chicago, IL). A $p$ value of $<0.05$ was considered statistically significant.

Results
Clinical characteristics of the patients with HBV-HCC
A total of 142 patients (116 with HBV-HCC, 15 with HCV-HCC and 11 with alcohol-related HCC) were enrolled in our study. A review of the patients was performed every 3 months for 3 years. Nine patients were lost during follow-up: one with HBV-HCC in the first year, one with HCV-HCC and four with HBV-HCC in the second year and one with HCV-HCC and two with HBV-HCC in the third year. The remaining 133 patients, including 109 patients with HBV-HCC, 13 with HCV-HCC and 11 with alcohol-related HCC, were assessed. None of these patients received adjuvant chemotherapy or radiation therapy following HCC resection. The relationship between the data collected during the 3-year follow-up and patients’ clinical characteristics was analyzed using the Kaplan–Meier method and the log-rank test. No differences were detected in overall survival time among patients with HBV-HCC, HCV-HCC and alcohol-related HCC; therefore, we assessed them together in further analyses. Sex and age were not statistically significant predictors of postoperative survival time; however, tumor size, tumor quantity, tumor stage, Child classification and portal vein thrombosis were correlated with survival time in these patients (Table 1). As expected, patients at different tumor stages had different 3-year survival rates, and rates of 41.12% for Stages I–II and 3.85% for Stages III were observed. The differences in survival time between these stages were determined to be significantly different ($p < 0.05$) using the log-rank test. Portal vein thrombosis and Child classification also showed significant association with overall survival time. Tumor size showed an association with survival time; however, tumor size, tumor quantity, tumor stage, Child classification and portal vein thrombosis were correlated with survival time in these patients (Table 1). As expected, patients at different tumor stages had different 3-year survival rates, and rates of 41.12% for Stages I–II and 3.85% for Stages III were observed. The differences in survival time between these stages were determined to be significantly different ($p < 0.05$) using the log-rank test. Portal vein thrombosis and Child classification also showed significant association with overall survival time. Tumor size showed an association with survival time; however, tumor size, tumor quantity, tumor stage, Child classification and portal vein thrombosis were correlated with survival time in these patients (Table 1). As expected, patients at different tumor stages had different 3-year survival rates, and rates of 41.12% for Stages I–II and 3.85% for Stages III were observed. The differences in survival time between these stages were determined to be significantly different ($p < 0.05$) using the log-rank test. Portal vein thrombosis and Child classification also showed significant association with overall survival time. Tumor size showed an association with survival time; however, tumor size, tumor quantity, tumor stage, Child classification and portal vein thrombosis were correlated with survival time in these patients (Table 1). As expected, patients at different tumor stages had different 3-year survival rates, and rates of 41.12% for Stages I–II and 3.85% for Stages III were observed. The differences in survival time between these stages were determined to be significantly different ($p < 0.05$) using the log-rank test. Portal vein thrombosis and Child classification also showed significant association with overall survival time.
longer survival time compared to that of patients with C/T and T/T alleles (CC vs. CT, \( p = 0.0054 \); CC vs. TT, \( p = 0.0107 \)). No difference exists between patients with C/T and T/T alleles referring to survival rate, and these data implied that the C allele seemed to affect survival time in a recessive manner (Fig. 2b).

We performed multivariate analysis with the Cox proportional hazards model for these predictive factors. Tumor stage, venous invasion, Child classification, tumor size and tumor quantity were divided into two groups as given in Table 1, and rs16917496 was divided into two groups as C/C versus C/T + T/T for analysis. As shown in Table 2, the rs16917496 SNP was identified as an independent predictor of HCC outcome (relative risk, \( 0.175 \); 95% CI \( 0.053–0.577 \); \( p = 0.004 \)). Tumor stage, venous invasion, Child classification and tumor size were also identified as independent predictive factors for HCC outcome. These data demonstrate the strong predictive power of the SET8 rs16917496 SNP on outcome in patients with HCC.

### SET8 expression on different rs16917496 genotypes and its correlation with HCC survival

To further evaluate the biological relevance of the rs16917496 SNP, we measured SET8 expression by immunostaining in all HCC tissues collected between 2007 and 2008 except for five HCC cases whose tissues were unavailable. Fifty-one cases were analyzed for SET8 staining, and the HSCORE was compared to Kaplan–Meier method subsequently; the patients with low levels of SET8 displayed longer survival length than that of the patients with high levels of SET8 (3-year survival rate, 52.94% vs. 29.41%, \( p = 0.0413 \)).

### Discussion

The SNP in the miR-502 binding site of the \( \text{SET8} \) 3’-UTR was examined for its predictive power relative to HCC outcomes. We showed that this SNP modulated \( \text{SET8} \) gene expression and that these changes in expression modified HCC survival time in a recessive manner. Our study is the first report for the role of a common SNP in \( \text{SET8} \) in the etiology of HCC. This SNP is particularly interesting because it is located in the \( \text{SET8} \) 3’-UTR seed region for miR-502 binding; the T to C transition of rs16917496 destroyed the G:C bond in miR-502 and \( \text{SET8} \) binding site so as to modulate \( \text{SET8} \) expression (Fig. 2a). Our data suggest that \( \text{SET8} \) modified HCC outcome by altering \( \text{SET8} \) expression, which depends, at least partly, on the binding affinity of miR-502. Consistent with the data of Song et al. that the CC genotype of \( \text{SET8} \) is associated with low expression at the RNA level, we show that the CC genotype is associated with low protein level and longer survival length of patients with HCC.

Univariate and multivariate analysis suggests that an SNP in the miRNA binding site of \( \text{SET8} \) is a prognostic factor in patients with HCC. This class of SNPs within the miRNA binding site, as well as polymorphisms in miRNAs themselves, are key factors in disease phenotypes.\(^{26,27}\) A small number of these SNPs were identified as a result of their associations with cancer risk and outcome.\(^{17,19,26,27}\)

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**Table 2. Multivariate analysis of prognostic factors associated with postoperational survival in patients with HCC with Cox proportional hazards model**

| Factors                      | Relative risk | 95% CI       | \( p \) value |
|------------------------------|---------------|--------------|---------------|
| Size of the tumor            | 2.237         | 1.339–3.737  | 0.002         |
| Tumor quantity               | 1.071         | 0.653–1.756  | 0.785         |
| TNM classification           | 3.064         | 1.751–5.361  | <0.001        |
| Child classification         | 0.267         | 0.124–0.576  | 0.001         |
| Portal vein thrombosis       | 1.706         | 1.057–2.752  | 0.029         |
| \( \text{SET8} \)            | 0.175         | 0.053–0.577  | 0.004         |

**Table 3. The distribution frequency of SET8 expressional levels for each genotype by \( \chi^2 \) test**

| Genotype | Low (number of cases) | High (number of cases) | \( p \)  |
|----------|-----------------------|------------------------|---------|
| C/C      | 5                     | 0                      | 0.002   |
| C/T      | 6                     | 13                     |         |
| T/T      | 6                     | 21                     |         |
As a methyltransferase, SET8 modulates p53 expression by specifically methylating at lysine 382 histones associated with the p53 genomic sequence. SET8 methylates lysine 382 in p53 and changes its transcriptional activity. Furthermore, depletion of SET8 augments the proapoptotic and checkpoint activation functions of p53, and SET8 expression is downregulated by DNA damage. The relationship between the methylation status of p53 in response to SET8 expression levels and HCC survival rates needs further study; however, SET8 knockdown has been shown to upregulate cells’ sensitivity to cell death and cell cycle arrest following DNA damage by suppressing the biological function of p53.

Using case–control association studies, Yu et al. found that 12 miRNA-binding site SNPs display an aberrant allelic frequency in human cancers. The SET8 miR-502-binding site SNP was identified as one of those 12 SNPs. Consequently, we compared the distribution frequency of this SNP in a case–control study, and we detected no statistical differences in the presence of the SET8 miR-502-binding site SNP; however, it is possible that this SNP may exhibit different effects in different kinds of tumors.

Although SNP studies in miRNA binding sites are at an early stage, our results are encouraging, as they indicate that miRNA allele SNPs have an effect on cancer risk and outcome. However, the results from our study require validation in other populations and in laboratory-based functional studies. miRNAs have been emphasized as a key factor in patients’ susceptibility to therapeutic response in many complex diseases, including cancer. In conclusion, an SNP in the SET8 miRNA binding site was found to be an independent prognostic marker for HCC outcomes. The analysis of genetic polymorphisms in miRNA binding sites may help to identify patient subgroups with poor prognoses and may, accordingly, help to refine therapeutic decisions regarding patients with HCC.

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