Liver Intestine-Cadherin (*CDH17*) Haplotype Is Associated with Increased Risk of Hepatocellular Carcinoma

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Abstract

Purpose: Hepatocellular carcinoma (HCC), the most common form of liver cancer, is a leading cause of cancer death worldwide. We previously showed that aberrant mRNA splicing of the liver intestine-cadherin gene *CDH17* in liver tissues was triggered by the specific constellation of two *CDH17* single nucleotide polymorphisms (651T and IVS6+35G). *CDH17* aberrant splicing was highly associated with tumor dissemination and shorter survival of HCC patients. Consequently, it is highly relevant to assess whether the presence of these single nucleotide polymorphisms in the general population represents a risk to the development of HCC.

Experimental Design: We conducted a case-control study including 164 HCC and 99 cirrhosis patients and 293 healthy controls. Genotyping was done by PCR and direct sequencing. Odds ratio (OR) and χ^2 analysis were used to analyze genotypes and haplotypes.

Results: Genotypes 651TT [OR, 2.62; 95% confidence interval (95% CI), 1.34-5.03] and IVS6+35 GG (OR, 1.95; 95% CI, 1.04-3.62) were highly associated with HCC disease. The 651T (C>T) and IVS6+35G (A>G) alleles were also overrepresented in HCC patients and, in particular, the T-G haplotype was the most prevalent in HCC patients when compared with healthy controls (OR, 1.57; 95% CI, 1.167-2.109; $P = 0.004$), which was in agreement with the aberrant splicing observed in tumor tissues. There was no significant difference in genotype and allele frequencies between cirrhosis patients and controls.

Conclusion: The functional T-G haplotype of *CDH17* (651 C>T and IVS6+35A>G) is a genetic susceptibility factor for the development of HCC in a Chinese population.

Hepatocellular carcinoma (HCC) is the most common liver malignancy, accounting for the third most common cause of cancer-related deaths worldwide and a leading cause of cancer death in Asia (1). It is well accepted that hepatitis B and C virus infections are the major risk factors of HCC. In this context, however, increasing evidence has revealed that genomic changes progressively alter the cellular phenotype to evolve from preneoplastic stage into HCC (2). Multiple gene alterations such as allelic deletion, mutation, and altered methylation are marked in HCC, resulting in heterogeneity in genetic and molecular aberrations (3). Studies also indicated, on the other hand, that aberrations in certain genes and molecular pathways are responsible for the certain clinical features of HCC (2). Thus, studies on combinations of aberrant genes and their regulatory pathways might provide implicative information of accurate molecular diagnosis and prognosis of HCC.

Single nucleotide polymorphisms (SNP) are the most common type of genomic sequence variations, which are thought to be associated with population diversity, susceptibility to diseases, and individual response to drug treatments (4). Many SNPs are silent, with no direct effect on the gene products but, by virtue of the linkage disequilibrium existing across the human genome, they can still be used as genetic markers to locate adjacent functional variants that contribute to the diseases. SNPs may also have functional consequences if they directly affect the coding or regulatory (usually promoter) regions of a gene. There have been cumulative studies on the associations between cancer risk and SNPs in selected candidate genes, and such information may shed light on the molecular and genetic basis of the polygenic nature of cancer.

Cadherins are a superfamily of transmembrane glycoproteins, which mediate calcium-dependent cell-cell adhesion (5). These adhesion molecules function in cell recognition, tissue morphogenesis, and tumor suppression. Abnormal expression of cadherins is often related to cancer proliferation, invasion, and metastasis; interestingly, it is often associated with dysregulated expression of cadherins in an aberrant spliced

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form. Alternative *E-cadherin* expression with exon 8 or exon 9 deletion, as well as frameshifted insertion, has been reported to be predominant in diffuse types of gastric carcinomas (6, 7). Another example is that wild-type cadherin-11 expression promotes epithelial differentiation of SKBR3 cell line, whereas the splice variant form of cadherin-11 enhances the invasive capacity of the cells (8).

We have recently shown for the first time that liver intestine-cadherin, a nonclassic member of the cadherin family, is over and aberrantly expressed in HCC patients (9). In addition, alternative splicing of liver intestine-cadherin was identified in 50% of HCC, which was strongly associated with poor prognosis such as venous infiltration and recurrences of liver tumor and, consequently, shorter overall survival (10). Moreover, our data indicated that aberrant expression of liver intestine-cadherin might play a role in HCC tumor pathogenesis and was related to the specific constellation of two liver intestine-cadherin gene (*CDH17*) SNPs alleles. Thus, we hypothesized that if these SNPs are implicated in the aberrant splicing observed in HCC patients, their frequency in those patients would be higher than that in healthy individuals. To investigate this possibility, we have analyzed 164 HCC and 99 cirrhosis patients, as well as 293 normal controls, for the *CDH17* SNPs. 651T, IVS6+35G allele and their associated genotype 651TT, and IVS6+35GG frequencies were overrepresented in HCC patients. There were no significant differences in genotype and allele frequencies between the cirrhosis and control groups, but increased allele frequency showed a significant trend toward the HCC group. Moreover, the combined T-G allele was highly prevalent in HCC patients, which was in support of our previous T-G minigene functional analysis (10) that the T-G haplotype altered the mRNA splicing of liver intestine-cadherin. Therefore, in this study, we present evidence that the presence of certain combination of SNP alleles may confer susceptibility to HCC. Of this study, we noticed that the sample sizes of HCC and cirrhosis patients were relatively small, and the preliminary results need further validation in a larger scale of investigation.

Materials and Methods

Subjects and samples. This study included 164 HCC patients and 99 cirrhosis patients in Queen Mary Hospital, Hong Kong. All HCC patients were diagnosed based on pathologic analyses and classified by the new tumor-node-metastasis staging system (11). Liver cirrhosis was diagnosed by either radiological evidence of cirrhosis on ultrasonography/computed tomography or histologically by liver biopsy (12). For the normal healthy control group, blood samples from 293 healthy individuals were obtained from the blood bank of the Hong Kong Red Cross. General information and clinical features of cases and controls are listed in Table 1. Genomic DNA was extracted from the peripheral blood leukocytes using the QIAamp blood kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. This research protocol was approved by the Institutional Review Board (UW 05-013 T/676).

Genotyping of CDH17 SNPs. The C/T polymorphism at nucleotide 651 in exon 6 (rs 3214050) and A/G polymorphism at IVS+35 in intron 6 (rs 2514813) were genotyped by PCR and sequencing analysis. A 750-bp fragment of *CDH17* spanning from exon 6 through intron 6 to exon 7 was generated with the primers as described before (10). The PCR reaction was carried out in a buffer containing 100 to 200 ng genomic DNA, 1× Taq buffer, 2 mmol/L $MgCl_2$, 100 μmol/L 4× deoxynucleotide triphosphate, 0.2 μmol/L each primer, and 1 unit of Platinum Taq polymerase (Invitrogen, Carlsbad, CA). The cycling conditions were 5 minutes at 94°C; followed by 30 cycles of 94°C for 40 seconds, 55°C for 40 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. The resulting PCR fragments were then subjected to DNA sequencing analysis by an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA) using the BigDye Terminator (v3.1) Cycle Sequencing Kit. The polymorphic alleles were further confirmed by sequencing with two direction primers.

Statistical analysis. SNP data were organized using Genotype Transposer (13). To observe deviation from Hardy-Weinberg equilibrium, observed and expected genotype frequencies were compared by χ^2 test. Comparison of genotype and allele frequencies between the groups was assessed by the odds ratio (OR) with 95% confidence interval (95% CI), which were calculated by unconditional logistic regression models and adjusted by age and gender using SAS 6.12 (SAS Institute, Cary, NC). The association between alleles and genotypes of *CDH17* with HCC and cirrhosis was made using healthy controls as comparison group.

The program PHASE v.2, which allows for recombination and decay of linkage disequilibrium with distance, was used for computational reconstruction of most likely haplotype pairs for each individual and for estimation of the haplotype frequencies in each group and case-control global statistics (14). The frequencies of each haplotype were compared between the cases and the controls using OR and χ^2 analysis. Clinical data analysis was done using the statistical analysis software SPSS for Windows (version 11, SPSS, Chicago, IL).

Results

The CDH17 651C/T and intronic (IVS6+35A/G) polymorphisms are associated with HCC. Our earlier observations have found two *CDH17* SNPs (651C/T and IVS6+35A/G) in the

Table 1. General information of controls, cirrhosis, and HCC groups

	Controls (*n* = 293)	Cirrhosis (*n* = 99)	HCC (*n* = 164)
Mean age ± SD (y)	47.31 ± 16.20	51.49 ± 12.32	54.92 ± 12.00
Gender			
Male (%)	54.90	73.74	82.93
Female (%)	45.10	26.26	17.07
HBsAg positive (%)		83.5	83.8
TNM stage I and II (%)			70.12
TNM stage III and IV (%)			29.88

Abbreviations: HBsAg, hepatitis B surface antigen; TNM, tumor-node-metastasis.

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Table 2. Frequency of *CDH17* alleles and genotypes in controls, cirrhosis, and HCC groups

Loci	Controls (n = 293)	Control vs cirrhosis (n = 99)			Control vs HCC (n = 164)		
	No (%)	No (%)	Adjusted OR* (95% CI)	P	No (%)	Adjusted OR* (95% CI)	P
651							
Allele							
C	422 (72.01)	136 (68.69)	1.00		201 (61.28)	1.00	
T	164 (27.99)	62 (31.31)	1.35 (0.94-2.01)	0.104	127 (38.72)	1.78 (1.32-2.43)	0.001†
Genotype							
CC	158 (53.92)	49 (49.50)	1.00		67 (40.85)	1.00	
CT	106 (36.18)	38 (38.38)	1.37 (0.82-2.34)	0.241	67 (40.85)	1.69 (1.05-2.81)	0.031†
TT	29 (9.89)	12 (12.12)	1.67 (0.72-3.58)	0.201	30 (18.29)	2.62 (1.34-5.03)	0.004†
IVS6+35							
Allele							
A	395 (67.41)	128 (64.65)	1.00		194 (59.15)	1.00	
G	191 (32.59)	70 (35.35)	1.31 (0.89-1.82)	0.194	134 (40.85)	1.47 (1.07-2.05)	0.019†
Genotype							
AA	140 (47.78)	44 (44.45)	1.00		63 (38.41)	1.00	
AG	115 (39.25)	40 (40.40)	1.23 (0.71-2.14)	0.518	68 (41.46)	1.40 (0.87-2.20)	0.179
GG	38 (12.97)	15 (15.15)	1.60 (0.73-3.41)	0.199	33 (20.12)	1.95 (1.04-3.62)	0.041†

*Adjusted by gender and age.
†P < 0.05 was considered significant.

transcripts of HCC specimens. To determine any allelic imbalance of *CDH17* in HCC, we analyzed the two *CDH17* SNPs in the peripheral blood leukocytes of 164 HCC patients, 99 cirrhosis patients, and 293 control cases. The genotype distributions for 651C/T and IVS6+35A/G in both controls and patients were under Hardy-Weinberg equilibrium. The frequency distribution of the alleles and genotypes for the two polymorphisms is presented in Table 2.

An overrepresentation of T allele at 651 was found in the HCC group compared with the control group (38.7% versus 27.9%). There was a significant risk for HCC associated to T-allele (OR, 1.78; 95% CI, 1.32-2.43) with P value at 0.001. The frequency of T allele–containing genotypes (TT and CT) was higher in HCC patients (59%) compared with normal controls (46%; P = 0.003). Both TT homozygotes (OR, 2.62; 95% CI, 1.34-5.03; P = 0.004) and CT heterozygotes (OR, 1.69; 95% CI, 1.05-2.81; P = 0.031) represented a risk to HCC disease, suggesting a possible dominant effect of this polymorphism on HCC. At IVS6+35, the G allele showed higher frequency in HCC patients than in normal controls (OR, 1.47; 95% CI, 1.07-2.05; P = 0.019). As for the G allele–containing genotypes, only the GG genotype was significantly higher in the HCC group than in the control group (OR, 1.95; 95% CI, 1.04-3.62; P = 0.041; Table 2).

Association of these two SNPs with cirrhosis patients was also examined. No significant differences in genotype or allele frequency were observed between the cirrhosis and control groups (Table 2). However, both frequencies of the variant 651T and IVS6+35G significantly increased from control, cirrhosis toward HCC, with 27.9%, 31.31%, and 38.72% in 651T (trend test, P = 0.001) and 33.14%, 35.35%, and 40.85% (trend test, P = 0.024) in IVS6+35G, respectively.

Functional T-G haplotype is prevalent in HCC. Our previous investigation showed that alternative splicing of liver intestine-cadherin with exon 7 skipping was frequently observed in HCC patients. As a result, it was suggested that the 651C/T and IVS6+35A/G SNPs would be responsible for the *CDH17* alternative splicing. This observation, together with the over-representations of the allele 651T and IVS6+35G in HCC patients, prompted us to investigate a possible role for the

Table 3. Haplotype pairs distribution of *CDH17* 651C/T and IVS6+35A/G in healthy controls and HCC patients

Haplotype/haplotype	Controls (n = 258)	HCC (n = 164)	χ^2 (Yates)	P (one sided)
	n (%)	n (%)		
C-A/C-A	121 (46.90)	63 (38.41)	2.93	0.087
C-A/C-G	18 (6.98)	4 (2.44)	4.18	0.041
C-A/T-G	85 (32.94)	64 (39.02)	1.62	0.203
C-G/C-G	0 (0)	0 (0)	—	—
C-G/T-G	9 (3.49)	3 (1.83)	2.50	0.114
T-G/T-G	25 (9.69)	30 (18.30)	6.55	0.010*

NOTE: Global distribution χ^2 = 13.32; 4 degrees of freedom; P = 0.0097.
*P < 0.05 was considered significant.

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Table 4. Haplotype frequency of *CDH17* 651C/T and IVS6+35A/G in healthy controls and HCC patients

Haplotype	Controls (n = 258)	HCC (n = 164), %	OR (95% CI)	P (Yates)
	n (%)	n (%)		
C-A	345 (67)	194 (59)	1	
C-G	27 (5)	7 (2)	0.46 (0.202-1.056)	1.101
T-G	144 (28)	127 (39)	1.57 (1.167-2.109)	0.004*

*P < 0.05 was considered significant.

haplotype of these SNPs in altering the liver intestine-cadherin mRNA splicing.

First, the linkage disequilibrium analysis showed that the allele 651T and IVS6+35G are closely linked, which was expected given their physical proximity. The distribution of the haplotype pairs comprising these two SNPs in the samples tested is listed in Table 3. The C-A/C-A, C-A/T-G, and T-G/T-G pairs were the most frequent combinations in descending order among both the HCC patients and control subjects. The C-G allele combination was very rare and there was no T-A either in patients or in the controls. Haplotype pairs were compared using the χ^2 analysis and the results indicated that the T-G/T-G pair was overrepresented in HCC patients compared with that of the controls (18.30% versus 9.69%; P = 0.01).

The reconstructed haplotype T-G was found highly prevalent in HCC patients than in normal controls (OR, 1.57; 95% CI, 1.167-2.109; P = 0.004; Table 4). In agreement with the observation that the T-G haplotype could be a pathogenic haplotype, in fact, T-G haplotype but not C-A haplotype minigene has been shown to promote the alternative splicing of liver intestine-cadherin with exon 7 skipping (10). We newly observed that the T-G haplotype minigene also resulted in both exon 6 and exon 7 skipping (data not shown), which was also identified in some of the HCC cases (Fig. 1, patients 3 and 4). The data suggested that the T-G haplotype, rather than the C-A haplotype, strongly promoted the alternative splicing. With regard to the relationship between the HCC patients with aberrant liver intestine-cadherin splicing and their polymorphisms, of 22 matched patients with alternative splicing in tumor mRNA, 16 (72.7%) patients had T-G haplotype whereas 6 (27.3%) patients were of C-A haplotype (P < 0.001). Therefore, it is conceivable that T-G haplotype was not only associated with HCC cancer risk but it also exerted an influence on the alternative splicing of liver intestine-cadherin.

Discussion

Overexpression of liver intestine-cadherin has been found in HCC (9, 10), gastric adenocarcinoma (15–17), and pancreatic cancers (18), although its pathogenesis roles in cancer are yet to be understood. *CDH17* mRNA is alternatively spliced to produce at least two variant transcripts, one of which was with exon 7 skipping and another with both exon 6 and exon 7 skipped (Fig. 1). The consequences of both splicing patterns would introduce premature translational stop codon in the reading frame, thereby producing either nonfunctional protein or potentially dominant negative protein. The alternative splicing of liver intestine-cadherin with exon 7 skipping occurred in 50% of HCC patients, which was clinically associated with shorter overall survival as well as tumor recurrences (10). The splicing of *CDH17* seemed to be mediated by C/T at 651 and A/G at IVS6+35 polymorphisms. We therefore investigated the frequency distribution of 651C/T and IVS6+35A/G in HCC and normal control cases and their associations with HCC.

Our study detected overrepresentations of the *CDH17* 651T allele (OR, 1.78; 95% CI, 1.32-2.43) and IVS+35G allele (OR, 1.47; 95% CI, 1.07-2.05) in HCC patients compared with healthy controls, although more significant association was detected with 651T (P = 0.001) than with IVS+35G (P = 0.019; Table 2). This suggests that *CDH17* 651T and IVS6+35G may represent the susceptibility alleles with relatively low penetrance. The 651T allele–containing genotypes were higher in HCC; especially the 651TT and IVS+35GG homozygotes seemed to be strongly associated with HCC. The present study, however, could not value the genetic risk of polymorphisms associated with the HBV-infectious factor, which has a high rate in HCC patients (Table 1), because the controls excluded HBV-positive cases from the blood bank. We also noticed that the sample size of patients was relatively small; thus, the positive results might need to be further proven in a larger scale.

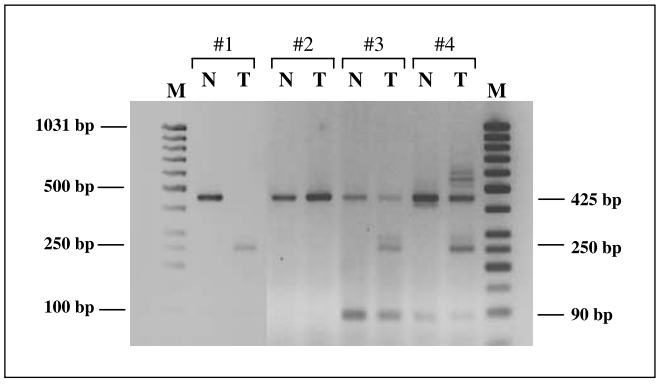

Fig. 1. Alternative mRNA splicing of liver intestine-cadherin in HCC tissues. Reverse transcription-PCR (10) on mRNA from HCC tissue (*T*) and peritumor tissue (*N*) showing a full-length fragment of 425 bp and two shorter fragments: 250 bp was altered transcript with exon 7 skipped and 90 bp was the fragment with both exon 6 and exon 7 skipped. Patient case number was indicated by asterisk. The *CDH17* genotypes of the above four cases were #1, 651C/T, IVS6+35A/G; #2, C/C, A/A; #3, T/T, G/G; and #4, C/T, A/G, respectively. M, marker. The molecular size is indicated on the left.

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It is estimated that 80% of the HCC cases develop in cirrhotic livers, thereby cirrhosis is considered to be the strongest predisposing factor (1). The mortality of HCC is also increasing in patients with compensated cirrhosis (19). Although allelic deletions in cirrhosis and chronic hepatitis are much lower than in either dysplastic hepatocytes or HCC (2), the linkage from the genetic evidence of these two diseases is not much known. The present data showed a significant increasing trend of 651T and IVS6+35G alleles from control, cirrhosis toward HCC ($P < 0.05$), which indicated that *CDH17* might play an important role in the initiation of HCC.

According to the haplotype reconstruction data (Table 3), the C-A and T-G haplotypes accounted for most allele combinations, whereas the combinations C-G and T-A were rare or absent, respectively. In this connection, the minigene *CDH17* constructs had covered all the possible haplotypes in the patients and controls. Thus, the haplotype T-G combination is more likely to promote the alternative splicing in a synergistic manner, especially in the case when both exon 6 and exon 7 were skipped. More importantly, haplotype reconstruction revealed that the HCC group had a statistically significantly higher T-G haplotype frequency than the normal control group, indicating that the T-G haplotype might represent a pathogenic combination to function on aberrant splicing of liver intestine-cadherin. Clinical relationship further proved that HCC patients with aberrant splicing had a significantly higher T-G haplotype of *CDH17*.

In conclusion, the *CDH17* T-G haplotype was overrepresented in HCC patients, which also potentially modified the splicing patterns of liver intestine-cadherin. This provides the first evidence that the functional T-G haplotype of *CDH17* (651 C>T and IVS6+35A>G) is a genetic susceptibility factor for the development of HCC in a Chinese population. These results may support the hypothesis that *CDH17* plays an important role in the carcinogenesis of HCC.

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