

# Alternative mRNA Splicing of Liver Intestine-Cadherin in Hepatocellular Carcinoma

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## ABSTRACT

**Purpose:** To identify alternative splicing of the liver intestine-cadherin (LI-cadherin) gene in hepatocellular carcinoma (HCC) and correlate its aberrant expression with clinical outcomes.

**Experimental Design:** Reverse transcription-PCR (RT-PCR) and quantitative real-time RT-PCR were used to examine alternative mRNA splicing and mRNA level of LI-cadherin in 50 paired tumor-peritumor tissues of 50 HCC and 8 normal liver specimens. The minigene exon-trapping strategy was employed to investigate the splicing mechanism introduced by nucleotide polymorphisms. Association of LI-cadherin splicing with tumor venous infiltration, first-year tumor recurrence, and overall survival after partial hepatectomy were determined.

**Results:** Alternative mRNA splicing of LI-cadherin was identified in half of the HCC specimens. Sequencing analysis indicated the loss of exon 7 in the spliced LI-cadherin gene. LI-cadherin mRNA was up-regulated from 2.58-fold to 800-fold in over 80% of HCC samples when compared with normal liver by quantitative PCR. Furthermore, nucleotide polymorphisms were identified in putative branch point at IVS6 + 35 (intron 6) as well as in coding sequence 651 (exon 6) in HCC tissues, which may affect alternative mRNA splicing. Clinically, those patients who harbored the alternative splicing of LI-cadherin were strongly associated with shorter overall survival time ( $P < 0.01$ ) as well as higher incidences of tumor recurrences and venous infiltration (both  $P < 0.05$ ) after hepatectomy.

**Conclusions:** Over-expression of LI-cadherin was frequently detected in liver cancer patients. Aberrant

alternative splicing of LI-cadherin was detected in 50% of HCC specimens and its clinical significance hinted at early tumor recurrence and poor overall survival of HCC patients.

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common liver malignancy and accounts for the third most common cause of cancer-related death worldwide (1). Apart from hepatectomy and liver transplantation, a variety of therapeutic interventions such as systemic and localized chemotherapy, experimental gene therapy, and new drug discovery are thought to be challenges for future HCC treatments. Not only hepatitis B virus and hepatitis C virus infections are involved in the molecular pathogenesis of HCC, many more studies have shown that multiple genetic alternations are associated with HCC. Thus, investigations on gene expression profiling, and subsequently, functional analysis will be important for better understanding of the pathogenesis of HCC, thereby providing molecular markers for early and accurate diagnosis, disease stage classification, and predicting the outcome of treatments of HCC.

Cadherins are a superfamily of transmembrane glycoproteins which mediate calcium-dependent cell adhesion in a variety of tissues (2). Many cadherins were found to be associated with cancers; for example, the epithelial E-cadherin, a putative tumor suppressor, is deregulated in gastric carcinoma (3–7), HCC (8), and breast carcinoma (9). By contrast, increased expression of other cadherins such as N-cadherin, P-cadherin, and cadherin-11 (OB-cadherin) are clinically correlated with breast carcinomas, colon, and gastric cancers (10, 11). Abnormal expression of certain cadherins is related to cancer proliferation, invasion, and metastasis. Interestingly, the malignancy is often associated with dysregulated expression of cadherin in an aberrant spliced form. Altered E-cadherin expression with exon 8 or exon 9 deletion is predominant in a diffuse type of gastric carcinoma (3) as well as frame-shifted transcript with insertion in gastric carcinoma cell lines (4). Cadherin-11 expression has been associated with an aggressive phenotype of breast carcinomas. Its wild-type expression promotes epithelial differentiation of the SKBR3 cell line, whereas its splice variant form enhances the invasive capacity of the cells (11).

Recently, liver intestine-cadherin (LI-cadherin), a non-classical member of the cadherin family, has been reported to be over-expressed in HCC (12), as well as in gastric (13, 14), and pancreatic cancer (15). However, little is known about its pathogenic role in carcinogenesis. In this study, we have identified and characterized the alternative mRNA splicing with exon 7 skipping of LI-cadherin in 50 HCC and 8 normal liver specimens. Over-expression and aberrant expression of LI-cadherin indicated a poor prognostic outcome of HCC patients.

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## MATERIALS AND METHOD

### Cell Lines and Clinical Specimens

HCC cell lines PLC/RAF/5, HepG2, Hep3B, HuH7, gastric adenocarcinoma cell line AGS, and kidney cell line COS-7 were all cultured in DMEM (Life Technologies, Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Life Technologies) at 37°C in 5% CO<sub>2</sub>, as previously reported (12). Clinical samples were collected according to protocols approved by the Ethics Committee of the University of Hong Kong. A total of 50 HCC tissues were collected immediately after hepatectomy from patients ages from 13 to 79 and diagnosed to have stage II to IVA disease by pathologic tumor-node-metastasis classification (16). Paired adjacent nontumorous tissues were also collected when available. Normal liver samples were obtained from liver transplant donors.

### Reverse transcription-PCR

RNA was isolated from frozen liver tumor tissues or HCC cell lines using RNeasy RNA isolation kit (Qiagen, Valencia, CA) following the manufacturer's instruction. Two micrograms of total RNA were used to synthesize first-strand cDNA with 0.5 µg of 15-mer oligo dT and reverse transcriptase (Promega, Madison, WI). The reverse transcription was first heated to 70°C for 10 minutes and iced for 5 minutes immediately afterward. After adding 200 units of reverse transcriptase, the reaction was carried out at 39°C for 1 hour. LI-cadherin transcripts were amplified with primers spanning exons 5 to 8 (forward 5'-ACAATC-GACCCACGTTTCTC-3', reverse 5'-ATATTGTGCACCGG-GATCAT-3'). PCR reaction was done in 40 cycles at 94°C for 40 seconds, 55°C for 40 seconds, and 72°C for 1 minute. The amplified products were run through 2% agarose gels with ethidium bromide and visualized with UV transillumination. Reverse transcription-PCR (RT-PCR) products were cloned into pCR2.1-TOPO vector using PCR cloning kit (Invitrogen) for further DNA sequencing analysis by an ABI 3100 genetics analyzer (Applied Biosystems, Foster City, CA) using the BigDye Terminator (v3.1) Cycle Sequencing Kit with T7 and M13 primers as described previously (17).

### Quantitative Real-Time RT-PCR

Total RNA was extracted as described above and quantitative real-time PCR for LI-cadherin mRNA as previously reported (12). Briefly, an equal amount of RNA treated with DNase I (Invitrogen) was used for reverse transcription using Random Hexamers and MutiScribe Reverse Transcriptase (Applied Biosystems). Two microliters of cDNA were subjected to AmpliTaq Gold DNA polymerase in 1× PCR buffer (25 µL) containing 200 µmol/L deoxynucleotide triphosphates, 1.5 mmol magnesium chloride, 0.5 µmol/L of primers (see above), and 200 nmol/L TaqMan probe (5'-CTCAGTAAGG CAGA-ACTCTC GCCCAGGA-3'). The thermal cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All the reactions were done at least in duplicate and analyzed using ABI Prism 7700 detection system (Applied Biosystems). The relative amount of LI-cadherin mRNA, normalized to an internal control ribosomal 18s and relative to a calibrator (normal liver sample), was calculated with the formula below:

$$\Delta\Delta C_T = (C_{T,LI-cadherin} - C_{T,r18s})_{HCCsample} - (C_{T,LI-cadherin} - C_{T,r18s})_{normalsample}$$

### Polymorphism Analysis

Genomic DNA was isolated from the liver tissues using the phenol extraction assay. Genomic DNA of LI-cadherin was amplified with forward primer 5'-TGGTTGGTTT-GGGTTTCAAT-3', and reverse primer 5'-CCATGATG-CATGGTCAAGTC-3' for 30 cycles at 94°C for 40 seconds, 55°C for 40 seconds, and 72°C for 1 minute. The resulting PCR products covered the entire length of exon 6, exon 7, and their intron/exon boundaries, were subjected to DNA sequencing analysis.

### pSPL3-LI-Cadherin Minigene Construction

A 750-bp sequence of LI-cadherin genomic DNA from intron 5 (-64 exon 6) through exon 7 to intron 8 was amplified from normal and HCC liver with respect to the IVS6 + 35A or IVS6 + 35G. The resulting PCR fragments were cloned into pCR 2.1-TOPO (Invitrogen). After sequencing verification, the DNA IVS6 + 35AA and IVS6 + 35GG fragments (IVS6 + 35A > G) were subcloned into the pSPL3 (kindly provided by Dr. Goodship, Newcastle University, United Kingdom), an exon-trapping vector through *Xho*I and *Bam*HI sites (18).

### Transfection and RT-PCR

The above two minigene constructs were then transiently transfected into Cos-7 cells using FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN). Twenty-four hours after transfection, total RNA was isolated from the cells using RNeasy RNA isolation kit (Qiagen). cDNA was generated using primer SA2 5'-ATCTCAGTGGTATTGTG-GAGC-3'. Thirty cycles of PCR was done using one vector primer SA4 (5'-CACCTGAGGAGTGAATTGGTTCG-3') in combination with LI-cadherin exon 6 primer (5'-ATGCCACA-GACCTGGATGAT-3'). The splicing products were confirmed by cloning into pCR2.1-TOPO and oligonucleotide sequencing as described above.

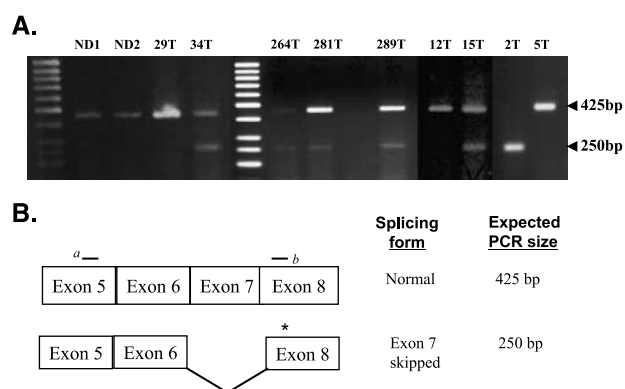
### Statistical Analysis

Statistical Package for the Social Sciences software v11.0 for Windows (SPSS, Inc., Chicago, IL) was used for statistical analysis. The association between alternative splicing variant of LI-cadherin and overall survival of HCC patients was analyzed using the Kaplan-Meier log-rank method. The Student's *t* test or  $\chi^2$  test was used to analyze the association between LI-cadherin splicing and clinical parameters (age, sex, tumor-node-metastasis stage, venous infiltration, and tumor recurrence) of the patients. All statistical tests were two-sided, and  $P < 0.05$  was regarded as statistically significant.

## RESULTS

### Altered LI-Cadherin mRNA in HCC Tissue

HCC, adjacent nontumorous (peritumor) tissues, and normal liver specimens were analyzed by RT-PCR with primers spanning exons 5 to 8 of LI-cadherin. The PCR products revealed normal size (450 bp) fragments and smaller (250 bp) fragments (Fig. 1A). Of the 50 HCC samples examined, 46



**Fig. 1** Alternative splicing of LI-cadherin in HCC. **A**, RT-PCR with primers spanning exons 5 to 8 on mRNA from HCC and normal tissue showing a full-length fragment of 425 bp and shorter fragment of 250 bp. ND1 and ND2 represented samples from healthy donor liver and 2T, 5T, 12T, 15T, 29T, 34T, 264T, 281T, and 289T represented samples from HCC tissues. **B**, Schematic diagram of alternative splicing of LI-cadherin between exon 5 and exon 8 identified in HCC tumor tissues. PCR products from (A) were subjected to DNA sequencing and revealed an out-of-frame skip of exon 7 in alternative spliced fragment. Lines, locations of forward (a, 482 bp) and reverse (b, 933 bp) primers. \*, premature stop codon found in exon 8.

(92%) yielded a normal size of RT-PCR fragment (450 bp), whereas 25 (50%) showed a shorter RT-PCR product of 250 bp in length. Among them 42% had both the normal and shorter PCR products, and 8% had only a 250-bp fragment (Table 1). For the 36 peritumor tissues tested, the short fragment was also observed in 11 (30%) samples, whereas the normal size was detected in all 36 cases. As a control, RT-PCR was carried out on total RNA from normal liver samples of healthy donors, and only normal size PCR products were seen in all of the eight samples (Fig. 1A, Table 1). Sequence analysis was then conducted to determine the composition of the altered RT-PCR product. As depicted in Fig. 1B, the 450-bp PCR product was the expected sequence of exon 6, exon 7, and part of exon 8 of LI-cadherin, whereas the 250-bp fragment was an alternatively spliced transcript with the exon 7 skipped. Loss of exon 7 disrupted the reading frame that introduced a premature stop codon within exon 8. The data suggested that there was a high frequency (50%) of alternative splicing of LI-cadherin resulting in exon 7-skipping transcript in HCC liver than in peritumor tissues (30%) and normal liver (0%). It is not clear, at this stage, how alternative transcripts of LI-cadherin are correlated with translated proteins, although multiple isoforms of LI-cadherin proteins were observed in the HCC samples.<sup>3</sup> These truncated proteins might have a potential role in the pathogenesis of HCC.

### Over-Expression of LI-Cadherin in HCC

Our previous study showed the over-expression of LI-cadherin protein in 72.2% of HCC tissues by Western blotting and immunohistochemistry (12). In this article, the transcript level of LI-cadherin was investigated through real-time quanti-

tative PCR in HCC samples calibrated by LI-cadherin expression of normal liver (Fig. 2). A total of 18 independent HCC samples and 3 normal liver tissues were tested. The relative ratio of LI-cadherin mRNA in HCC was normalized by normal samples. It was observed that LI-cadherin mRNA up-regulated from 2.58-fold to 800-fold in 15 out of 18 HCC samples, and 4 samples reached > 50-fold to 800-fold higher than the normal. For peritumor tissues, the average fold ratio was 2.105. In accord with our previous findings, the data indicated that expression of LI-cadherin was highly up-regulated at the mRNA level in tumorous tissues of HCC patients.

### Polymorphisms at 651 and Putative Branch Site of Intron 6 of LI-Cadherin Gene

RNA splicing involves excision of introns from the primary mRNA transcripts and ligation of exons to produce mature mRNA for translating into proteins. Accurate splicing requires recognition of *cis*-acting consensus sequences including intron-exon boundaries, the branch site and auxiliary elements such as intronic or exonic splicing enhancers or silencers (19, 20). To identify the cause of exon 7 skipping, we carried out genomic sequencing analysis of exon 6, exon 7, and the adjacent intronic sequence. We designed two primers at intron 5 (-64 of exon 6) and intron 7 + 177 to amplify genomic DNA from tumor liver by PCR. The PCR product(s) would cover the boundaries of intron 5 and exon 6, the whole of intron 6, and the boundary of exon 7 and intron 7. The PCR fragment was subcloned into pCR2.1-TOPO vector, followed by sequence analysis. No mutation was found at the 5' or 3' splicing sites. However, at intron 6 + 35, we identified a GG or AG single nucleotide polymorphism (Fig. 3A). Intron 6 is a small intron. There seems no classic polyT tract, instead, several rich C sequences were found close to the 3' splice site. Upstream of the rich Cs, we found that an adenine base and its surrounded consensus sequences (TCTCGAT) could be a putative lariat branch point (YNYTRAY) region (20), although it was not a perfect match (Fig. 3B). At this adenine (IVS6 + 35), GG and AG polymorphisms were identified in 16 out of 22 HCC samples (Table 2). In the same patients, TT and CT polymorphisms were also observed at 651 (Table 2). The samples were either normal or harboring polymorphisms at both sites: 651 (exon 6) and IVS6 + 35. In the control group, genomic DNA from normal livers showed "wild-type" genotype (651 CC, IVS6 + 35 AA) at the above variation sites (Table 2).

### T Allele at 651 and G Allele at IVS6 + 35 Allele G Results in Exon 7 Skipping

To confirm the impact above two polymorphisms on splicing efficiency, we constructed a wild-type allele (651 C,

**Table 1** Frequency of alternative splicing of LI-cadherin in HCC, peritumor, and normal liver tissues by RT-PCR

Liver tissues	No.	PCR Product sizes (% distribution)*		
		450 bp only	450 + 250 bp	250 bp only
Healthy donor	8	8 (100%)	0 (0%)	0 (0%)
Peritumor tissue	36	25 (70%)	11 (30%)	0 (0%)
HCC	50	25 (50%)	21 (42%)	4 (8%)

\*The expected normal fragment of LI-cadherin PCR product is 450 bp. Alternative splicing of LI-cadherin results in a shorter fragment of 250 bp.

<sup>3</sup> X.Q. Wang, B.W. Wong, and J.M. Luk, unpublished observations.

Table 2 LI-cadherin gene polymorphisms in HCC and normal liver genomic DNA

Genotypes	HCC liver, n = 22 (%)	Normal liver, n = 5 (%)
651 (Exon 6) CC IVS6 (+ 35) AA	6 (27%)	5 (100%)
651 (Exon 6) TT IVS6 + 35 GG	5 (23%)	0
651 (Exon 6) CT IVS6 + 35 AG	11 (50%)	0

IVS6 + 35 A) or a “mutant” allele (651 T, IVS6 + 35 G) minigenes into pSPL3, an exon-trapping vector (Fig. 4A). The constructs were transfected into Cos-7 cells for analyzing splicing events *in vivo*. The 651 C and IVS6 + 35 A minigene produced a band that reflected inclusion of exon 6 and exon 7 (450 bp), whereas the 651 T and IVS6 + 35 G minigene generated two products, one was of the correct size, and another (200 bp) with exon 7 skipped (Fig. 4B). Both splicing fragments were verified by DNA sequencing. These data strongly suggested that skipping of exon 7 in an alternative transcript resulted from 651 T and putative branch point IVS6 + 35 G polymorphisms. It was noteworthy that the “mutated” minigene still generated a normal spliced product, as 5' or 3' splice sites were functional. Moreover, these two polymorphisms were not strong enough to abolish the normal splicing pattern and only promoted alternative splicing.

#### Alternative Splicing in HCC Cell Lines

We tested alternative splicing of LI-cadherin in PLC/PRF/5, Hep3B, HepG2, and Huh7, four HCC cell lines obtained by the same method. Altered transcript with exon 7 skipping was shown in PLC/PRF/5 cell line (Fig. 5) and codon 651 TT, IVS6 + 35 GG polymorphism in genomic DNA (data not shown). Alternative splicing was absent in the other three HCC cell lines. The gastric adenocarcinoma AGS cell line also did not show any alternative splicing of LI-cadherin. In addition, our previous study did not

find altered RT-PCR products of LI-cadherin in gastric tumor samples (data not shown). Alternative splicing of LI-cadherin (exon 7 skipped) has thus far been observed only in liver cancer.

#### Correlation of Altered Splicing of LI-Cadherin With Clinical Outcomes of HCC

Because aberrant splicing is often associated with cancer progression, neoplasia and metastasis (21, 22), the next question to ask is whether the observed alternative splicing of LI-cadherin in HCC would be related to any designated clinical parameters such as sex, age, tumor-node-metastasis stage, venous infiltration, recurrence, and survival. The presence of spliced mRNA of LI-cadherin was significantly associated with the decreased overall survival time in HCC patients ( $P < 0.001$ ; Fig. 6), venous infiltration ( $P < 0.05$ ) and the first-year intrahepatic or extrahepatic recurrences ( $P < 0.05$ ) (Table 3). In brief, patients with alternative splicing had a higher rate of venous infiltration and recurrence of tumor after liver tumor resection as well as shorter survival time. The data suggested that the poor prognosis for those patients might have resulted from cancer metastasis.

#### DISCUSSION

LI-cadherin is a nonclassical member of the cadherin superfamily. It has distinct structure in both the extracellular and the cytoplasmic portion. The cytoplasmic domain of LI-cadherin consists of only 20 amino acids, whereas other classic cadherins contain 150 to 160 highly conserved amino acids through forming complexes with catenins (2). Cell-cell adhesion by LI-cadherin is apparently independent of any interaction with cytoskeleton components (23). For members of the classic cadherin superfamily, down-regulation of E-cadherin has been strongly linked to metastasis and decreased survival in gastric and breast cancers, indicating its role as a putative tumor suppressor gene (3, 9). LI-cadherin was originally found to be expressed in rat intestine and liver tissue (24), although being exclusively expressed in the intestinal but not in liver epithelial cells in human (2). However, recent studies have reported that

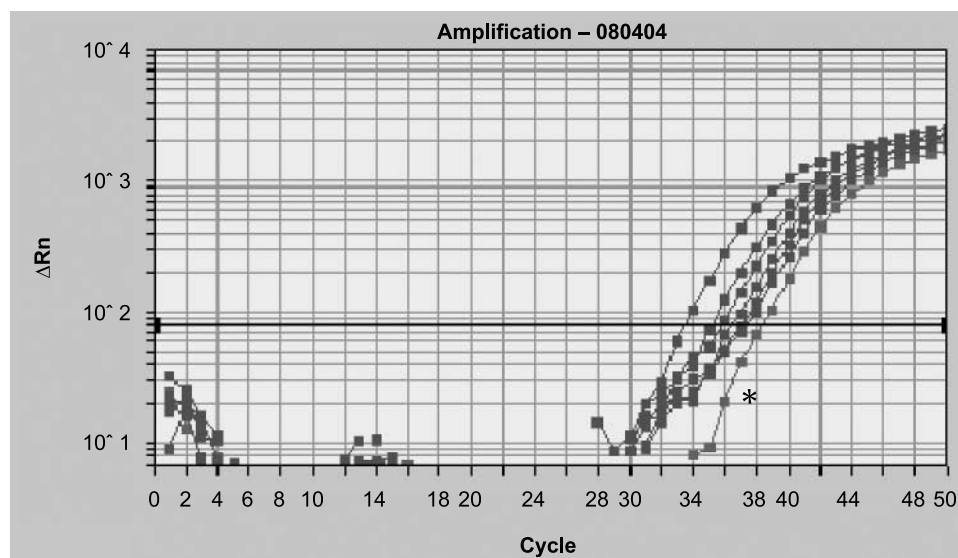
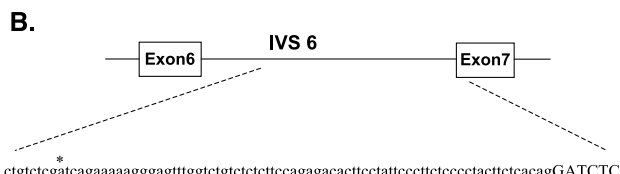
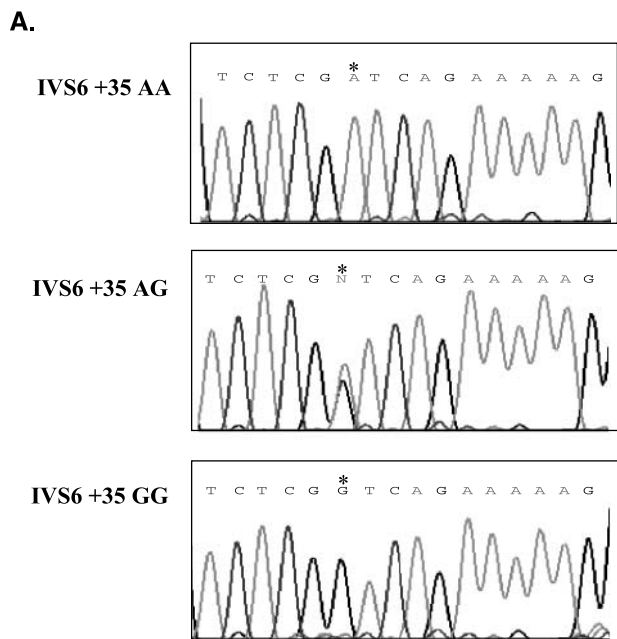


Fig. 2 Real-time quantitative PCR plot of LI-cadherin in HCC specimens. Emission intensity of the reporter FAM dye ( $\Delta R_n$ , Y-axis) is plotted versus cycle number (X-axis). \*, normal liver sample; all other were HCC samples. Experiments were repeated twice and data were similar. Mean expression level of LI-cadherin mRNA in HCC was normalized to calibrator (normal liver sample), and it was up-regulated from 2.58-fold to 800-fold as described in the text.



**Fig. 3** A, genomic DNA sequences of IVS6 + 35. The genotypes of polymorphism were presented AA (two alleles of A), AG (one allele A, one allele G), and GG (two alleles of G). B, schematic of the region of LI-cadherin exon 6, intron 6, and exon 7. Lower case, intronic sequence; upper case, exon sequence; underlined, the putative branch site sequence; \*, the adenine base.

over-expression of LI-cadherin is associated with several types of cancers such as early gastric metaplasia and neoplasia (13, 14), adenocarcinoma of the pancreas (15), and HCCs (12). Apart from its known distinct characteristics from other cadherins, there are still many unknowns about the LI-cadherin, especially in its involvement in cancer development. The present study provides the first evidence of high frequency of alternatively spliced LI-cadherin transcript in liver tumor tissues, and the altered expression correlating with survival of HCC patients and early recurrence of the disease. As such, the alternative splicing of LI-cadherin, which is not hitherto identified in other cancers (gastric and pancreatic malignancies) may hint at the strong potential risk to HCC. In spite of this, the molecular pathogenesis mechanisms of LI-cadherin and its splicing variants in liver cancer remain to be unequivocally defined. A more definitive assessment of LI-cadherin as a potential indicator for poor clinical outcomes will require a larger series of cases for expression and clinical analysis.

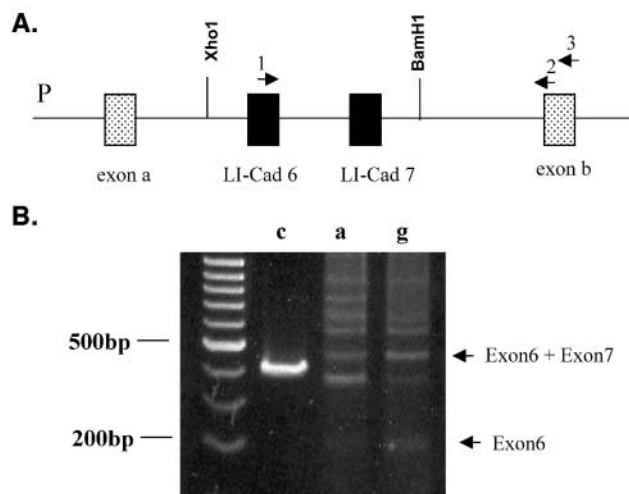
Alternative splicing is a common event in certain genes and this natural process consequently results in different proteins with different functions. However, for many genes, alternations in splicing patterns are often associated with neoplasia and metastasis through affecting regulators of apoptosis, hormones,

**Table 3** Relationship between alternative splicing of LI-cadherin and clinicopathologic features of HCC

Clinical parameters	LI-cadherin expression		P
	With splicing (n = 19)	No splicing (n = 23)	
Age	57.5 ± 10.4	56.5 ± 9.5	0.760
Sex			
Male	16	22	0.209
Female	3	1	
Tumor-node-metastasis stage			
I-II	4	5	0.957
III-IV	15	18	
Venous infiltration			
Present	14	10	0.049
Absent	5	13	
Tumor recurrence*			
Present	12	5	0.044
Absent	6	13	

\*First-year intrahepatic or extrahepatic recurrence after liver tumor resection. A total of 36 HCC tissues were available with defined clinically data for this association study.

and cell adhesion molecules (21, 22). Thus, it is important to distinguish “alternative” splicing, a common process not necessarily associated with malignancy, from “aberrant” splicing, which is often associated with malignancy (25). In our study, the presence of spliced transcript of LI-cadherin in both tumorous and adjacent nontumorous liver tissues (albeit to lower frequency) of HCC patients, but its absence in eight normal samples implied that this alternative splicing seems to be aberrant splicing. Spliced transcript with exon 7 loss would



**Fig. 4** Exon-trapping assay showed that IVS6 + 35 A > G affected splicing of LI-cadherin gene. A, IVS6 + 35 AA and IVS6 + 35GG were cloned into pSPL3 vector with XhoI and BamHI sites. Exon a and exon b, exonic sequences; 1, LI-cadherin exon 6 primer; 2, SA4 primer; 3, SA2 primer; P, promoter. B, RT-PCR products from Cos-7 transfectants with primer 1 and 2. The first lane was DNA marker; c, product from transfection of pSPL3 vector; a, product from transfection of IVS6 + 35 AA construct; g, product from transfection of IVS6 + 35 GG construct. The 450-bp band corresponded to exon 7 inclusion, and the 200-bp band corresponded to exon 7 skipping. Both bands were verified by sequencing analysis.

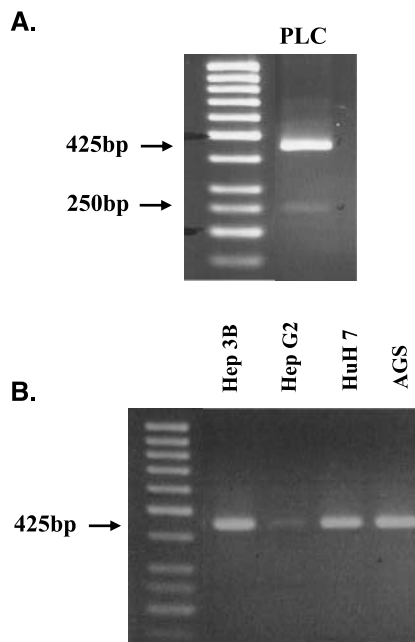


Fig. 5 RT-PCR analysis of LI-cadherin splicing with the loss of exon 7. A, PLC/RAF/5 cells; B, Hep3B, HepG2, Huh7, and AGS cell lines. The 250 bp spliced transcript was only found in PLC/RAF/5 cells.

introduce a premature stop codon in the open-reading frame. Predicted LI-cadherin protein by premature stop codon would be potentially nonfunctional. The wild-type LI-cadherin plays the important role of mediating cell-cell homotypic interaction that promotes tissue organization, perhaps also acting as an important regulator of invasive cell growth. Therefore, it is speculative that over-expression of the splice variant may have a pathogenic role as a dominant inhibitor of the wild-type function. There are cumulating evidences to support the concept that the malignant behavior of some tumors is sustained by the aberrant expression of cell adhesion splicing variants. As shown by Byers' group (11), the presence of an alternatively spliced cadherin-11 variant enhanced the invasion of cadherin-11-positive human breast cancer cell. In this study, the high prevalence of LI-cadherin splice variant in HCC tissues was associated with tumor infiltration and recurrence that may lead to shorter overall survival of liver cancer patients. Intriguingly, the four HCC cell lines described in this study have different metastasis potentials. Coexpression of wild-type LI-cadherin and the splice variant in the PLC/RAF/5 cell line seemed to have more intrahepatic tumor nodules in xenograft nude mice studies. Further confirmation of these observations is under way using stable transfectants over-expressing the LI-cadherin splice variant and/or the wild-type form.

Gene expression involves multiple regulatory steps that affect the biosynthetic process from functional gene to active protein(s). A great deal of evidence indicates that mutations that directly affect RNA processing can cause and modify human diseases (21, 22). In recent years, many mutations of 5' and 3' consensus splice sites have been reported to be related to various cancers, and have been shown to affect exon definition and pre-mRNA splicing. But few mutations of the branch site have been

identified. In the search of mutations that might result in exon 7 deletion, we found LI-cadherin 651 TT or CT and IVS6 + 35 GG or AG polymorphism in the same HCC patients. The two polymorphism sites were located closely, it was therefore possible that the two sites linked together as chromosomes segregated. LI-cadherin 651 polymorphism might have effects on alternative splicing, but the cDNA sequence did not show partial exon 6 deletion by using a cryptic splice site. An adenine at IVS6 + 35 and its consensus sequences were not close to the 3' splice site, but it seemed to be a better putative branch site compared with other adenines (Fig. 3B). The evidences from the minigene assay suggested that this putative branch site AA (wild-type) reflected a normal splicing pattern, whereas GG produced a normal and alternative splicing pattern effecting the exon 7 skipping. Whether the nucleotide polymorphism at sites 651 and IVS6 would have synergistic effects on alternative splicing remain to be further elucidated. In short, this is the first study to show polymorphisms at the intron branch site, and possibly at the exon region, having an effect on the mRNA splicing efficiency. In addition, the two polymorphisms are similarly detected in genomic DNA from blood lymphocytes of HCC patients.<sup>4</sup> It is of great interest to investigate the contribution of this polymorphism in HCC and healthy control populations to decide whether LI-cadherin is a pathogenesis variant, acting as a disease marker of HCC.

HCC recurrence, which often happens in liver cancer patients, is one of the leading causes of death. To date, no information is available about alternative splicing of LI-cadherin gene in HCC and its association with the patient's prognosis. The present study, however, has indicated that alternative splicing of LI-cadherin in HCC is correlated with venous infiltration and recurrences of liver tumor (Table 3), which consequently and unfortunately may shorten the overall survival time (Fig. 6), having significant impacts on clinical outcomes. Further investigation is undertaken to understand how and in what aspects the normal and the aberrant LI-cadherin contribute to the pathogenesis of HCC.

Taken together, LI-cadherin is a potential disease marker of HCC for its unchecked expression at mRNA and protein levels frequently detected in tumor tissues. Moreover, a hitherto unidentified splicing variant of LI-cadherin with the loss of exon 7 was found in half of the HCC specimens that were tested. Molecular characterization of the LI-cadherin gene reveals A/G polymorphism at a putative branch site of intron 6 as well as 651C/T (exon 6) in the coding sequence, which may cause the alternative mRNA splicing in HCC. Clinically, those patients with aberrant expression of LI-cadherin displayed a significantly poor prognosis, including earlier tumor recurrence as well as shorter overall survival time. The presence of alternative splicing of LI-cadherin in HCC tissues or peripheral blood may have an implication to detect recurrences in follow-up disease monitoring or to strengthen the diagnosis of HCC, although its pathogenic role in HCC development and progression has yet to be unequivocally defined.

<sup>4</sup> X.Q. Wang, J.M. Luk, P.P. Leung, and S.T. Fan, unpublished observations.

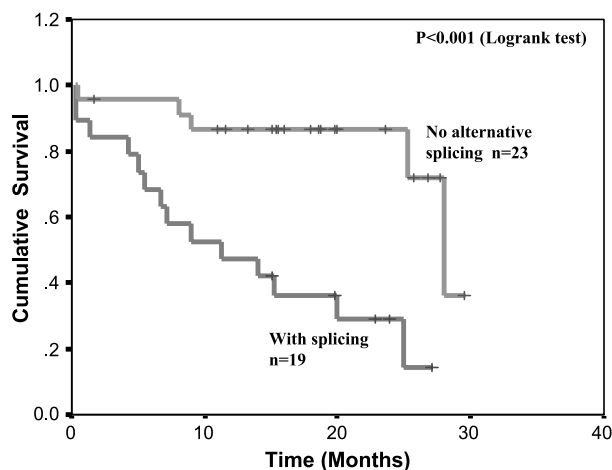


Fig. 6 Univariate Kaplan-Meier analysis for overall survival for two groups of liver cancer patients with or without LI-cadherin splicing in HCC tissues.

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