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Isolation and Sequence Analysis of Human Cadherin-6 Complementary DNA for the Full Coding Sequence and Its Expression in Human Carcinoma Cells[1]

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ABSTRACT

The expression pattern of E- and P-cadherin in human carcinomas has been reported by many laboratories. However, little is known about the involvement of other cadherin types in human carcinomas. cDNA clones for a cadherin molecule were isolated from a cDNA library of human hepatocellular carcinoma cells which lacked E- and P-cadherin expression but exhibited cell aggregation activity mediated by an unknown cadherin, and they were subjected to sequence analysis. The overlapped clones covered 4315 nucleotides and were found to encode a typical cadherin molecule consisting of 790 amino acids. Since the deduced amino acid sequence was identical to a partially available human cadherin-6 sequence except for two amino acid residues, the clones were considered to be human cadherin-6 cDNAs encoding the entire open reading frame. The deduced amino acid sequence also showed extremely high homology with recently reported rat K-cadherin, 97% for the putative mature protein, suggesting that cadherin-6 is the human counterpart of rat K-cadherin. Expression of cadherin-6 in various human normal tissues and carcinoma cells was examined by Northern blot analysis using a specific probe corresponding to the signal and precursor sequence. Among normal tissues examined, brain, cerebellum, and kidney showed strong expression of cadherin-6, whereas lung, pancreas, and gastric mucosa showed weak expression. Transcripts of cadherin-6 were not detected in normal liver, whereas four of six hepatocellular carcinoma cell lines examined expressed cadherin-6 abundantly. As reported for rat K-cadherin, three renal carcinoma cell lines also expressed cadherin-6 strongly. The most interesting finding was obtained for small cell lung carcinoma lines. Among 15 of such cell lines examined, all of 11 cadherin-6-positive lines were classified into the classic type, whereas the negative cell lines were all of the variant type. The present results suggest that besides E- and P-cadherin, other cadherin molecules are expressed in human cancers and are responsible for additional biological properties of the carcinoma cells.

INTRODUCTION

Cadherin is a Ca^{2+}-dependent cell-cell adhesion molecule which mediates cell-cell binding in a homophilic manner. Cadherin constitutes a gene family and consists of subclasses with individual binding specificities. Initial studies identified three subclasses, epithelial (E-), neural (N-) and placental (P-) cadherin, and further characterization revealed that they played a key role in many morphogenetic events as well as in the maintenance of orderly structures such as epithelium (reviewed in Refs. 1 and 2). We started studying human cadherins, focusing on their possible involvement in the biological properties of cancer, since it was conceivable that metastasis and invasion of cancer could not occur without disruption of the mutual adhesion of cancer cells (3–6).

To date, a considerable volume of experimental and clinical evidence has been obtained to indicate that dysfunction of E-cadherin, a major cadherin molecule expressed in both epithelium and cancer, leads at least to enhanced invasiveness and progression of cancer (4, 5, 7–12), and it has become apparent that there are a number of mechanisms responsible for the E-cadherin dysfunction in cancer, including loss or reduction of expression, mutations (13, 14), and aberrations of catenins (6, 15–22), which connect cadherin to the cytoskeleton network.

Besides the three subclasses mentioned above, many molecules with characteristics of cadherin have been identified over the past few years. These include the typical cadherins, which possess the same domain structure (23–26), a truncated-type cadherin (27), cadherin-related proteins with more than five extracellular subdomains (28), and desmosomal cadherins (29–31), indicating the existence of a cadherin superfamily. In addition, a PCR technique based on the high conservation among cadherin family members has facilitated the molecular cloning of various cadherins (32–34). Because E- and P-cadherin are thus far the only two cadherins that have been well characterized in cancer, it would be of interest to determine whether other cadherins are expressed in cancer and affect its biological properties. Before undertaking the present study, we had noticed some cancer cell lines exhibiting strong cell aggregation activity after trypsin/Ca^{2+} treatment (35) without expression of E- and P-cadherin (data not shown), suggesting that other cadherin molecule(s) were present in them. In this report, we describe the molecular cloning and sequence analysis of a cadherin molecule from one of those lines, a human hepatocellular carcinoma cell line Li21 (36), and its unique expression pattern in cancer cells.

MATERIALS AND METHODS

PCR. To obtain a cDNA fragment of the unknown cadherin expressed in Li21 cells, PCR was performed based on a strategy similar to that described by Suzuki *et al.* (32). The primer pair used was the upstream primer 5′-GGA-ATTCAC(ACGT)GC(ACGT)CC(ACGT)CC(ACGT)TA(CT)GA-3′ and the downstream primer GGAATTCTC(ACGT)GC(ACGT)A(AG)(CT)TT(CT)-TT(AG)AA-3′, both of which contained an *Eco*RI recognition site at their 5′ ends. Poly(A)$^{+}$[3] RNA was prepared from C-Li21 cells as described by Okayama *et al.* (37). Single-strand cDNA was synthesized using Superscript II (GIBCO-BRL, Gaithersburg, MD) and subjected as a template to 35 cycles of PCR (94°C, 55°C, and 72°C for 1.5, 2, and 3 min, respectively). The PCR product, about 160 bp long, was purified, digested with *Eco*RI, and subcloned into the *Eco*RI site of the pBluescript II SK(−) phagemid vector. This fragment was used as a probe for cDNA cloning as described below.

Construction and Screening of cDNA Library. An oligo-dT-primed cDNA was synthesized from poly(A)$^{+}$ RNA of Li21 cells using a cDNA synthesis system plus kit (Amersham, Buckinghamshire, United Kingdom), ligated to the arms of λgt10 via *Eco*RI adaptors, and packaged *in vitro* using the cDNA cloning system, λgt10 (Amersham). The phage plaques were transferred to nylon filters. The filters were hybridized at 42°C in a buffer containing 50% formamide, 0.65 M NaCl, and the above PCR fragment labeled with [α-^{32}P]dCTP, washed twice with 0.1X SSC-0.1% SDS at 65°C for 30 min, and exposed to Kodak XAR-5 film (Rochester, NY) at −70°C with an intensifying screen. Positive clones were plaque purified under the same conditions as those above.

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[3] The abbreviations used are: poly(A)$^{+}$, polyadenylated; bp, base pair(s); SCLC, small

DNA Sequence Analysis. The inserts of the positive clones were subcloned into pBluescript II SK(−) or pUC118 (Takara Shuzo, Kyoto, Japan). The phagemids were denatured by alkali treatment and subjected to sequence analysis using a 7-deaza-GTP Sequenase version 2 kit (United States Biochemicals, Cleveland, OH). The DNA sequence was determined on both strands successively using oligonucleotide primers which anneal the vector and insert DNA sequences. The nucleotide and amino acid sequences were analyzed using the GeneWorks software package (IntelliGenetics, CA).

Northern Blot Analysis. Total RNA was prepared from various human tissues and cancer cell lines by acid guanidinium thiocyanate-phenol-chloroform extraction (38), and poly(A)$^+$ RNA was isolated using oligo-dT latex beads (Oligotex-dT30 Super; Daiichikagaku, Tokyo, Japan). Human brain, cerebellum, and pancreas poly(A)$^+$ RNAs were purchased from Clontech (Palo Alto, CA). Poly(A)$^+$ RNAs (2 μg/lane) were separated in 1% agarose/formaldehyde gels, transferred to nitrocellulose filters, and hybridized at 42°C in a buffer containing 50% formamide and 0.65 M NaCl with a specific PCR probe described below. To avoid cross-hybridization with other cadherin subclasses, a 182-bp nucleotide sequence (positions 116–297 in Fig. 1) encoding the signal peptide and precursor region, where significant homologies have not been reported among different subclasses, was amplified by a PCR-labeling procedure (13) and used as a probe. We confirmed that this probe did not detect either E- or P-cadherin (data not shown). The filters were washed twice with 2X SSC, 20 mM sodium phosphate buffer, 0.06% sodium PP_i, and 0.05% SDS at 65°C for 30 min and exposed to XAR-5 film at −70°C with an intensifying screen.

RESULTS

Amplification and Isolation of a Cadherin cDNA Fragment. A PCR method was used to isolate a cDNA fragment of an unknown cadherin expressed in Li21 cells using a set of two degenerate oligonucleotide primers encoding highly conserved sequences in the cytoplasmic domain of known cadherins (32), and a fragment about 160 bp long was obtained (data not shown). This fragment was subcloned into pBluescript II SK(−) and subjected to sequence analysis. The DNA sequence consisted of 155 nucleotides and showed significant homology with reported cDNA sequences of known cadherins (data not shown).

cDNA Cloning and Sequencing. The λgt10 cDNA library constructed from Li21 poly(A)$^+$ RNA was screened with the 155-bp fragment. Approximately 4×10^4 recombinants were screened, and only one positive clone, designated AL1-1, was isolated. This clone contained a cDNA insert of 3787 bp with the poly(A) sequence, but comparison with cDNA sequences of known cadherins revealed that it lacked the translation initiation codon and the following nucleotides encoding the signal peptide, precursor region, and most of extracellular domain 1. In order to identify this sequence, the library was screened again using a 321-bp nucleotide located at the 5′-end of AL1–1 (positions 650–970 in Fig. 1) amplified by PCR as a probe. Approximately 2×10^5 recombinants were rescreened, and three positive clones were isolated. Among these clones, one, designated B1-1, proved to contain the entire open reading frame. Although B1-1 also had a poly(A) tail at the 3′-end, the addition site was different from that of AL1–1. Fig. 1 shows the two clones schematically with representative restriction endonuclease sites.

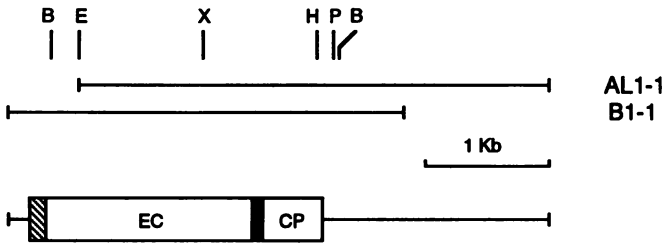

Fig. 1. Two cDNA clones, AL1-1 and B1-1, and their restriction map with a schematic drawing of cadherin structure. The *box* indicates the coding region. *Hatched, solid,* and *clear* areas represent the signal and precursor sequence, the transmembrane domain, and the extracellular (*EC*) and cytoplasmic (*CP*) domains, respectively. *B, Bam*HI; *E, Eco*RI;

The overlapping nucleotide sequence, 4315 bp in length, is shown in Fig. 2. The open reading frame begins with an ATG codon at position 121–123, terminates at a TAA codon at position 2491–2493, and consists of 2370 nucleotides encoding 790 amino acids. A polyadenylation signal, ATTAAA, is identified at position 3101–3106 for clone B1-1, but no obvious signal is present for AL1–1. The deduced amino acid sequence contains two hydrophobic sequences corresponding to the signal peptide and transmembrane domain, the long extracellular domain, and the short cytoplasmic domain, which are structures common to typical cadherins (1). This polypeptide is expected to undergo posttranslational modifications including proteolytic cleavage at amino acid position 53–54 (39) and to be expressed at the cell surface as the mature protein, which is 737 amino acids long with 5 consensus sites for *N*-linked glycosylation (40).

Comparison with Other Cadherins. A homology search with cadherin sequences reported previously revealed that this cadherin shows striking identity with two cadherin sequences. One of these is cadherin-6, which has been cloned from a human fetal brain library and partially sequenced (32). The amino acid sequence of the present cadherin from position 377 to 790 completely agrees with the reported 424 amino acids of cadherin-6, except for only two amino acid residues (Val at position 421 and Thr at position 425 are both Ile in cadherin-6), strongly suggesting that the clones presented here encode cadherin-6. To avoid confusion, the cadherin cloned in the present study will be referred to as cadherin-6 hereafter. The other cadherin highly homologous with this cadherin-6 is rat K-cadherin, which was recently isolated from a rat renal cell carcinoma library (41). Homologies of cadherin-6 with rat K-cadherin and other known human cadherins are summarized in Table 1. Homology between cadherin-6 and rat K-cadherin is extremely high, being 97% for the putative mature protein, as compared with those between cadherin-6 and known human cadherins. Even for the signal and precursor sequences, where each cadherin subclass shows a characteristic sequence, the two cadherins are markedly homologous, suggesting that cadherin-6 is the human counterpart of rat K-cadherin.

Comparison of cadherin-6 with other human cadherin subclasses reveals that there are two groups of cadherin on the basis of resemblance to cadherin-6. One group includes cadherin-8, -11 and -12, which exhibit over 60% homology with cadherin-6, and when aligned with cadherin-6, few gaps are required. The other group includes E-, N- and P-cadherin, and cadherin-4 and -5, which show lower homology, 30–40%, with cadherin-6. Cadherin-13, which lacks the transmembrane and cytoplasmic regions, also shows lower homology, 30%, for the extracellular part.

Expression of Cadherin-6 in Human Tissues and Carcinoma Cell Lines. To examine cadherin-6 expression by RNA blotting without cross-hybridization with other cadherin species, we chose a 182-bp fragment corresponding to the signal and precursor sequences as a probe. Fig. 3 shows cadherin-6 expression in Li21 cells and various normal human tissues. Cadherin-6 transcripts were detected as four bands intensely in Li21 cells, brain, cerebellum, and kidney and faintly in lung, pancreas, and gastric mucosa, with approximate molecular sizes of 9, 4.1, 3.4, and 2.7 kb. This result is inconsistent with the finding by Xiang *et al.* (41) that K-cadherin was scarcely expressed in adult rat tissues and also the report by Suzuki *et al.* (32) that brain expressed cadherin-6 very faintly. Interestingly, expression of the 9-kb cadherin-6 transcript was very faint in Li21 cells and kidney but intense in brain and cerebellum, and expression of the 2.7-kb

SEQUENCE AND EXPRESSION OF HUMAN CADHERIN-6

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   1 TGAGAGCCAAGCAAAGAACATTAAGGAAGGAAGGAGGAATGAGGCTGGATACGGTGCAGTGAAAAAGGCACTTCCAAGAGTGGGGCACTCACTACGCACAGACTCGACGGTGCCATCAGC
 121 ATGAGAACTTACCGCTACTTCTTGCTGCTCTTTTGGGTGGGCCAGCCCTACCCAACTCTCTCAACTCCACTATCAAAGAGGACTAGTGGTTTCCCAGCAAAGAAAAGGGCCCTGGAGCTC
   1 M  R  T  Y  R  Y  F  L  L  L  F  W  V  G  Q  P  Y  P  T  L  S  T  P  L  S  K  R  T  S  G  F  P  A  K  K  R  A  L  E  L
                                                                F     N                                          R
 241 TCTGGAAACAGCAAAAATGAGCTGAACCGTTCAAAAAGGAGCTGGATGTGGAATCAGTTCTTTCTCCTGGAGGAATACACAGGATCCGATTATCAGTATGTGGGCAAGTTACATTCAGAC
  41 S  G  N  S  K  N  E  L  N  R  S  K  R  S  W  M  W  N  Q  F  F  L  L  E  E  Y  T  G  S  D  Y  Q  Y  V  G  K  L  H  S  D
        A        R           S           ▲
361 CAGGATAGAGGAGATGGATCACTTAAATATATCCTTTCAGGAGATGGAGCAGGAGATCTCTTCATTATTAATGAAAACACAGGCGACATACAGGCCACCAAGAGGCTGGACAGGGAAGAA
 81 Q  D  R  G  D  G  S  L  K  Y  I  L  S  G  D  G  A  G  D  L  F  I  I  N  E  N  T  G  D  I  Q  A  T  K  R  L  D  R  E  E

481 AAACCCGTTTACATCCTTCGAGCTCAAGCTATAAACAGAAGGACAGGGAGACCCGTGGAGCCCGAGTCTGAATTCATCATCAAGATCCATGACATCAATGACAATGAACCAATATTCACC
121 K  P  V  Y  I  L  R  A  Q  A  I  N  R  R  T  G  R  P  V  E  P  E  S  E  F  I  I  K  I  H  D  I  N  D  N  E  P  I  F  T

601 AAGGAGGTTTACACAGCCACTGTCCCTGAAATGTCTGATGTCGGTACATTTGTTGTCCAAGTCACTGCGACGGATGCAGATGATCCAACATATGGGAACAGTGCTAAAGTTGTCTACAGT
161 K  E  V  Y  T  A  T  V  P  E  M  S  D  V  G  T  F  V  V  Q  V  T  A  T  D  A  D  D  P  T  Y  G  N  S  A  K  V  V  Y  S
       D                       A
721 ATTCTACAGGGACAGCCCTATTTTTCAGTTGAATCAGAAACAGGTATTATCAAGACAGCTTTGCTCAACATGGATCGAGAAAACAGGGAGCAGTACCAAGTGGTGATTCAAGCCAAGGAT
201 I  L  Q  G  Q  P  Y  F  S  V  E  S  E  T  G  I  I  K  T  A  L  L  N  M  D  R  E  N  R  E  Q  Y  Q  V  V  I  Q  A  K  D

841 ATGGGCGGCCAGATGGGAGGATTATCTGGGACCACCACCGTGAACATCACACTGACTGATGTCAACGACAACCCTCCCCCGATTCCCCCAGAGTACATACCAGTTTAAAACTCCTGAATCT
241 M  G  G  Q  M  G  G  L  S  G  T  T  T  V  N  I  T  L  T  D  V  N  D  N  P  P  R  F  P  Q  S  T  Y  Q  F  K  T  P  E  S
                                              Δ
961 TCTCCACCGGGGACACCAATTGGCAGAATCAAAGCCAGCGACGCTGATGTGGGAGAAAATGCTGAAATTGAGTACAGCATCACAGACGGTGAGGGGCTGGATATGTTTGATGTCATCACC
281 S  P  P  G  T  P  I  G  R  I  K  A  S  D  A  D  V  G  E  N  A  E  I  E  Y  S  I  T  D  G  E  G  L  D  M  F  D  V  I  T
                                                                                                H
1081 GACCAGGAAACCCAGGAAGGGATTATAACTGTCAAAAAGCTCTTGGACTTTGAAAAGAAGAAAGTGTATACCCTTAAAGTGGAAGCCTCCAATCCTTATGTTGAGCCACGATTTCTCTAC
 321 D  Q  E  T  Q  E  G  I  I  T  V  K  K  L  L  D  F  E  K  K  K  V  Y  T  L  K  V  E  A  S  N  P  Y  V  E  P  R  F  L  Y
                                                                R                 H  I
1201 TTGGGGCCTTTCAAAGATTCAGCCACGGTTAGAATTGTGGTGGAGGATGTAGATGAGCCACCTGTCTTCAGCAAACTGGCCTACATCTTACAAATAAGAGAAGATGCTCAGATAAACACC
 361 L  G  P  F  K  D  S  A  T  V  R  I  V  V  E  D  V  D  E  P  P  V  F  S  K  L  A  Y  I  L  Q  I  R  E  D  A  Q  I  N  T
                                              D                                                                    Δ
1321 ACAATAGGCTCCGTCACAGCCCAAGATCCAGATGCTGCCAGGAATCCTGTCAAGTACTCTGTAGATCGACACACAGATATGGACAGAATATTCAACATTGATTCTGGAAATGGTTCGATT
 401 T  I  G  S  V  T  A  Q  D  P  D  A  A  R  N  P  V  K  Y  S  V  D  R  H  T  D  M  D  R  I  F  N  I  D  S  G  N  G  S  I
                   A                                                   i        i                       Δ
1441 TTTACATCGAAACTTCTTGACCGAGAAACACTGCTATGGCACAACATTACAGTGATAGCAACAGAGATCAATAATCCAAAGCAAAGTAGTCGAGTACCTCTATATATTAAAGTTCTAGAT
 441 F  T  S  K  L  L  D  R  E  T  L  L  W  H  N  I  T  V  I  A  T  E  I  N  N  P  K  Q  S  S  R  V  P  L  Y  I  K  V  L  D
                                              Δ
1561 GTCAATGACAACGCCCCAGAATTTGCTGAGTTCTATGAAACTTTTGTCTGTGAAAAAGCAAAGGCAGATCAGTTGATTCAGACCCTGCATGCTGTTGACAAGGATGACCCTTATAGTGGA
 481 V  N  D  N  A  P  E  F  A  E  F  Y  E  T  F  V  C  E  K  A  K  A  D  Q  L  I  Q  T  L  H  A  V  D  K  D  D  P  Y  S  G

1681 CACCAATTTTCGTTTTCCTTGGCCCCTGAAGCAGCCAGTGGCTCAAACTTTACCATTCAAGACAACAAAGACAACACGGCGGGAATCTTAACTCGGAAAAATGGCTATAATAGACACGAG
 521 H  Q  F  S  F  S  L  A  P  E  A  A  S  G  S  N  F  T  I  Q  D  N  K  D  N  T  A  G  I  L  T  R  K  N  G  Y  N  R  H  E
                                                    Δ
1801 ATGAGCACCTATCTCTTGCCTGTGGTCATTTCAGACAACGACTACCCAGTTCAAAGCAGCACTGGGACAGTGACTGTCCGGGTCTGTGCATGTGACCACCACGGGAACATGCAATCCTGC
 561 M  S  T  Y  L  L  P  V  V  I  S  D  N  D  Y  P  V  Q  S  S  T  G  T  V  T  V  R  V  C  A  C  D  H  H  G  N  M  Q  S  C

1921 CATGCGGAGGCGCTCATCCACCCCACGGGACTGAGCACGGGGGCTCTGGTTGCCATCCTTCTGTGCATCGTGATCCTACTAGTGACAGTGGTGCTGTTTGCAGCTCTGAGGCGGCAGCGA
 601 H  A  E  A  L  I  H  P  T  G  L  S  T  G  A  L  V  A  I  L  L  C  I  V  I  L  L  V  T  V  V  L  F  A  A  L  R  R  Q  R

2041 AAAAAAGAGCCTTTGATCATTTCCAAAGAGGACATCAGAGATAACATTGTCAGTTACAACGACGAAGGTGGTGGAGAGGAGGACACCCAGGCTTTTGATATCGGCACCCTGAGGAATCCT
 641 K  K  E  P  L  I  I  S  K  E  D  I  R  D  N  I  V  S  Y  N  D  E  G  G  G  E  E  D  T  Q  A  F  D  I  G  T  L  R  N  P

2161 GAAGCCATAGAGGACAACAAATTACGAAGGGACATTGTGCCCGAAGCCCTTTTCCTACCCCGACGGACTCCAACAGCTCGCGACAACACCGATGTCAGAGATTTCATTAACCAAAGGTTA
 681 E  A  I  E  D  N  K  L  R  R  D  I  V  P  E  A  L  F  L  P  R  R  T  P  T  A  R  D  N  T  D  V  R  D  F  I  N  Q  R  L
       K  P  W  R  Q  Q  S  -           M                                                                   S
2281 AAGGAAAATGACACGGACCCCACTGCCCCGCCATACGACTCCTTGGCCACTTACGCCTATGAAGGCACTGGCTCCGTGGCGGATTCCCTGAGCTCGCTGGAGTCAGTGACCACGGATGCA
 721 K  E  N  D  T  D  P  T  A  P  P  Y  D  S  L  A  T  Y  A  Y  E  G  T  G  S  V  A  D  S  L  S  S  L  E  S  V  T  T  D  A
       R  K  M  N                                                                                                   G
2401 GATCAAGACTATGATTACCTTAGTGACTGGGGACCTCGATTCAAAAAGCTTGCAGATATGTATGGAGGAGTGGACAGTGACAAAGACTCCTAATCTGTTGCCTTTTTCATTTTCCAATAC
 761 D  Q  D  Y  D  Y  L  S  D  W  G  P  R  F  K  K  L  A  D  M  Y  G  G  V  D  S  D  K  D  S  *
                G                                                      M
2521 GACACTGAAATATGTGAAGTGGCTATTTCTTTATATTTATCCACTACTCCGTGAAGGCTTCTCTGTTCTACCCGTTCCAAAAGCCAATGGCTGCAGTCCGTGTGGATCCAATGTTAGAGA
2641 CTTTTTTCTAGTACACTTTTATGAGCTTCCAAGGGGCAAATTTTTATTTTTTAGTGCATCCAGTTAACCAAGTCAGCCCAACAGGCAGGTGCCGGAGGGGAGGACAGGGAACAGTATTTC
2761 CACTTGTTCTCAGGGCAGCGTGCCCGCTTCCGCTGTCCTGGTGTTTTACTACACTCCATGTCAGGTCAGCCAACTGCCCTAACTGTACATTTCACAGGCTAATGGGATAAAGGACTGTGC
2881 TTTAAAGATAAAAATATCATCATAGTAAAAGAAATGAGGGCATATCGGCTCACAAAGAGATAAACTACATAGGGGTGTTTATTTGTGTCACAAAGAATTTAAAATAACACTTGCCCATGC
3001 TATTTGTTCTTCAAGAACTTTCTCTGCCATCAACTACTATTCAAAACCTCAAATCCACCCATATGTTAAAATTCTCATTACTCTTAAGGAATAGAAGCAAATTAAACGGTAACATCCAAA
3121 AGCAACCACAAACCTAGTACGACTTCATTCCTTCCACTAACTCATAGTTTGTTATATCCTAGACTAGACATGCGAAAGTTTGCCTTTGTACCATATAAAGGGGGAGGGAAATAGCTAATA
3241 ATGTTAACCAAGGAAATATATTTTACCATACATTTAAAGTTTTGGCCACCACATGTATCACGGGTCACTTGAAATTCTTTCAGCTATCAGTAGGCTAATGTCAAAATTGTTTAAAAATTC
3361 TTGAAAGAATTTTCCTGAGACAAATTTTAACTTCTTGTCTATAGTTGTCAGTATTATTCTACTATACTGTACATGAAAGTAGCAGTGTGAAGTACAATAATTCATATTCTTCATATCCTT
3481 CTTACACGACTAAGTTGAATTAGTAAAGTTAGATTAAATAAAACTTAAATCTCACTCTAGGAGTTCAGTGGAGAGGTTAGAGCCAGCCACACTTGAACCTAATACCCTGCCCTTGACATC
3601 TGGAAACCTCTACATATTTATATAACGTGATACATTTGGATAAACAACATTGAGATTATGATGAAAACCTACATATTCCATGTTTGGAAGACCCTTGGAAGAGGAAAATTGGATTCCCTT
3721 AAACAAAAGTGTTTAAGATTGTAATTAAAATGATAGTTGATTTTCAAAAGCATTAATTTTTTTTCATTGTTTTTAACTTTGCTTTCATGACCATCCTGCCATCCTTGACTTTGAACTAAT
3841 GATAAAGTAATGATCTCAAACTATGACAGAAAAGTAATGTAAAATCCATCCAATCTATTATTTCTCTAATTATGCAATTAGCCTCATAGTTATTATCCAGAGGACCCAACTGAACTGAAC
3961 TAATCCTTCTGGCAGATTCAAATCGTTTATTTCACACGCTGTTCTAATGGCACTTATCATTAGAATCTTACCTTGTGCAGTCATCAGAAATTCCAGCGTACTATAATGAAAACATCCTTG
4081 TTTTGAAAACCTAAAAGACAGGCTCTGTATATATATATACTTAAGAATATGCTGACTTCACTTATTAGTCTTAGGGATTTATTTTCAATTAATATTAATTTTCTACAAATAATTTTAGTG
4201 TCATTTCCATTTGGGGATATTGTCATATCAGCACATATTTTCTGTTTGGAAACACACTGTTGTTTAGTTAAGTTTTAAATAGGTGTATTACCCAAGAAGTAAAGATGGAAACGTT(A)n
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tissue or cell type might show preferential selection of the polyadenylation site. Cadherin-6 mRNA was not detected in poly(A)$^+$ RNA isolated from heart, liver, and colonic mucosa.

Cadherin-6 expression in various human carcinoma cell lines was then examined using an approach similar to that described above. RNA blotting of five hepatocellular carcinoma cell lines, except for Li21, is shown in Fig. 4*A*. Li22, Li23, and Li24 cells (36) strongly expressed 4.1-, 3.4-, and 2.7-kb cadherin-6 transcripts. In contrast, no bands were detected in Li7 (42) and HepG2 (43). Consequently, four of six hepatoma lines strongly expressed cadherin-6. Among four renal cell carcinoma cell lines examined, three expressed cadherin-6 (Fig. 4*B*), which is consistent with the previous finding that K-cadherin was expressed in one of three human renal cell carcinomas (41). Most of the SCLC cell lines examined (11 of 15) also expressed cadherin-6 with various degrees of intensity, although cadherin-6 mRNA was not detected in four lines, Lu24v, Lu135v, H82, and N417 (Refs. 44 and 45; Fig. 4*C*). Besides the human carcinoma cells described here, we examined cadherin-6 expression in various human cell lines including five colon adenocarcinomas, four gastric adenocarcinomas, two pancreatic adenocarcinomas, and one cell line each of lung adenocarcinoma, esophageal squamous carcinoma, vulvar squamous carcinoma, cervical carcinoma, bladder carcinoma, retinoblastoma, and transformed endothelium. Cadherin-6 transcripts were not, or only very weakly detected, in poly(A)$^+$ RNAs from these lines (data not shown).

DISCUSSION

We have reported here cloning of a human cadherin molecule from a cDNA library of a hepatocellular carcinoma cell line, Li21, which does not express either E- or P-cadherin but exhibits cell aggregation mediated by an unknown cadherin. The deduced amino acid sequence is essentially identical to that of partially identified human cadherin-6 (32), except that Val at amino acid position 421 and Thr at 425 are both replaced by Ile in the latter. Since it is unlikely that two different genes separately encode the two molecules, we conclude that the cDNA clones presented here encode human cadherin-6 and that these are the first reported clones which cover the entire open reading frame. It has been reported that human N-cadherin cDNAs isolated from different libraries show minor differences from one another (46–48). The two amino acid substitutions found here may be explained by polymorphism, mutation, or cloning artifacts. It is noteworthy that, at these two positions, a cDNA clone of rat K-cadherin, isolated from a renal cell carcinoma library (41), encodes the same amino acids as AL1-1 and B1-1, suggesting that the two amino acid substitutions may be a characteristic of cancer-specific cadherin-6.

Suzuki *et al.* (32) and Tanihara *et al.* (34) have proposed that typical cadherins can be divided into two types on the basis of structural similarities: type I including E-, N-, P-, B- (23), R- (24), EP- (25), and M-cadherin (26), and cadherin-4; and type II cadherins including cadherin-5, -8, -11, and -12. They also suggested that cadherin-6, -7, -9, and -10, for which only restricted sequences are available, might be classified as type II cadherins (32, 34). The entire cadherin-6 sequence presented here shows higher homology with cadherin-8, -11, and -12 than with other human cadherins (Table 1), confirming that cadherin-6 should be a type II cadherin. However, only cadherin-5 among type II cadherins shows a low similarity to cadherin-6, comparable to the similarity between cadherin-6 and type I cadherins (Table 1). This is consistent with the previous finding that cadherin-5 shows rather low similarity to other type II cadherins (32), suggesting that cadherin-5 is a unique molecule among the type II cadherins.

Since human cadherin-6 shows extremely high similarity to rat K-cadherin (41), even in the signal peptide and precursor regions, it is considered to be the human counterpart of rat K-cadherin. Interestingly, the most diversified region between the two cadherins is the cytoplasmic domain, where two clusters of amino acids different from each other are present, whereas the two cadherins are almost identical in the extracellular and transmembrane domains with only a few conservative amino acid changes (Fig. 2). In contrast, the classical-type cadherins, E-, N- and P-cadherin, show the highest degree of interspecific conservation in the cytoplasmic domain (48–51), which is linked to the cytoskeleton via catenins. When each subdomain of cadherin-6 is compared with that of other type II molecules, the most conserved subdomain is not the cytoplasmic domain but the extracellular domain 2 (Table 1). As for the extracellular domain of cadherin-6, three potential sequences involved in cell adhesion recognition, PPI, GAD and HAV, proposed by Blaschuk *et al.* (52), are absent or partially changed in cadherin-6. In contrast, possible Ca^{2+}-binding sites (53, 54) are well conserved in cadherin-6, and four cysteine residues involved in the mechanism of cadherin adhesion (55) are also conserved in cadherin-6 at amino acid positions 497, 589, 591, and 600 (Fig. 1). These features are also the case for the rat homologue, K-cadherin (41). As mentioned above, cadherin-6 and other type II cadherins have structural characteristics not only similar to but also different from classic cadherins. It is thus of considerable interest to investigate whether cadherin-6 and other type II cadherins function in the same manner as classic cadherins.

Analysis of the expression pattern of cadherin-6 revealed in this study provided some interesting findings. Both normal kidney and renal cell carcinomas expressed cadherin-6. In contrast, although four of six hepatocellular carcinoma cell lines examined strongly expressed cadherin-6, and indeed cadherin-6 cDNAs were isolated from a hepatocellular carcinoma library, cadherin-6 expression was not detected in normal liver tissue. The significance of the cadherin-6 expression in hepatocellular carcinoma cells is still unclear; therefore, it will be important in the future to examine cadherin-6 expression in surgically resected hepatomas together with the morphological and clinical features of the cancers.

Since brain and cerebellum strongly expressed cadherin-6, we focused on cadherin-6 expression in SCLC cells, which are known to show features of differentiation similar to neuroendocrine cells. SCLC cells can be subdivided into two types, the classic type and the variant type, according to the expression pattern of neuroendocrine biomarkers, and separately subgrouped into four types based on their appearance: type 1, tightly packed spherical aggregates; type 2, relatively densely packed aggregates; type 3, very loosely adherent aggregates; and type 4, cells attached to the substrate (45). As shown in Fig. 4*C*, cadherin-6 expression was detected in 11 of 15 SCLC lines examined. Surprisingly, all of these 11 cadherin-6-positive cell lines were of the classic type and morphologically type 1 or 2. In contrast, all four of the cadherin-6-negative cell lines were classified as the variant type and were morphologically type 3 (only Lu24v cells grow as type 2–3;

Fig. 2. Nucleotide sequence (*upper*) and deduced amino acid sequence (*lower*) of a cadherin molecule cloned from a Li21 cDNA library. Amino acid sequence is shown in *one-letter code*. The stop codon is marked by an *asterisk*. The addition site of the poly(A) tail and the polyadenylation signal of clone B1–1 are shown by single and double underlining, respectively. The putative signal peptide and transmembrane region are also indicated by *underlining*. The *solid triangle* and *clear triangles* show the possible proteolytic cleavage site (39) and *N*-linked glycosylation sites, respectively. *Capital* and *small letters* below the deduced amino acid sequence indicate amino acid residues of rat K-cadherin (41) and human cadherin-6 partially sequenced by Suzuki *et al.* (32), respectively, which differ from the sequence presented here. The nucleotide sequence data will appear in the GSDB/DDBJ/

Refs. 44 and 45). These findings suggest that the expression of cadherin-6 is associated with neuroendocrine differentiation and that cadherin-6 may be a useful marker for distinguishing between the classic and variant types; furthermore, cadherin-6 expression may be responsible for the tighter cell-cell adhesion evident in SCLC cell lines positive for the molecule than in those lacking it. A similar finding has been reported for the Ca^{2+}-independent cell-cell adhesion molecule N-CAM, also called cluster 1 SCLC antigen; N-CAM was abundantly expressed in classic-type SCLCs, whereas the expression level tended to be lower in the variant type (56). It has also been reported that in kidney development, the expression pattern of K-cadherin, a homologue of cadherin-6, is similar to that of N-CAM (41). However, it remains to be elucidated whether the expressions of both cadherin-6 and N-CAM are regulated by the same mechanisms and whether their expression actually affects the morphological appearance and biological properties of SCLC cells.

In conclusion, we have isolated a full-length human cadherin-6 cDNA from a hepatocellular carcinoma library and demonstrated its unique expression in cancer cells. However, the exact functions and roles of cadherin-6 during normal morphogenesis and cancer development remains to be elucidated. We hope that this report will promote further studies on the roles of various cadherin molecules in cancer development and spread.

Table 1 *Homologies between cadherin-6 and other cadherins for separate regions and the putative mature protein*

The extracellular domain is divided into five subregions according to Tanihara *et al.* (34). rK, E, N, P, 4, 5, 8, 11, 12, and 13 represent percentage homology between human cadherin-6 and rat K-cadherin (41), human E-cadherin (49), N-cadherin (46), P-cadherin (50), and cadherin-4, -5, -8, -11, -12, and -13 (32, 34), respectively. EC, extracellular domain; TM, transmembrane domain; CP, cytoplasmic domain.

	EC1	2	3	4	5	TM	CP	Total
rK	100	98	97	98	100	100	89	97
E	29	43	31	29	23	29	50	35
N	34	47	27	31	24	50	48	37
P	29	37	33	21	17	26	49	32
4	31	46	30	29	24	49	46	36
5	38	49	37	41	37	41	35	39
8	60	75	57	57	51	64	60	60
11	62	78	52	53	48	73	62	60
12	70	78	62	43	56	73	71	64
13	32	38	30	28	21			30[a]

[a] Because cadherin-13 lacks the transmembrane and cytoplasmic domains, only the homology in the extracellular domain is shown.

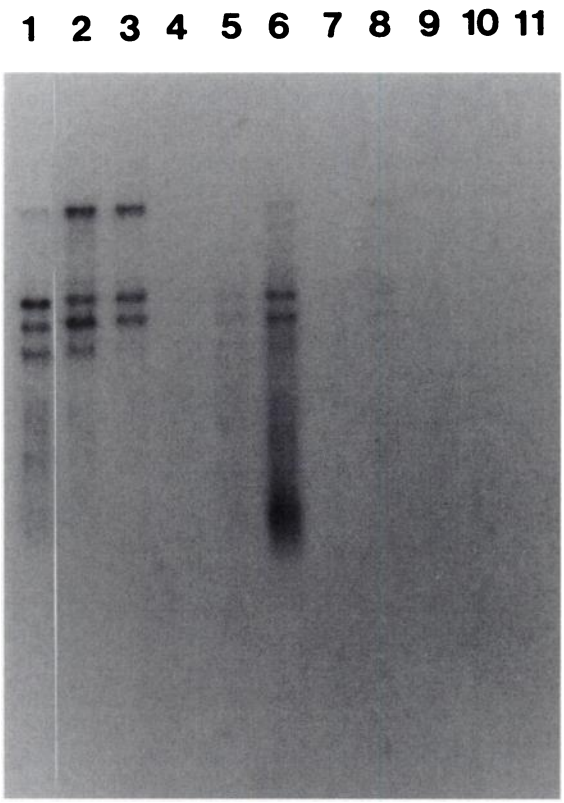

Fig. 3. Expression of cadherin-6 in Li21 cells and normal human tissues examined by Northern blot analysis. Poly(A)$^+$ RNA from Li21 cells (*Lane 1*), human brain (*Lane 2*), cerebellum (*Lane 3*), heart (*Lane 4*), lung (*Lane 5*), kidney (*Lane 6*), liver (*Lane 7*), pancreas (*Lane 8*), gastric mucosa (*Lane 9*), colonic mucosa (*Lane 10*), and placenta (*Lane 11*) were hybridized with the 182-bp PCR probe. The RNA sample in *Lane 6* appears to be degraded in comparison with other samples, probably because of the prolonged ischemic state of the kidney tissue during surgical resection. *Bars* show positions of molecular size marker mRNAs of 9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kb from the

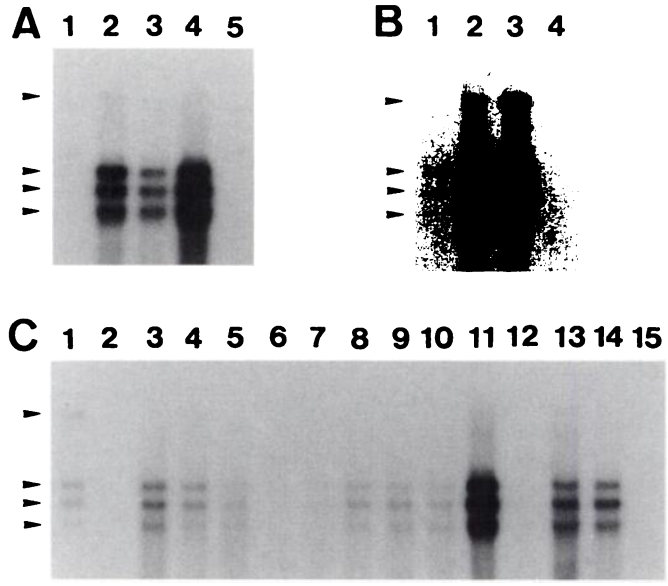

Fig. 4. Expression of cadherin-6 in hepatocellular carcinoma cell lines (*A*), renal cell carcinoma cell lines (*B*) and SCLC cell lines (*C*) examined by Northern blot analysis. *A: Lane 1,* Li7; *Lane 2,* Li22; *Lane 3,* Li23; *Lane 4,* Li24 (36, 42); *Lane 5,* Hep G2 (43). *B: Lane 1,* KT1A; *Lane 2,* KT4; *Lane 3,* KT12 (57); *Lane 4,* KT35. *C: Lane 1,* Lu24; *Lane 2,* Lu24v; *Lane 3,* Lu130; *Lane 4,* Lu134A; *Lane 5,* Lu134B; *Lane 6,* Lu135v; *Lane 7,* Lu139; *Lane 8,* Lu140; *Lane 9,* Lu141; *Lane 10,* Lu143 (44); *Lane 11,* H69; *Lane 12,* H82; *Lane 13,* N230; *Lane 14,* N231; *Lane 15,* N417 (45). *Arrowheads,* positions of cadherin-6 transcripts.

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