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E-Cadherin Gene Mutations Provide Clues to Diffuse Type Gastric Carcinomas

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ABSTRACT

The calcium-dependent homophilic cell adhesion molecule and candidate suppressor gene, E (epithelial)-cadherin, plays a major role in the organization and integrity of most epithelial tissues. Diffusely growing gastric carcinomas show markedly reduced homophilic cell-to-cell interactions. We speculated that mutations in the E-cadherin gene may be responsible for the scattered phenotype of this type of carcinoma. For that reason we have examined E-cadherin in 26 diffuse type, 20 intestinal type and 7 mixed gastric carcinomas (Laurén's classification) at the DNA, RNA, and protein levels.

Reverse transcription polymerase chain reaction and direct sequencing of amplified E-cadherin complementary DNA fragments revealed in-frame skipping of either exon 8 or exon 9 in 10 patients with diffuse tumors and an exon 9 deletion in one patient with a mixed carcinoma; both exons encode putative calcium binding domains. These alterations were not seen in nontumorous gastric tissues. Splice site mutations responsible for the exon deletions were identified in six of these patients, eliminating the possibility of alternative splicing mechanisms. Five of these splice site alterations were confirmed as somatic mutations. Non-splice site mutations were observed in three diffuse type tumors, namely a 69-base pair deletion of exon 10 and two point mutations, one of which destroys a putative calcium binding region. Immunohistochemical evaluation showed E-cadherin immunoreactivity in tumors and lymph node metastases of patients expressing abnormal mRNA. The allelic status of the E-cadherin gene was analyzed in one patient, revealing loss of heterozygosity with retention of a mutated E-cadherin allele. Overall, E-cadherin mutations were identified in 50% (13 of 26) of the diffuse type and in 14% (1 of 7) of the mixed carcinomas. In contrast, two silent E-cadherin mutations (not changing the amino acid sequence) were detected in two tumors of the intestinal type.

Our study provides strong *in vivo* evidence that E-cadherin gene mutations may contribute to the development of diffusely growing gastric carcinomas and support a tumor/metastasis suppressor gene hypothesis.

INTRODUCTION

The cadherins, a family of calcium dependent cell adhesion molecules, have been implicated in numerous cellular functions, including establishing and maintaining intercellular connections, controlling cell polarity, and morphogenesis (1). *In vitro* studies indicate a potential role for one member of this family, E (epithelial)-cadherin, as an invasion suppressor (2). Pathological examinations have shown that reduced E-cadherin immunoreactivity is associated with dedifferentiation and metastasis in primary human tumors *in vivo*, including carcinomas of head and neck (3), breast (4), and prostate (5).

The correlation between reduced or loss of E-cadherin expression and invasive properties may not be a general phenomenon, since invasivness of cells and dedifferentiation of carcinomas can occur even in the presence of E-cadherin. Madin-Darby canine kidney epithelial cells transformed by a temperature-sensitive v-*src* gene obtain an invasive phenotype at the permissive temperature, although E-cadherin is still maintained (6). Conflicting results with regard to E-cadherin immunoreactivity and tumor dedifferentiation have been reported for gastric carcinomas: two studies showed strong E-cadherin immunoreactivity in 68% of diffuse type tumors (7) and in 30% of signet ring cell carcinomas (a morphological unique form of diffuse tumors) (8), respectively; whereas a third reported no detectable E-cadherin immunoreactivity in all 21 diffuse type tumors examined (9). E-cadherin immunoreactivity has also been analyzed in lymph node metastases of gastric carcinomas. In 72% (41 of 57) of the metastases, E-cadherin immunoreactivity showed the same extent and intensity as in the individual primary tumor (7, 8). It is important to notice, however, that in these studies the expression of E-cadherin was detected by immunohistochemical techniques only and, therefore, there is no indication about its functional activity. Defects affecting critical regions of the E-cadherin gene may account for these discrepancies. As a first step to answer this question we have analyzed the integrity of E-cadherin cDNA.[2] We chose to analyze E-cadherin in gastric carcinomas because tumor cells lacking homophilic cell-to-cell interactions are often encountered (diffuse or scattered tumors) and because no clear picture has emerged from immunohistochemical analysis of E-cadherin in this type of tumor. Preliminary characterization of E-cadherin alterations in diffuse type gastric carcinomas using reverse transcription PCR and direct sequencing of amplified cDNA fragments resulted in the identification of abnormal mRNA in 4 of 14 patients, resulting from in-frame skipping of either exon 8 or exon 9 (10).

We report here E-cadherin alterations occurring in 50% of diffuse type gastric carcinomas, the identification of splice site mutations in the E-cadherin gene eliminating single exons, and two amino acid substitutions, one of which destroys a putative calcium binding site. In addition, LOH with retention of a mutated E-cadherin allele was detected in one case, supporting a tumor/metastasis suppressor gene hypothesis. Our results may help to solve the anomaly of diffusely growing tumor cells expressing E-cadherin but not showing homophilic cell-to-cell adhesion.

MATERIALS AND METHODS

Tissues. Fresh cancer tissues, nontumorous mucosa, and lymph node metastases were obtained at surgery, snap frozen, and stored in liquid nitrogen. The tumors included 26 diffuse type, 20 intestinal type, and 7 mixed carcinomas according to Laurén's classification (11).

Northern Blot. Total RNA was isolated by the guanidinium isothiocyanate and CsCl centrifugation method (12). Northern blots were prepared and hybridized as described (13), except that 8 to 40 μg total RNA per lane were used to approximate signal intensity. The human E-cadherin cDNA probe used (HC6-1) was kindly provided by Professor Birchmeier, Essen, Germany, and previously shown to be E-cadherin specific (2).

Sequences of Primers Used in This Study. Underlined nucleotides represent recognition sites for restriction enzymes not present at these positions in

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[2] The abbreviations used are: cDNA, complementary DNA; PCR, polymerase chain reaction; LOH, loss of heterozygosity; nt, nucleotide.

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the human E-cadherin sequence. Nucleotide positions refer to a published human E-cadherin cDNA sequence (14).

Ex5	5′-ACAGAGCCTCTGGATAGAGAACGC (nt 743–766)
Ex7	5′-CTTCTGCAGACCTCTGTGATGGAGGTC (nt 929–946)
Ex8	5′-CTTCTGCAGCTACGTATACCCTGGTGG (nt 1110–1128)
Ex8/2	5′-CTTCTGCAGATCACAGTCACTGACACC (nt 1181–1198)
rEx8	5′-GGGATTGAAGATCGGAGGATTATC (nt 1202–1225)
rEx8/2	5′-TGTTGTGCTTAACCCCTCACCTTG (nt 1145–1168)
Ex9	5′-CTGAAAGTGACTGATGCTG (nt 1280–1298)
Ex9/2a	5′-CAGCGTGGGAGGCTGTATACAC) (nt 1314–1335)
rEx9	5′-TTTCAGTGTGGTGATTACGAC (nt 1265–1285)
rEx10/2	5′-CCACATTCGTCACTGCTACG (nt 1453–1472)
rEx11	5′-TGTGTACGTGCTGTTCTTCAC (nt 1760–1780)
Ex13	5′-GGCGTCTGTAGGAAGGCACAG (nt 2171–2191)
r3prime	5′-CCAGCACATGGGTCTGGG (nt 2764–2781)
Intron8	5′-CTCAGCTCTGCTAGCAGTCTTG
rIntron9	5′-GTATGAACAGCTGTGAGGATGC

Screening for Deletions in the E-Cadherin cDNA. All patients were examined for E-cadherin cDNA deletions (Table 1). E-cadherin cDNA was amplified after reverse transcription of mRNA as described previously (10) using primer pairs Ex7-rEx10/2 and Ex9/2a-rEx11. PCR products were visualized by agarose gel electrophoresis. Abnormal fragments still observed after a second independent PCR amplification were excised (10) and sequenced using internal primers to determine the boundaries of the deletions.

Analysis of Splice Site Mutations in the E-Cadherin Gene. From 6 patients (patients 10, 12, 16, 34, 52, and 53; see Table 1) displaying loss of either exon 8 or 9 in the E-cadherin cDNA (see Fig. 1 and Ref. 10), genomic DNA from noncancerous gastric epithelial tissues and tumors was isolated as described (12). Nontumorous material from patient 12 was not available for analysis. DNA (100 ng) was used for amplification of introns 7 to 9. The following primer pairs were used (see Fig. 3*A*): Ex7 and rEx8 (amplification of intron 7); Ex8 and rEx9 (amplification of intron 8); Ex9 and rEx10/2 (amplification of intron 9). Cycle conditions were: 1 min at 94°C; 1 min at 55°C; 2 min at 72°C. Amplification products were purified and sequenced as described (10) using primers rEx8/2, Ex8/2, rEx9, Ex9/2a, and rEx10/2. In tumor tissues from patients 12, 34, and 52, normal and mutated sequences could not be separated in the autoradiographs; therefore, amplification products obtained from a second independent PCR from these patients as well as those from patients 16 and 53 were sequenced after subcloning into a T-tailed pBluescript vector (15). PCR products from all nontumorous tissues and from patient 10 obtained from a second independent amplification were directly sequenced without subcloning.

Screening for Mutations in Putative Calcium Binding Domains and in the Cytoplasmic Region of E-Cadherin. Patients examined with this technique are shown in Table 1. The mRNA was reverse transcribed (10) and then subjected to 40 cycles of amplification using primers Ex5-rEx10/2 and Ex9/2a-rEx11 (calcium binding domains; see Fig. 7*A*). The overlapping amplified regions are located between nt 743 and 1780 (14). Primer pair Ex13-r3prime was used to amplify the cytoplasmic region of E-cadherin (nt 2171–2781) (see Fig. 7*A*). Amplification conditions for all reactions were: 1.5 min at 94°C; 1.5 min at 55°C; 1.5 min at 72°C. The amplified E-cadherin cDNA fragments were purified and sequenced in their entire length as described (10) using internal primers. Mutations were confirmed by a second independent cDNA amplification and sequence analysis.

Touch Preparation Used for Detection of LOH. To obtain single diffusely growing carcinoma cells free of stromal cells or lymphocytes we used the touch preparation method. The original protocol (16) was slightly modified. Microscopic slides were lightly pressed onto the freshly cut surface of a frozen lymph node metastase of patient 10 at −20°C in a cryostat. Cells adhering to the slides were stained with toluidine blue/methylene blue (16). Approximately 100 single tumor cells (identified by cytological inspection) were transferred into 10 μl of 5% dextrose in a microfuge tube. To detect possible LOH, PCR was used to introduce a recognition sequence of a restriction enzyme in an allele specific manner (17, 18). The cells were lysed for 5 min at 94°C prior to a nested PCR amplification. In the first step primers Intron8 and rIntron9 were used to amplify exon 9 and adjacent intronic sequences. The cycle conditions were: 30 s at 94°C; 30 s at 55°C; 30 s, 72°C. Two μl of the first step PCR were used as template in the second step PCR. The upstream primer was Ex9/2a. Cycle conditions were the same as used for the first step. By designing the downstream primer (5′-GCA TCT TGC CAG GTA CCA TAC AAG) to have a nontemplate G nucleotide at its 3′-terminus, we cause the amplified wild type allele to contain the sequence 5′-AAGGCTT. The mutated allele, lacking a G nucleotide, is amplified with the sequence AAGCTT, in which in both cases the C is the complementary base to the nontemplate G nucleotide of the downstream primer. Since AAGCTT is the recognition sequence for *Hin*dIII restriction endonuclease, the mutated allele will now be cut by *Hin*dIII. The wild type allele does not contain this

Table 1 *Methods used for E-cadherin mutation analysis*

Patient	Histology (Laurén)	Screening for cDNA deletions[a]	Analysis of splice site mutations[b]	Screening for point mutations[c]
1	Diffuse	+[d]	ND	+(A)
2	Intestinal	+	ND	+
3	Diffuse	+	ND	+
4	Diffuse	+	ND	+
5	Intestinal	+	ND	+
6	Mixed	+	ND	+
7	Intestinal	+	ND	+
8	Mixed	+	ND	+
9	Diffuse	+	ND	+
10	Diffuse	+(A)	+(A)	ND
11	Intestinal	+	ND	+
12	Diffuse	+(A)	+(A)	ND
13	Intestinal	+	ND	+(A)
14	Intestinal	+	ND	+
15	Intestinal	+	ND	+
16	Diffuse	+(A)	+(A)	ND
17	Intestinal	+	ND	+
18	Intestinal	+	ND	+
19	Mixed	+	ND	+
20	Diffuse	+	ND	+(A)
21	Intestinal	+	ND	+
22	Intestinal	+	ND	+
23	Diffuse	+(A)	ND	ND
24	Intestinal	+	ND	+
25	Mixed	+	ND	+
26	Mixed	+	ND	+
27	Intestinal	+	ND	+
28	Diffuse	+(A)	ND	ND
29	Mixed	+(A)	ND	ND
30	Diffuse	+(A)	ND	ND
31	Diffuse	+	ND	+
32	Intestinal	+	ND	+
33	Diffuse	+	ND	+
34	Diffuse	+(A)	+(A)	ND
35	Diffuse	+	ND	+
36	Diffuse	+	ND	+
37	Diffuse	+	ND	+
38	Intestinal	+	ND	+
39	Intestinal	+	ND	+
40	Diffuse	+	ND	+
41	Diffuse	+	ND	+
42	Intestinal	+	ND	+
43	Diffuse	+(A)	ND	ND
44	Diffuse	+	ND	+
45	Diffuse	+	ND	+
46[e]				
47	Intestinal	+	ND	+
48	Mixed	+	ND	+
49	Intestinal	+	ND	+(A)
50	Diffuse	+(A)	ND	ND
51	Diffuse	+	ND	+
52	Diffuse	+(A)	+(A)	ND
53	Diffuse	+(A)	+(A)	ND
54	Intestinal	+	ND	+

[a] E-cadherin cDNA sequences [nt 929–1472 and nt 1314–1780 (14)] were amplified and visualized by agarose gel electrophoresis.

[b] Splice sites surrounding exons 8 and 9 were sequenced.

[c] The regions of the E-cadherin cDNA subjected to direct sequencing were nt 743–1780 and nt 2171–2781 (14).

[d] +, patient analyzed with this method; +(A), alteration observed (see Table 2 for details); ND, not determined.

[e] Patient 46 was excluded from the study because this patient had an adenocarcinoma in a Barrett's esophagus. Therefore, the total number of cases is 53.

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recognition sequence and will not be cut, allowing the discrimination between normal and mutated E-cadherin alleles after *Hin*dIII restriction and agarose gel electrophoresis (2.5% agarose).

Immunohistochemistry. Frozen sections were fixed with acetone at room temperature and incubated with E-cadherin specific monoclonal antibodies DECMA-1 (19) (dilution, 1:1600; Sigma Chemical Co., St. Louis, MO) and 6F9 (2) (dilution, 1:20; Bissendorf Biochemicals, Hannover, Germany). The alkaline phosphatase anti-alkaline phosphatase method described by Cordell *et al.* (20) was used for immunostaining.

RESULTS

The E-cadherin cell adhesion molecule was examined in gastric carcinomas at the mRNA, genomic DNA, and protein level. Preliminary results had already revealed altered E-cadherin cDNA molecules in 4 of 14 diffuse type gastric tumors (10). In this study we have extended our E-cadherin mRNA analysis, determined the underlying gene mutations most likely responsible for the alterations of the mRNA in six patients, and analyzed the allelic status of the E-cadherin gene in one patient. In addition, E-cadherin immunoreactivity was analyzed in those tumors expressing altered mRNA.

The E-cadherin exon 7–10 cDNA region from all patients (n = 53) was amplified and abnormal PCR products (n = 12) directly sequenced. In the cDNA from tumor tissues of 5 patients (4 diffuse and 1 mixed carcinoma) exon 8 sequence is followed directly by exon 10, skipping exon 9. In two additional diffuse type tumors exon 8 is missing from the mRNA, resulting in adjoining exons 7 and 9. These alterations were not seen in any of noncancerous gastric tissues analyzed (n = 51) (Fig. 1). Four cases (patients 10, 12, 16, and 52) displaying loss of either exon 8 or exon 9 besides those shown in Fig. 1 have been described previously (10). Exons 8 and 9 both contain highly conserved domains proposed to be necessary for the calcium binding and adhesive function of E-cadherin (21). In tumor tissue but not in adjacent nontumorous mucosa of an additional patient, 69 nucleotides of exon 10 were shown to be deleted.

The wild type E-cadherin cDNA fragment was absent from the tumor tissue in 5 cases of diffuse tumors (this study and Ref. 10). This may be an indication for a possible LOH at the E-cadherin locus in these patients. In 7 additional patients (one of whom had a mixed type carcinoma), the wild type fragment was present, leaving the possibility that in these cases the tumor cells themselves are heterogenous or the analysed material was a mixture of tumor cells and normal gastric epithelium. In 4 patients with both the wild type and exon 9-deleted E-cadherin fragments a third PCR product, migrating between the normal and mutated fragments, was visible on the agarose gel. Isolation and sequencing of this band revealed a heteroduplex molecule containing normal as well as mutated E-cadherin sequences (Fig. 1).

To further strengthen the PCR and sequencing results Northern blot experiments were performed. Exon 9 deletions (resulting in the loss of 183 nt from an approximately 4.5 kilobase E-cadherin mRNA transcript) should be visible on Northern blots, whereas exon 8 deletions (loss of 129 nt) may be to small to be resolved. Two patients (patients 10 and 16) previously shown to lack exon 9 in the E-cadherin cDNA were selected because the wild type fragment was not seen in the PCR analysis (10) and because sufficient RNA was available from tumor tissue, nontumorous epithelium, and lymph node metastases. An E-cadherin specific cDNA probe detected an approximately 4.5-kilobase mRNA transcript in nontumorous gastric epithelium. The E-cadherin gene transcript, however, was noticeably shortened in the tumor tissues and lymph node metastases of both patients (Fig. 2), confirming the PCR analysis.

Mutations in the E-cadherin gene may be responsible for the exon losses observed in 12 patients (11 cases with either exon 8 or 9 deletions, 1 patient with a partial deletion of exon 10), since there is

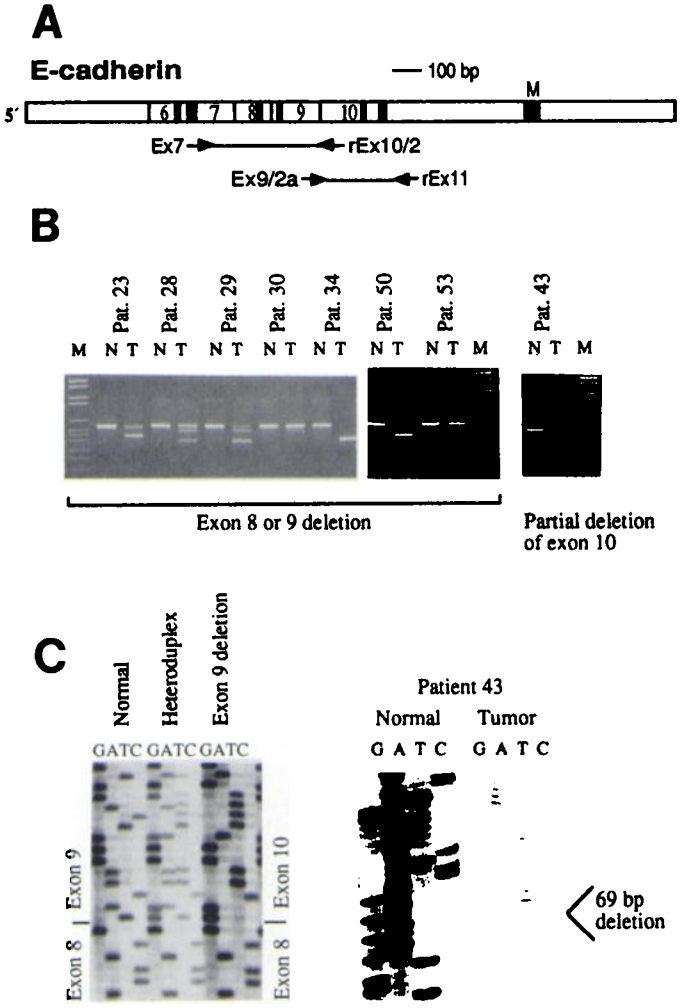

Fig. 1. Detection of abnormal E-cadherin cDNA in gastric carcinomas. *A*, E-cadherin cDNA regions amplified by PCR. *M*, transmembrane domain; *vertical black bars*, putative calcium binding sites; *6–10*, exons. *B*, ethidium bromide stained agarose gel showing the amplification products of noncancerous (*N*) and tumor (*T*) tissues from eight patients. Patient (*Pat.*) designation is shown above the gel. In tumor tissues either one (patients 34 and 50), two (patients 23 and 43), or three (patients 28, 29, 30, 53) bands are seen; these PCR products correspond to either mutated; normal and mutated; or normal, heteroduplex, and mutated molecules, respectively. The primers used for PCR were Ex7-rEx10/2 (exon 8 and 9 deletion) or primers Ex9/2a-rEx11 (partial deletion of exon 10). (Agarose gels of four patients with either exon 8 or exon 9 deletions have been published previously (10) and are not shown here). *M*, molecular weight marker VI, Boehringer, Germany. *C*, sequencing gel of a heteroduplex formed between normal and exon 9 deleted cDNA resulting in a composite sequence (*middle*). A deletion found in patient 43 removes the first 69 base pairs (*bp*) of exon 10 (the complementary strand of the cDNA is shown).

no evidence for alternative splicing mechanisms within the mouse E-cadherin gene (22) and no length alterations of the human E-cadherin mRNA were found in noncancerous epithelial tissues (see Fig. 1). The molecular basis for these mutations could result either from exon deletions in the E-cadherin gene or from skipping of exons during mRNA processing. To discriminate between these two possibilities we analyzed genomic DNA corresponding to E-cadherin exons 8 and 9 from six patients (patients 10, 12, 16, 34, 52, and 53) expressing abnormal E-cadherin mRNA (this study and Ref. 10). Using exon specific primers we first amplified introns 7–9 of the E-cadherin gene from noncancerous gastric epithelial tissues from patients 10, 16, 34, 52, and 53 and determine the exon/intron boundaries (Fig. 3). (Normal tissue was not available for analysis from patient 12.) Locations of the exon/intron borders are conserved between mouse (22) and human E-cadherin. Wild type sequences for the *cis*-acting elements known to influence RNA splicing (23) were

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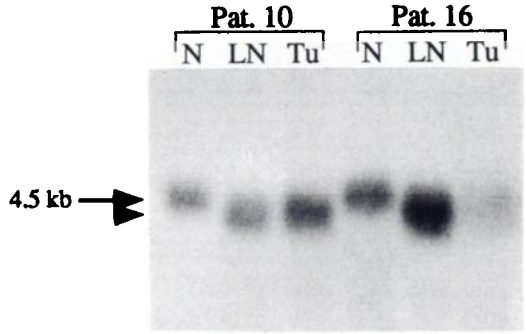

Fig. 2. Northern hybridization of an E-cadherin specific probe to nontumorous gastric tissues (*N*), lymph node metastases (*LN*), and tumor tissues (*Tu*). Patient designations are shown above the blot; *arrow*, length of the wild type E-cadherin mRNA; *arrowhead*, abnormal E-cadherin mRNA in lymph node metastases and tumors. Both patients displayed altered E-cadherin PCR products (10). *kb*, kilobases.

observed in all of the tested nontumorous materials. However, unique mutations affecting these elements were found in the tumor tissues in all six patients analyzed (Fig. 4); in the tumor of patient 10 a G nucleotide was lacking, changing the exon 9/intron 9 boundary from AAAG/gttt to AAAG/ttt, resulting in a composite sequence shifted by one nucleotide. The wild type sequence also detected in the tumor sample results either from an unaltered second allele or from stromal cell or lymphocyte DNA (compare Fig. 5). In patients 16 and 53 a G nucleotide at the same site was substituted by a T or a C, respectively. Twenty nucleotides were deleted from the intron 8/exon 9 border in patient 34. Deletions of 37 and 3 nucleotides were observed in the intron 7/exon 8 border in patients 12 and 52, respectively. The alterations in patients 10, 16, 34, 52, and 53 most likely arose as somatic mutations and are not due to polymorphic variations, since wild type sequences were observed in noncancerous tissues from these cases (Fig. 4). These results provide strong evidence that skipping either exon 8 or exon 9, at least in these patients, is due to mutations of genomic DNA sequences that influence splice site selection.

In carcinoma cells of a lymph node metastasis from patient 10 the allelic status of the E-cadherin gene was determined. A major problem in the molecular analysis of diffusely growing carcinomas is the presence of DNA from noncancerous cells. To investigate possible LOH in tumor cells free of stromal cell and lymphocyte contamination, we isolated carcinoma cells from the metastasis by touch preparation and designed a sensitive PCR-based restriction enzyme digestion assay. This assay allowed us to distingish wild type and mutated E-cadherin alleles (see "Materials and Methods"). In normal gastric epithelium from the same patient only the wild type E-cadherin allele was seen. However, in the lymph node DNA derived almost exclusively from metastasizing carcinoma cells the mutated allele is very prominent, indicating loss of the wild type allele from the tumor cells (Fig. 5). The faint band corresponding to the wild type allele probably results from the few lymphocytes that are still present in the touch preparations. In genomic DNA isolated from a segment of the cellularly heterogeneous lymph node metastase without enrichment of tumor cells, the mutated allele is detectable only as a faint band. The prominent normal allele presumably is derived from stromal cell or lymphocyte DNA. Stromal cells and lymphocytes do not, however, express E-cadherin.

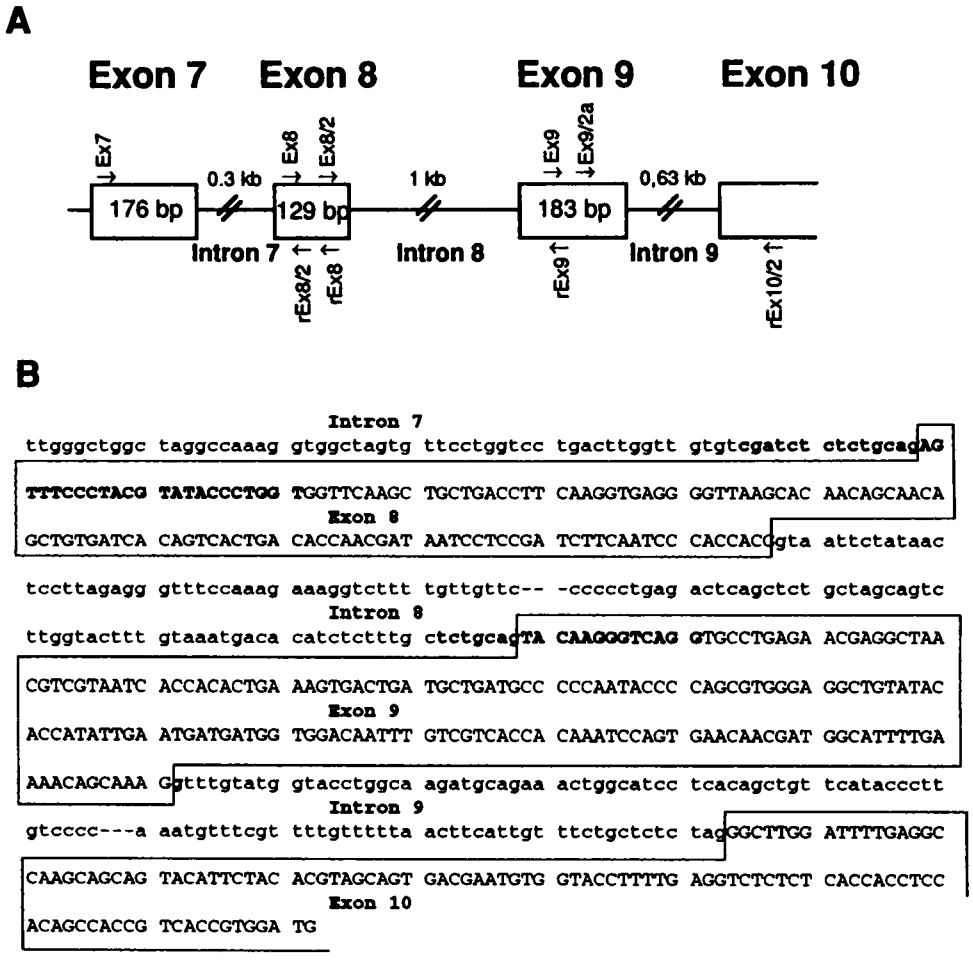

Fig. 3. E-cadherin genomic DNA sequence in the region around exon 8 and 9. *A*, schematic representation of genomic DNA fragments amplified by PCR. Numbering of exons refers to homologies between mouse (22) and human sequences. *B*, wild type sequence of a portion illustrated in *A*. Exon and intron DNA sequences are shown in *UPPERCASE* and *lowercase*, respectively. **Boldface** nucleotides represent divergent sequences detected in tumor tissues. *kb*, kilobases.

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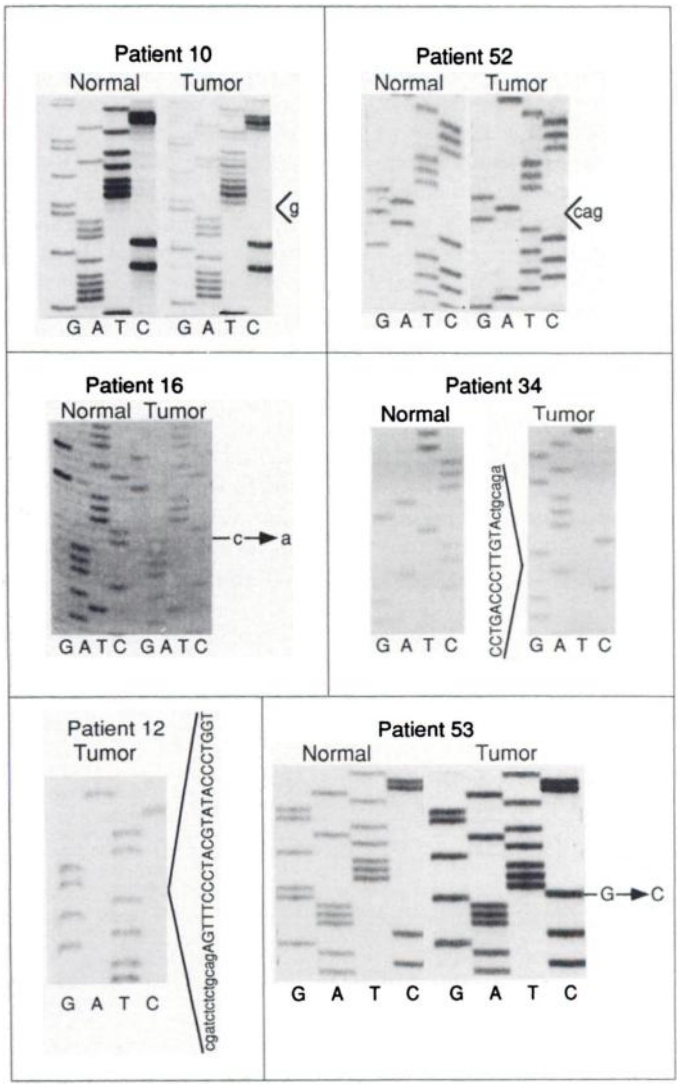

Fig. 4. Mutations in the E-cadherin gene affecting exon/intron boundaries. Autoradiographs of sequencing gels from six patients are shown. In patient 10 a G nucleotide at the exon 9/intron 9 border has been removed in tumor DNA but not in DNA from adjacent nontumorous mucosa. In patient 16 and 53 this G nucleotide is substituted by a T (C-to-A transversion is indicated because the complementary strand is shown) or a C, respectively. Twenty nucleotides at the intron 8/exon 9 border were lost in tumor tissue from patient 34 (the complementary strand is shown). In patients 12 and 52, 37- and 3-base pair deletions at the intron 7/exon 8 boundary were found in the tumor DNA, respectively. (DNA from nontumorous mucosa was not available from patient 12.)

Deletion of either exon 8 or 9 does not change the reading frame of the E-cadherin transcript as both exons start and end with complete codons. The altered transcripts encode a protein lacking either 43 or 61 amino acids from the extracellular portion. The 69-nucleotide deletion from exon 10 found in one patient also does not alter the protein's reading frame. Using two different monoclonal antibodies, we detected E-cadherin immunoreactivity in tumor and lymph node metastases of all patients expressing mutated E-cadherin mRNA (Fig. 6).

After having found E-cadherin mRNAs lacking putative calcium binding sites in 11 patients (and a 69-nucleotide deletion in another case), we reasoned that mutations within sequences necessary for the adhesive function are also likely in gastric carcinomas. From 41 patients displaying a normal sized E-cadherin amplification product we directly sequenced PCR amplified cDNA fragments spanning 6 putative calcium binding sites and the cytoplasmic domain (see Table 1 for the patients analyzed with this method). In patient 1, a point mutation in codon 473 (GTC to GAC) resulting in the substitution of valine to aspartic acid was detected in exon 10 (Fig. 7). This amino acid is located adjacent to a putative calcium binding site. The alteration is presumably a somatic mutation because it was not seen in noncancerous tissue from the same patient. In another patient (patient 20) we observed an A to C transversion in codon 370 in exon 8. The corresponding amino acid alteration is a substitution of alanine for a highly conserved aspartic acid (D) in the motif DTNDN. Similar motifs have been found in other cadherin molecules and are thought to directly complex calcium (21). The wild type sequence was also present in PCR amplified cDNA from this tumor (see Fig. 7). We cannot distingish between the possibilities of whether the tumor tissue analyzed contained nontumorous epithelium, the tumor tissue was heterogeneous, or the second E-cadherin allele was unaltered at this position; both the mutated and wild type form of E-cadherin were present in adjacent nontumorous tissue as well as in a lymph node metastase of this tumor, suggesting the presence of one wild type (with regard to this nucleotide position) and one mutated E-cadherin allele.

Mutations were also detected in intestinal type tumors; 2 of 20 patients were found to have a point mutation. In contrast to the mutations found in diffuse or mixed type carcinomas, which affect the structure of E-cadherin, mutations in the intestinal type tumors will not alter the protein structure. Both of the changes were silent mutations caused by C to T transitions at CpG dinucleotides, one of which

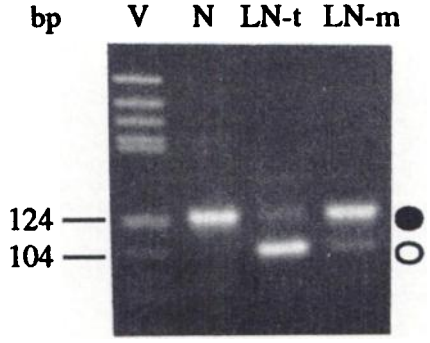

Fig. 5. Loss of the wild type and retention of a mutated allele in metastatic tumor cells. Agarose gel after *Hin*dIII restriction showing loss of the wild type allele in carcinoma cells of a lymph node metastasis from patient 10. The positions of the wild type and mutated alleles are marked by *filled* and *open ovals*, respectively. *N*, normal gastric epithelium from the same patient; *LN-t*, DNA almost exclusively from metastasizing carcinoma cells obtained by the touch preparation method; *LN-m*, DNA from a segment of the cellularly heterogeneous lymph node metastasis as a mass without isolation of tumor cells; *V*, DNA molecular weight marker V, Boehringer, Germany. *bp*, base pairs.

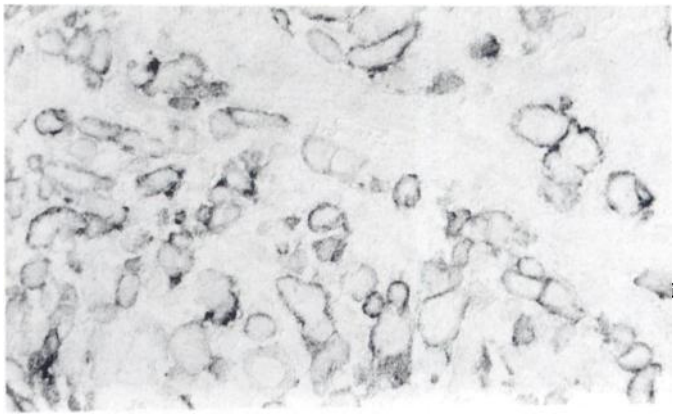

Fig. 6. Detection of E-cadherin immunoreactivity in patients expressing mutated E-cadherin mRNA. Immunohistochemical staining of tumor cells of patient 10 lacking exon 9 in the E-cadherin mRNA is shown using monoclonal antibody 6F9.

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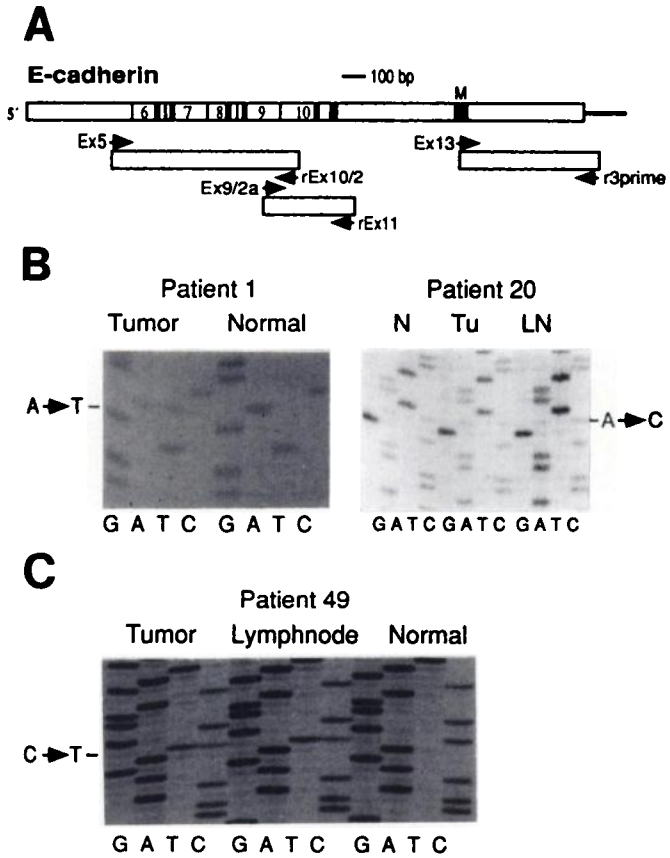

Fig. 7. Screening for E-cadherin mutations in 6 putative calcium binding domains and in the cytoplasmic region by direct cDNA sequencing. *A*, positioning of the amplified human sequences by homology comparison (14) (22). *Vertical thick black bars*, putative calcium binding sites; *horizontal thick black bar*, 3'-nontranslated sequence. *M*, membrane spanning domain. Exons 7–10 are numbered. *B, C*, autoradiographs of sequencing gels showing point mutations in the E-cadherin cDNA. In patient 1 a T to A transversion was found in exon 10 (the complementary sequence is shown). In patient 20 an A to C transversion was detected, destroying a putative calcium binding site in exon 8. A silent C to T transition was observed in patient 49. *bp*, base pairs; *N*, noncancerous; *Tu*, tumorous; *LN*, lymph node metastasis.

is located in a calcium binding site in exon 7 (GAC GCG to GAT GCG; both codons GAC and GAT code for aspartic acid) (Fig. 7). The other mutation is located in exon 14 (AAC GTT to AAT GTT; the codons AAC and AAT both code for asparagine). Only the wild type sequence was found in noncancerous tissue from case 49 (exon 7), excluding polymorphic alleles. Normal tissue from patient 13 was not available; a sequence polymorphism cannot be ruled out. E-cadherin mutations detected in gastric cancer are summarized in Table 2 and Fig. 8. In 50% of diffuse type and in 14% of mixed gastric tumors E-cadherin mutations were found. In intestinal tumors, however, only two silent point mutations were seen.

DISCUSSION

Gastric carcinoma is one of the most common malignancies worldwide. There is a need to elucidate the molecular mechanisms of gastric tumorigenesis and to correlate gene mutations with the biological behavior of these tumors. It has long been known that tumor cells of diffuse type gastric carcinomas, cancers with a worse prognosis when they become advanced (24), show diminished homophilic cell-to-cell cohesion (25). The reason for these findings were not clear at that time.

Since these observations were made, molecules involved in mediating cell-to-cell interactions have been identified and characterized. Intercellular adhesion molecules can be subdivided into two main groups depending upon calcium dependency of the adhesion mechanism (26). Homophilic adhesion mediated by calcium dependent molecules, the cadherins, have been shown to be tighter than adhesion mediated by calcium independent cell adhesion molecules (1). In the last few years E (epithelial)-cadherin has been analyzed in a variety of human carcinomas. In most studies the immunoreactivity of E-cadherin has been shown to be inversely correlated with tumor differention; *e.g.*, loss or reduced immunoreactivity has been found in poorly differentiated tumors compared with the nontumorous epithelium from the same patients (27).

In gastric cancer none of 21 diffuse type gastric carcinomas examined showed any E-cadherin immunoreactivity (9). In contrast, almost 30% (5 of 17) of signet ring cell carcinomas (which belong to the diffuse type tumors) were E-cadherin immunoreactive (8). Shimoyama *et al.* (7) reported that 68% (19 of 28) of scattered (diffuse) type carcinomas showed strong E-cadherin immunoreactivity; nevertheless, the tumor cells do not have the ability to form aggregates. It should be noted that E-cadherin immunoreactivity does not provide information about the gene integrity.

In contrast to most E-cadherin studies on human tumors, we have analyzed the cell adhesion molecule in much more detail and focused our analysis on the detection of structural changes of the E-cadherin molecule *in vivo*. In 50% of diffuse type and 14% of mixed gastric

Table 2 *E-cadherin gene mutations in gastric carcinomas*

Patient	Laurén	Mutation[a] (codon)	Effect of mutation
1	Diffuse	GTC to GAC (473)	Val to Asp
10[b]	Diffuse	AAAG/gttt to AAAG/ttt	Skipping exon 9[c]
12	Diffuse	37-base pair deletion	Skipping exon 8[c]
13	Intestinal	AAC to AAT (751)	
16	Diffuse	AAAG/gttt to AAAG/tttt	Skipping exon 9[c]
20	Diffuse	GAT to GCT (370)	Asp to Ala (calcium binding region)
23	Diffuse	ND[d]	Skipping exon 8
28	Diffuse	ND	Skipping exon 9
29	Diffuse	ND	Skipping exon 9
30	Mixed	ND	Skipping exon 9
34	Diffuse	20-base pair deletion	Skipping exon 9
43	Diffuse	ND	Deletion of the first 69 bp of exon 10
49	Intestinal	GAC to GAT (288)	
50	Diffuse	ND	Skipping exon 8
52	Diffuse	tctct**gcag**/AGTT to tctct**g**/AGTT[e]	Skipping exon 8[c]
53	Diffuse	AAAG/gttt to AAAG/ttt	Skipping exon 9

[a] **Boldface** and **underlined** nucleotides were mutant; lowercase letters, introns; uppercase letters, exons.
[b] LOH was detected in a lymph node metastasis from this patient.
[c] These exon deletions have been reported previously (10).
[d] ND, not determined.
[e] The deleted sequence can be either gca or cag.

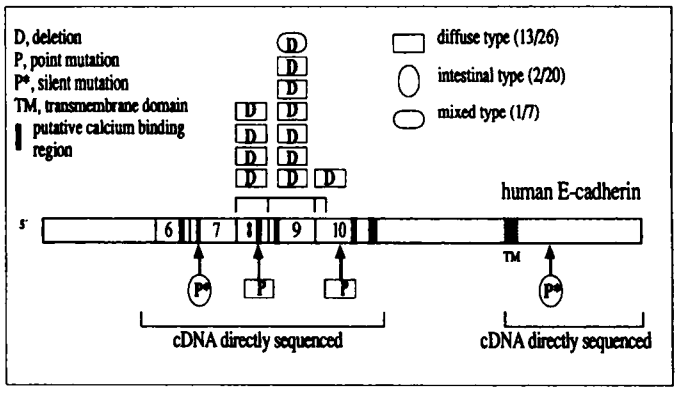

Fig. 8. Overview. E-cadherin mutations in gastric carcinomas.

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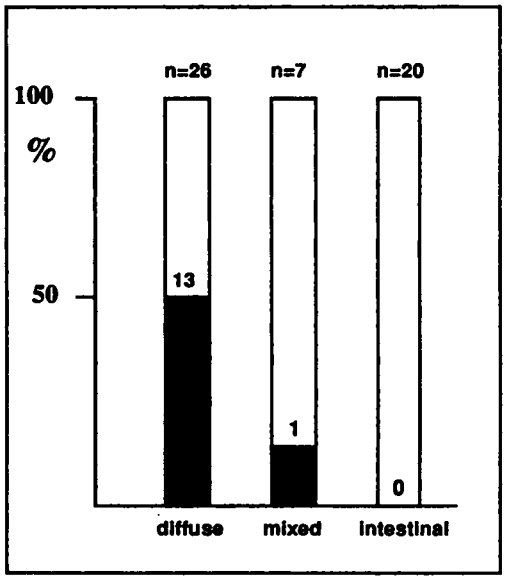

Fig. 9. Frequency of E-cadherin alterations affecting its structure in gastric carcinomas.

carcinomas E-cadherin alterations affecting its structure were detected (Fig. 9). In 11 patients either exon 8 or exon 9 was missing from the E-cadherin mRNA, removing potential sites determining adhesive capacity. It is reasonable to speculate that E-cadherin molecules lacking either exon 8 or exon 9 are functionally inert, since amino acid substitutions affecting a single calcium binding site have been shown to abolish the adhesive function (28). Removal of complete calcium binding domains due to loss of these exons would be expected to generate inactive E-cadherin adhesion receptors, facilitating scattering of carcinoma cells. In six of these patients splice site mutations responsible for the exon deletions were found. Interestingly, a remarkably large proportion (16 of 38) of mutations in the dihydrofolate reductase gene in cultured Chinese hamster ovary cells were induced by a polycyclic aromatic hydrocarbon derivative at pre-mRNA consensus splice sites, resulting in splicing defects (29). Similar chemicals are a widespread class of environmental pollutants. Such splice site mutagens, possibly present in the diet, may induce genetic alterations in gastric mucosal cells.

In addition to exon skipping, several point mutations were also found in the E-cadherin cDNA. However, only the two mutations detected in diffuse tumors would result in a change in the structure of the E-cadherin protein. In one case the mutated sequence was also found in adjacent nontumorous gastric mucosa. It will be interesting to see whether the second E-cadherin allele is intact over its entire sequence in this tumor. The two point mutations in intestinal type tumors have no effect on the amino acid sequence of E-cadherin and may be considered to be silent. The prevalence of mutations in diffuse carcinomas provides strong *in vivo* evidence that E-cadherin alterations play a major role in the development of this type of cancer. On the other hand, 50% of the diffuse tumors analyzed *did not* contain mutations of E-cadherin. Other mechanisms leading to a scattered phenotype of diffuse type gastric carcinomas should be considered: (*a*) other parts of E-cadherin that were not analyzed in this study (compare Fig. 8) could carry mutations affecting its adhesive function; (*b*) down-regulation of E-cadherin could be responsible for the weak mutual adhesiveness of the tumor cells (9); (*c*) alterations or down-regulation of membrane associated molecules interacting with cadherins, the catenins, may negate the adhesive action of E-cadherin. Catenins transmit the adhesion signal from cadherins to the cytoskeleton (30). In a human lung carcinoma cell line, PC9, showing reduced cadherin-mediated aggregation activity part of the α-catenin gene has been found to be deleted (31). Furthermore, loss of α-catenin immunoreactivity due to a homozygous deletion of the α-catenin gene was observed in a human prostate cancer cell line (32); (*d*) tyrosine phosphorylation of β-catenin may lead to reduced adhesivness of carcinoma cells (33).

In one patient the allelic status of the E-cadherin gene was analyzed. Here the wild type E-cadherin cDNA fragment was missing in both the primary tumor and in a lymph node metastasis. In nontumorous mucosa only the wild type allele was seen, whereas in tumor cells obtained by touch preparation of a lymph node metastasis the mutated E-cadherin allele (lacking a G nucleotide at the exon 9/intron 9 border) was the most prominent. This finding indicates that in the primary tumor and the metastasis of this patient the wild type allele was lost and the mutated allele was retained. The concept of E-cadherin as an invasion and metastasis suppressor (27, 33, 34) has, therefore, now been further strengthened.

LOH of the *uvo* locus at chromosome 16q22.1, in close proximity to the location of the E-cadherin gene (35), has been observed in hepatocellular carcinomas, where it was correlated with tumor progression (36). In prostate, breast carcinomas, and Wilm's tumor frequent LOH has also been detected on the long arm of chromosome 16 and provides a further indication for a tumor suppressor gene at this location (37–39). The detection of E-cadherin gene mutations and LOH in gastric carcinomas now provides more evidence supporting its categorization as a tumor suppressor gene. Recent studies reporting interactions of β-catenin with the APC tumor suppressor protein conform to this hypothesis (40, 41). The association of the *APC* gene product with catenins may indicate an important link between tumorigenesis and cell adhesion.

Cell adhesion molecules have recently been postulated to be encoded by tumor suppressor genes: the *fat* gene, a cadherin homologue in *Drosophila*, is required for correct morphogenesis and functions as a tumor suppressor gene (42); the *DCC* (deleted in colon carcinoma) gene, a member of the immunoglobulin superfamily of adhesion receptors, is located on a region of chromosome 18q that is often lost in malignant colorectal carcinomas (43) and in intestinal type gastric carcinoma (diffuse cancers were not examined) (44); the recently isolated von Hippel-Lindau disease tumor suppressor gene may be involved in signal transduction or cell adhesion (45). Submembranous junctional plaque proteins may function as tumor suppressor genes as well (46).

We describe here the first E-cadherin gene mutations in human cancer *in vivo*. Since in one patient LOH with retention of a mutated allele was detected and only the mutated but not normal gene transcript was seen in four other diffuse type gastric carcinomas and lymph node metastases, it is tempting to speculate that E-cadherin may be encoded by a tumor/metastasis suppressor gene in this type of tumor.

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