Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 2316–2321, March 1999
Medical Sciences

Mutated epithelial cadherin is associated with increased tumorigenicity and loss of adhesion and of responsiveness to the motogenic trefoil factor 2 in colon carcinoma cells

J. A. Efstathiou*†, D. Liu†‡, J. M. D. Wheeler*†, H. C. Kim*, N. E. Beck*, M. Ilyas*, A. J. Karayiannakis‡, N. J. McC. Mortensen§, W. Kmiot‡, R. J. Playford‡, M. Pignatelli‡, and W. F. Bodmer*¶

*Cancer and Immunogenetics Laboratory, Imperial Cancer Research Fund, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom; ‡Division of Investigative Science, Imperial College of Science, Technology and Medicine, Hammersmith Campus, Du Cane Road, London W12 ONN, United Kingdom; and §Department of Colorectal Surgery, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

Contributed by Walter F. Bodmer, December 18, 1998

ABSTRACT **Epithelial (E)-cadherin and its associated cytoplasmic proteins (α-, β-, and γ-catenins) are important mediators of epithelial cell–cell adhesion and intracellular signaling. Much evidence exists suggesting a tumor/invasion suppressor role for E-cadherin, and loss of expression, as well as mutations, has been described in a number of epithelial cancers. To investigate whether E-cadherin gene (*CDH1*) mutations occur in colorectal cancer, we screened 49 human colon carcinoma cell lines from 43 patients by single-strand conformation polymorphism (SSCP) analysis and direct sequencing. In addition to silent changes, polymorphisms, and intronic variants in a number of the cell lines, we detected frameshift single-base deletions in repeat regions of exon 3 (codons 120 and 126) causing premature truncations at codon 216 in four replication-error-positive (RER+) cell lines (LS174T, HCT116, GP2d, and GP5d) derived from 3 patients. In LS174T such a mutation inevitably contributes to its lack of E-cadherin protein expression and function. Transfection of full-length E-cadherin cDNA into LS174T cells enhanced intercellular adhesion, induced differentiation, retarded proliferation, inhibited tumorigenicity, and restored responsiveness to the migratory effects induced by the motogenic trefoil factor 2 (human spasmolytic polypeptide). These results indicate that, although inactivating E-cadherin mutations occur relatively infrequently in colorectal cancer cell lines overall (3/43 = 7%), they are more common in cells with an RER+ phenotype (3/10 = 30%) and may contribute to the dysfunction of the E-cadherin–catenin-mediated adhesion/signaling system commonly seen in these tumors. These results also indicate that normal E-cadherin-mediated cell adhesion can restore the ability of colonic tumor cells to respond to trefoil factor 2.**

Cell-to-cell adhesion plays a critical role in the development and maintenance of complex differentiated epithelial tissues and structures in multicellular organisms. Interference with cell attachment, independence of growth control, and increased migration have long been implicated during the neoplastic process (1, 2). It is now apparent that the hallmarks of epithelial malignancy, that is to say, uncontrolled proliferation, derangement of cellular and morphological differentiation, invasion, and colonization to distant organs, can be explained, at least in part, by alterations in the adhesive properties of neoplastic cells.

The cadherins constitute a large family of calcium-dependent cell adhesion molecules (3). The human epithelial (E)-cadherin gene (*CDH1*) maps to chromosome 16q22 and encompasses 16 exons (4). It encodes a 120-kDa transmembrane glycoprotein (5) that localizes mainly to the zonula adherens junctions and serves as the prime mediator of epithelial cell-to-cell adhesion through homotypic interactions of its extracellular domain. E-cadherin's cytoplasmic carboxyl tail links to the actin microfilament network of the cellular cytoskeleton through a group of closely related but distinct membrane undercoat proteins, termed the catenins (α, β, and γ) (6, 7). Such binding is essential for the adhesive function of E-cadherin (8). The catenins also act as important components of intracellular signal transduction pathways and have been found to associate with molecules that have growth-regulatory and signaling functions, such as the epidermal growth factor (EGF) receptor (9), the oncogene c-*erbB2* (10), the product of the adenomatous polyposis coli (APC) tumor suppressor gene (11), the negative regulator of the wnt-signaling pathway, axin, and the Tcf-Lef transcription factors (12). APC competes directly with E-cadherin for binding to β-catenin (13) and may, therefore, be an indirect regulator of E-cadherin-mediated adhesion. Mutations in APC and β-catenin are common events during colorectal cancer and affect the stoichiometric balance between the various catenin complexes.

A number of studies have confirmed that structural and functional integrity of the components of the E-cadherin/catenin complex are necessary not only during cell adhesion but also during embryogenesis, normal cell cycling, polarization, and differentiation (14). Furthermore, perturbation of E-cadherin/catenin-mediated cell adhesion is associated with cell migration and the early reparative response of epithelial restitution after mucosal ulceration in inflammatory bowel disease (15). Cell–cell junctional proteins, when promoting cell motility, appear to be common targets for soluble growth factors and cytokines, such as the epidermal growth factor (EGF), the transforming growth factor α (TGFα), the hepatocyte growth factor/scatter factor (HGF/SF), as well as the trefoil peptides [pS2, intestinal trefoil factor, and human spasmolytic polypeptide (hSP)] (16).

Loss of function or expression of any of the E-cadherin/catenin complex components appears to be associated with loss of epithelial differentiation and normal tissue architecture and the acquisition of a motile, invasive phenotype (14). Abnormalities in the expression and cellular localization of E-cadherin are frequently associated with high tumor grade, infiltrative growth, and lymph node metastasis in a variety of human malignancies, including prostate, breast, bladder, pancreas, stomach, and colon tumors (17–21).

There are overwhelming genetic data to support the role of E-cadherin as a tumor/invasion suppressor in epithelial cells. Loss of heterozygosity on 16q is detected frequently in metastasizing malignancies derived from the liver, prostate, and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviations: E-cadherin, epithelial cadherin; APC, adenomatous polyposis coli; hSP, human spasmolytic polypeptide; RER, replication-error; SSCP, single-strand conformational polymorphism.

†J.A.E., D.L., and J.M.D.W. contributed equally to this work.
¶To whom reprint requests should be addressed. e-mail: walter.bodmer@hertford.ox.ac.uk.

breast (22–24). Mutations in *CDH1* have been described in a number of human cancers, including breast, stomach, endometrium, ovary, and thyroid (25). Inactivating mutations are commonly found in two histological subtypes of poorly cohesive tumors, namely lobular breast and diffuse-type gastric carcinomas (26, 27). It has also been shown that loss of E-cadherin expression in a transgenic mouse model is associated with the development of invasive carcinoma from well differentiated adenomas (28). In addition, germ-line mutations have recently been reported in two studies of early-onset, diffuse-type stomach cancers (29, 30).

Despite many studies documenting loss of E-cadherin expression in colorectal cancer (21, 31, 32), reports of allelic loss at the *CDH1* locus have been rare and, to our knowledge, no mutations have been described. In this study we screened a panel of 49 human colorectal carcinoma cell lines, derived from 43 patients, for mutations in all of the E-cadherin exons and the corresponding intron–exon junctions. Having identified a truncating mutation in LS174T (along with a splice site mutation), which lacks E-cadherin protein expression, we introduced full-length E-cadherin cDNA into the cell line to examine the effects on adhesion, differentiation, proliferation, tumorigenicity, and cytokine-stimulated migration.

MATERIALS AND METHODS

Cell Lines, Tissue Culture, and DNA Extraction. The 49 human colorectal carcinoma cell lines derived from 43 patients used in this study are listed in Table 1. Cells were maintained in 25-cm^2 sterile Falcon tissue culture flasks (Becton Dickinson) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in a 10% CO_2/90% air atmosphere. Genomic DNA was extracted by standard techniques.

Monoclonal Antibodies and hSP. A mouse monoclonal antibody against human E-cadherin (HECD-1) was kindly provided by M. Takeichi (Kyoto University, Kyoto, Japan), and the anti-mouse E-cadherin antibodies (ECCD-2 and DECMA-1) were obtained from Takara Shuzo (Kyoto) and Sigma (U.K.), respectively. Purified mouse monoclonal antibodies against human α-, β-, and γ-catenins were obtained from Transduction Laboratories (Lexington, KY).

Recombinant hSP was produced in a yeast vector system and purified as described previously (33). On the basis of our previous experience regarding its activity (34), the glycosylated form of hSP was used in this study at a dose of 100 mg/ml (6.8 mM).

Polymerase Chain Reaction (PCR) Amplification. Using previously described intron-complementary primers, we amplified all 16 exons (including the intron–exon boundaries) of the E-cadherin gene. All reactions were carried out in a total volume of 50 μl with final reaction concentrations of 1× standard PCR buffer (Promega), 200 mM dNTPs, 1.5 mM Mg^{2+}, 0.2 mM each primer, and 1 unit of *Taq* DNA polymerase. Because of the high G+C content of exon 1, 5% (vol/vol) dimethyl sulfoxide was added and an annealing temperature of 70°C was used. To reduce the size of the amplicon spanning exons 4–5 [to a size appropriate for single-strand conformational polymorphism (SSCP) analysis], the PCR product was digested by adding 2 units of *Rsa*I enzyme to the PCR mixture and incubating at 37°C for 2 h.

SSCP Analysis. This analysis was performed as previously described (35). To evaluate whether any observed SSCP band shifts in the cell lines were polymorphisms, we screened 89 samples of genomic DNA prepared from the peripheral blood of random human controls, in the regions of interest.

Direct Sequencing of the E-Cadherin Gene. The nucleotide sequences of the PCR products showing an abnormal electrophoretic mobility on SSCP analysis were determined by direct sequencing. The sequences obtained from our experiments were compared with the published sequences of the E-cadherin exons (GenBank accession nos. D49685 and Z35402–Z35415) by using SEQUENCHER 3.0 software (Applied Biosystems).

Table 1. Forty-nine human colorectal cancer cell lines with their replication-error (RER) status and the presence of E-cadherin mutations and polymorphisms

Cell line	RER	Codon	Nucleotide	Consequence
HRA19	−			
VACO5	+			
VACO4S/4A	−			
VACO10MS	?	B		
T84	−	B		
SW948	−	B		
SW837	?	B		
SW620/480	−			
SW48	+	B		
SW403	−	B		
SW1417	−	B*		
SW1222	?			
SCKO-1	−	C		
PC JW	−	B*		
LS411	+	B*		
LS174T	+	126,	GCCCCCCG → GCCCCCG	Single-bp deletion Stop (codon 216)
		Intron 8 + 1	ACG**g**taa → ACG**a**taa	
		B		
LS1034	−	B		
LOVO	+	B		
LIM1863	−	A		
		B		
HT29/WIDR	−	B*		
HCT116	+	120	TGGG**G**C → TGGGC	Single-bp deletion Stop (codon 216)
		B		
HCA7	+			
HCA46	−			
DLD1/HCT15	+	B*		
COLO320DM	−	B		
COLO201/205/206	−	Intron 1 + 15	tcccc**t**ga → tcccga	Two-bp deletion
CC20	−	B*		
CC07	−			
CACO2	−	B		
C99	−			
C84	−	B*		
C80	−	B		
C75	−	A		
C70	−	C		
C32	−	C		
C106	−	B		
C10	−			
SW1116	−	B		
GP2d/5d	+	126	GCCCCCCG → GCCCCCG	Single-bp deletion Stop (codon 216)
		B		
LS180	+			
COLO741	?	C		
HT55	−	B*		

?, Borderline RER; A, intron 4 + 10 gtagagaaa**g** → gtagagaaa**c**; B, codon 692 GC**C** → GC**T** Ala → Ala; C, codon 751 AA**C** → AA**T** Asn → Asn; *, homozygous.

Determination of the Replication-Error (RER) Status of the Cell Lines. To determine the RER status of the cell lines, we amplified BAT-26 (36), a single poly(A) tract, by using fluorescently labeled primers and PCR conditions similar to those previously described. Three microliters of diluted (1:10) PCR product were combined with 2 μl of formamide loading buffer and 0.5 μl of Rox size standard. This mixture was denatured at 96°C for 5 min and quenched on ice prior to gel loading (4.25% denaturing acrylamide) on a 377 Prism fluorescence-based, semiautomated DNA sequencer (Applied Biosystems). Results were analyzed with GENESCAN software (version 2.0.2). All PCRs and analyses were repeated at least in duplicate; any cell lines presenting ambiguous results were further investigated with BAT-25; and all RER+ designations were confirmed by using the CA repeat marker D15S58.

Transfection of Mouse E-Cadherin cDNA into LS174T Cells. LS174T cells were cotransfected with mouse full-length E-cadherin cDNA in the expression plasmid pBATEM2 (kind gift from M. Takeichi) together with the hygromycin-resistant gene in the expression plasmid pBabeHygro (37) using the Lipofectin reagent (GIBCO/BRL). pBabeHygro was transfected simultaneously to serve as a vector control. Cultures were selected in 0.8 mg/ml hygromycin B (Boehringer, U.K.). Resistant colonies were ring-cloned from the cultures for analysis and tested for mycoplasma.

Western Blotting, Immunoprecipitation, and Immunofluorescence. These procedures were performed as described previously (38) (see Fig. 1*A*).

Cell–Cell Aggregation Assay. This assay was performed as described previously (39). The degree of cell–cell adhesion was evaluated by calculating the cell aggregation index N_t/N_0, where N_0 is the total number of single cells before incubation and N_t is the total number of single cells after incubation for t min ($N_t/N_0 = 1$: no cell–cell adhesion; $N_t/N_0 < 1$: specific cell–cell adhesion). For the blocking experiment, LSBAT17 (transfected LS174T) cells were preincubated with the antibody DECMA-1 (20 μg/ml) for 1 hr before the assay.

Growth in Soft Agar. Autoclaved agar (1.6%; Sigma) was mixed with an equal volume of 2× DMEM/20% FCS and plated onto 35-mm Petri dishes (Nunc). Cells (5×10^4) were resuspended in 0.6% autoclaved agar and mixed with an equal volume of 2× DMEM/20% FCS before they were overlaid on the basal gel. After 14 days of culture at 37°C in a 10% CO_2 atmosphere, the colonies (>30 cells) were counted under a phase-contrast microscope (Olympus). Triplicate dishes were used in each experiment.

Three-Dimensional Collagen Gel Assay. This assay was performed as previously described (40). After 4–10 days, the gels were fixed with 10% neutral buffered formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin/eosin.

Production of Xenograft Tumors. Congenitally athymic female nude mice between 5 and 6 weeks old were used (Clare Hall Laboratories, Imperial Cancer Research Fund). Approximately 10^6 cells of the E-cadherin transfectant cell line (LSBAT17) or the parental cell line (LS174T) were resuspended in 0.2 ml of phosphate-buffered saline (PBS) and inoculated into one subcutaneous site on each mouse. Six mice were used for each cell line. Tumor masses were measured every other week until 35 weeks. Student's t test was performed to analyze the significance of the difference between the LS174T and LSBAT17 cell groups.

Transwell Motility Assay. Falcon transwell chemotaxis chambers (Becton Dickinson) with 12-mm-diameter, 8-μm pore polycarbonate filters were used for the assay. Cells (1×10^5) from the above cell lines, suspended in 1.0 ml of DMEM/2% FCS, were added to the upper chamber, and 1.0 ml of DMEM/2% FCS containing 100 μg of recombinant hSP was placed in the lower compartment. Wells in which the lower part was filled with culture medium alone were used as controls. Cells were incubated for 72 hr and each experiment was performed in triplicate. At the end of the incubation, the nonmotile cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters containing the migrated cells were fixed in methanol, stained in Diff-Quick (Baxter Diagnostics), and mounted on glass slides. Migrated cells were counted across two diameters and values are expressed as a percentage of the total number of cells seeded in the upper compartment.

RESULTS

SSCP and Sequence Analysis. We detected exon 3 frameshift deletion mutations causing a downstream premature stop (TGA) at codon 216 in four cell lines. In LS174T and the GP2d and GP5d cells (which displayed similar SSCP band shifts and come from the same patient, Fig. 2) this stop codon was produced by a single-base deletion in a C_6 tract at codon 126; in HCT116 it was caused by a single-base deletion in a G_4 tract at codon 120 (Table 1). In all cases, the presence of the wild-type sequence indicates that these cell lines are heterozygous for their respective mutations. No similar bandshifts were detected in exon 3 in the 89 human random controls examined. A single base substitution (ACG**g**taa → ACG**a**taa) at the exon 8 splice donor site was also detected in LS174T, and it was presumed that this was the "second hit."

In COLO201, COLO205, and COLO206, all of which are derived from the same patient and grow as semi-adherent lines, we detected a heterozygous two-base deletion in a C_4 tract of intron 1 (12 bases in from exon 1). Again the corresponding SSCP band shift was not present in control samples.

We also identified various polymorphisms in a number of our cell lines and in our human random controls. These genetic variants included an intron 4 change (gtagagaaa**g** → gtagagaaa**c**), an intron 12 alteration (a**t**t → a**c**t), and silent changes in exon 13 at codon 692 (GC**T** → GC**C**; Ala → Ala) and in exon 14 at codon 751 (AA**C** → AA**T**; Asn → Asn). The polymorphic variants in intron 4 and at codons 692 and 751 have been described by other groups (26, 27, 41). It is worth noting that in one of our human random controls, we also detected a substitution (**G**CA → **T**CA) in exon 13 at codon 698 causing an Ala-to-Ser amino acid change. It would be interesting to establish whether this might be associated, for example, with any increased risk of colon cancer as is the case of the APC missense variants (42).

RER Status of the Cell Lines. The RER statuses of the cell lines are listed in Table 1. Using BAT-26, we were able to classify our cell lines into three groups: RER+, RER−, and borderline. These results are in agreement with previous published papers on the RER status in some of the above cell lines. Twenty-three percent of our cell lines are RER+, including the four lines with the exon 3 *CDH1* frameshift deletions.

Expression of Mouse E-Cadherin cDNA in Human Colorectal Carcinoma Cells. HCT116, GP2d, and GP5d have been shown to express the E-cadherin protein, whereas LS174T has been shown to be E-cadherin negative. For this reason the LS174T cell line was selected for wild-type E-cadherin transfection. Twenty-one colonies of E-cadherin transfectants were isolated after selection in hygromycin B, and three clones (LSBAT1, LSBAT10, and LSBAT17) proved to be positive by Western blotting, immunofluorescence, and Southern blotting (data not shown) for stable integration and expression of the E-cadherin expression vector. All three cell lines were tested and found to be negative for mycoplasma contamination. LSBAT17 cells expressed higher levels of E-cadherin than the other two colonies of transfectants (LSBAT1 and -10). This finding was confirmed by fluorescence-activated cell sorting (FACS) analysis (data not shown). Positive cells in the LSBAT17 cell line were increased by 70% after two cycles of immunofluorescence-labeled cell sorting. Therefore the LSBAT17 cell line was selected for further experiments.

Intercellular Adhesion of E-Cadherin Transfectants. To investigate the function of exogenous E-cadherin in the transfectants, intercellular adhesion was determined with a cell–cell

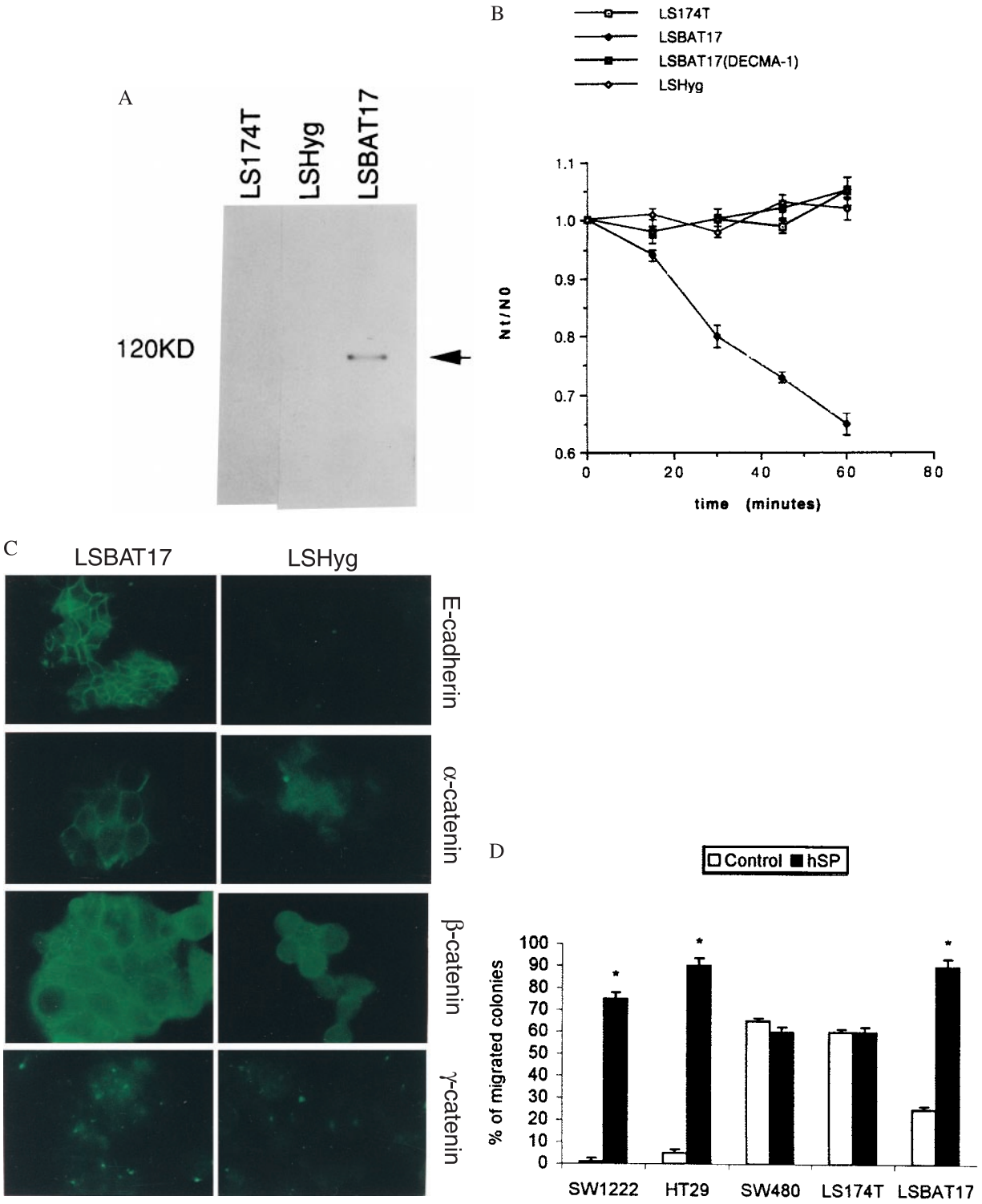

FIG. 1. (*A*) Western blotting of LS174T, LSHyg, and the transfectant LSBAT17 cells that express the 120-kDa E-cadherin protein. (*B*) Cell–cell aggregation assay. N_0 is the total number of single cells before incubation and N_t is the total number of single cells after incubation for t min. DECMA-1 (anti-mouse E-cadherin antibody) was used to block LSBAT17, which inhibited the increased cell-to-cell adhesion seen in these cells. (*C*) Immunofluorescence staining showing expression of E-cadherin and catenins. LSBAT17 cells showed membranous expression of α-catenin, whereas the control transfectants LSHyg did not. LSBAT17 cells showed greater β-catenin staining compared to LSHyg cells. Both LSBAT17 and LSHyg cells showed low levels of γ-catenin expression. (*D*) Transwell motility assay. At the final time point, 85% of LSBAT17 cells had migrated through the membrane, suggesting that the stimulatory effect of hSP is associated with E-cadherin expression.

aggregation assay. The number of single cells in LSBAT17 decreased after incubation for 1 hr, indicating that intercellular adhesion was enhanced in these cells. This increased cell–cell adhesion was inhibited by blocking with anti-mouse E-cadherin antibody DECMA-1, implying that the exogenous E-cadherin was functional. The control transfected cell line LSHyg did not show changes in intercellular adhesion (Fig. 1*B*).

Induction and Redistribution of Catenin Expression in E-Cadherin Transfectants. To evaluate whether transfection of E-cadherin altered the expression of endogenous catenins, α-, β-,

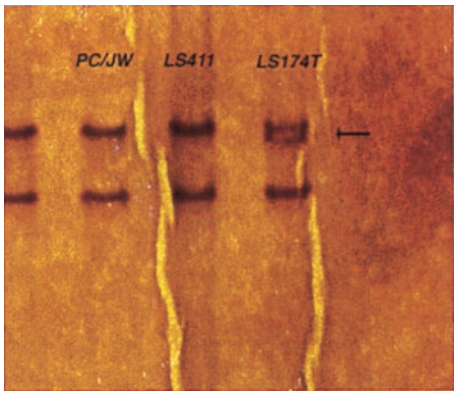

FIG. 2. SSCP analysis of exon 3 of E-cadherin, showing the heterozygous band mobility of LS174T (arrow). Sequencing showed this to be a single base pair deletion causing a premature stop at codon 216.

and γ-catenin localization was evaluated by immunofluorescence staining (Fig. 1*C*). LSBAT17 cells showed membranous expression of α-catenin, whereas the control transfectants LSHyg did not express α-catenin. LSBAT17 cells showed β-catenin intercellular staining compared with the LSHyg vector control, which expressed only low levels of β-catenin in the cytoplasm. There were similar low levels of γ-catenin expression in LSBAT17 and LSHyg cells.

Induction of Cell Polarity of E-Cadherin Transfectants in Collagen Gel. To evaluate whether transfection of E-cadherin can alter morphology, the E-cadherin transfectants LSBAT17 and parental cell line LS174T were grown in three-dimensional collagen gel and monolayer culture. The LS174T cell line showed poorly differentiated and unpolarized morphology with a poorly cohesive growth pattern under both conditions. In contrast, the E-cadherin transfectant LSBAT17 cells were more cohesive and tightly connected with each other in monolayer, and formed well organized, cohesive aggregates with polarization of nuclei toward the periphery in a collagen gel (data not shown).

Growth of E-Cadherin Transfectants in Soft Agar. LSBAT17 and the vector control LSHyg cells were grown in anchorage-independent (soft agar) conditions. The number of colonies in LSBAT17 cells showed a 2.7-fold reduction compared with LSHyg cells. Inhibition of cell growth was confirmed in anchorage-dependent conditions by using a colorimetric assay (data not shown).

Inhibition of Tumorigenicity in E-Cadherin Transfectants in Nude Mice. To further evaluate the effect of E-cadherin on tumorigenicity the LSBAT17 and LS174T cells were injected into nude mice. The tumor volume of the E-cadherin transfectant LSBAT17 group significantly decreased in comparison with the parental cell line LS174T group at day 14 ($P = 0.004$) and day 35 ($P = 0.004$). The results are summarized in Table 2.

Transwell Motility Assay. We have previously shown that LS174T cells are unresponsive to the migratory effects of hSP (34). To investigate whether transfection of wild type E-cadherin gene restored the hSP responsiveness, LSBAT17 and LSHyg cells were incubated with hSP. After 72 hr of incubation less than 5% of control cells migrated through the filters. Addition of recombinant hSP resulted in migration of 75% of the E-cadherin-positive HT29 cells ($P < 0.001$). There were no differences in the proportion (5%) of migrated LS174T cells as compared with the controls ($P = 1.0$). However, transfection of this cell line with E-cadherin cDNA increased cell migration significantly. At the final time point, 85% of LSBAT17 cells had migrated through the membrane ($P < 0.001$). These findings suggest that the stimulatory effect of hSP is associated with E-cadherin expression (Fig. 1*D*).

DISCUSSION

In this study, we screened 49 human colorectal cancer cell lines derived from 43 patients for E-cadherin mutations. Single-base deletion mutations were found in nucleotide repeat regions at codons 120 and 126, resulting in frameshifts and premature stops in four RER+ colorectal cancer cell lines (LS174T, HCT116, GP2d, and GP5d) derived from three patients. These truncations lie in the N-terminal, extracellular domain of the protein and are predicted to generate small, secreted E-cadherin fragments with loss of surface expression and no cellular adhesive capacity. Such small products may even interfere with the normal function of full-length E-cadherin molecules. These frameshift mutations were present as heterozygotes, although an additional single-base substitution was detected at the exon 8 splice donor site in LS174T, with apparent retention of a wild-type allele in HCT116, GP2d, and GP5d. It is not clear whether the presumed haploinsufficiency in the latter cases has any function, such as selective significance, or whether the mutations in these lines are simply bystanders due to their RER+ status. A single-nucleotide insertion mutation, in the same C_6 region where the deletions in LS174T, GP2d, and GP5d are found, and unaccompanied by allelic loss, has been described in endometrial cancer (41).

Overall our results suggest that inactivating E-cadherin mutations are not common genetic mechanisms in colorectal tumorigenesis. Only 7% (3/43) of the cell lines from different patients exhibited such events, and in only one allele for two of them. The fact that E-cadherin expression and function is often perturbed in colorectal cancer may be explained by nonmutational mechanisms such as transcriptional inactivation of the gene as a result of aberrant CpG methylation (43, 44) around the promoter region or through disruption of other members of the E-cadherin/catenin complex, such as aberrant tyrosine phosphorylation of β-catenin (45).

Our results do indicate, however, that inactivating E-cadherin mutations occur relatively frequently in a subset of colorectal tumors, namely those with an RER+ phenotype (3/10 = 30%).

Table 2. Tumor volume in nude mice

Mouse no.	Cell line	Tumor volume, cm^3		Cell line	Tumor volume, cm^3	
		Day 14	Day 35		Day 14	Day 35
1	LS174T	0.032	0.11	LSBAT17	0	0
2	LS174T	0.032	0.8	LSBAT17	0	0
3	LS174T	0.064	1.1	LSBAT17	0	0
4	LS174T	0	0.8	LSBAT17	0.005	0.008
5	LS174T	0.036	0	LSBAT17	0.008	0.036
6	LS174T	0.064	1	LSBAT17	0.008	0.036
7	LS174T	0.064	1	LSBAT17	0	0
8	LS174T	0	0	LSBAT17	0.008	0.036
Mean		0.036 ± 0.03	0.6 ± 0.48		0.004 ± 0.004	0.015 ± 0.018

LSBAT17 and LS174T cells were injected into nude mice. The tumour volume of the LSBAT17 group was significantly decreased compared with the LS174T group at day 14 and day 35. Means are given ±SD.

Ten to 20% of sporadic colorectal cancers, and nearly all hereditary non-polyposis colorectal cancers (HNPCC) have DNA mismatch repair defects, leading to replication errors (RERs) (46, 47). It has also been suggested that RER+ tumors follow a slightly different pathway than RER− tumors. Mutations in genes with mononucleotide repeat sequences, such as TGFβRII, have already been described (48), though their functional consequences are not clear (49). The presence of a region in the *CDH1* gene susceptible to mutation in RER+ tumors may increase the probability of functionally relevant E-cadherin changes in these as compared with RER− tumors. Since frequent E-cadherin mutations have been described in a subset of sporadic breast and gastric tumors (26, 27)—i.e., lobular breast and diffuse gastric—it will be also worth investigating colorectal carcinomas with similarly poorly cohesive histological features, such as signet cell, mucinous, and ulcerative-colitis-associated cancers for E-cadherin mutations and expression.

Cases of down-regulation of E-cadherin without mutations in *CDH1* may, of course be due to, for example, methylation of key CpG sites in the promoter region. The frameshift and splice-site mutations in the *CDH1* gene in LS174T clearly explain its lack of E-cadherin protein expression. The reversal of LS174T to an essentially nontransformed phenotype after transfection of the *CDH1* gene, and so expression of E-cadherin, confirms the functional significance of these changes for the evolution of the tumor from which LS174T was derived. It is notable that LS174T has no APC mutation, which suggests that in this tumor the E-cadherin change may have been an alternative to APC expression loss.

The dependence of the stimulatory effect of hSP on cell migration and E-cadherin expression suggests another cell property that may be relevant to tumorigenesis which is altered by changes in E-cadherin expression.

In conclusion, our data lend further support to the important invasion/tumor-suppressor role of E-cadherin and emphasizes the case for further investigation of the role of E-cadherin changes in specific subtypes of tumors.

1. Pignatelli, M. & Vessey, C. J. (1994) *Hum. Pathol.* **25,** 849–856.
2. Pignatelli, M. (1996) *Yale J. Biol. Med.* **69,** 131–135.
3. Takeichi, M. (1988) *Development (Cambridge, U.K.)* **102,** 639–655.
4. Bussemakers, M. J., van Bokhoven, A., Mees, S. G., Kemler, R. & Schalken, J. A. (1993) *Mol. Biol. Rep.* **17,** 123–128.
5. Takeichi, M. (1991) *Science* **251,** 1451–1455.
6. Gumbiner, B. M. & McCrea, P. D. (1993) *J. Cell Sci. Suppl.* **17,** 155–158.
7. Nieset, J. E., Redfield, A. R., Jin, F., Knudsen, K. A., Johnson, K. R. & Wheelock, M. J. (1997) *J. Cell Sci.* **110,** 1013–1022.
8. Shimoyama, Y., Nagafuchi, A., Fujita, S., Gotoh, M., Takeichi, M., Tsukita, S. & Hirohashi, S. (1992) *Cancer Res.* **52,** 5770–5774.
9. Hoschuetzky, H., Aberle, H. & Kemler, R. (1994) *J. Cell Biol.* **127,** 1375–1380.
10. Ochiai, A., Akimoto, S., Kanai, Y., Shibata, T., Oyama, T. & Hirohashi, S. (1994) *Biochem. Biophys. Res. Commun.* **205,** 73–78.
11. Su, L. K., Vogelstein, B. & Kinzler, K. W. (1993) *Science* **262,** 1734–1737.
12. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. & Clevers, H. (1997) *Science* **275,** 1784–1787.
13. Hulsken, J., Birchmeier, W. & Behrens, J. (1994) *J. Cell Biol.* **127,** 2061–2069.
14. Pignatelli, M., Karayiannakis, A. J., Noda, M., Efstathiou, J. & Kmiot, W. A. (1997) in *The Gut as a Model in Cell and Molecular Biology,* eds. Halter, F., Winton, D. & Wright, N. A. (Kluwer, Boston), pp. 194–203.
15. Hanby, A. M., Chinery, R., Poulsom, R., Playford, R. J. & Pignatelli, M. (1996) *Am. J. Pathol.* **148,** 723–729.
16. Alison, M. R., Chinery, R., Poulsom, R., Stamp, G., Ashwood, P., Longcroft, J. M. & Wright, N. A. (1995) *J. Pathol.* **175,** 405–414.
17. Umbas, R., Schalken, J. A., Aalders, T. W., Carter, B. S., Karthaus, H. F., Schaafsma, H. E., Debruyne, F. M. & Isaacs, W. B. (1992) *Cancer Res.* **52,** 5104–5109.
18. Gamallo, C., Palacios, J., Suarez, A., Pizarro, A., Navarro, P., Quintanilla, M. & Cano, A. (1993) *Am. J. Pathol.* **142,** 987–993.
19. Pignatelli, M., Ansari, T. W., Gunther, P., Liu, D., Hirano, S., Takeichi, M., Kloppel, G. & Lemoine, N. R. (1994) *J. Pathol.* **174,** 243–248.
20. Mayer, B., Johnson, J. P., Leitl, F., Jauch, K. W., Heiss, M. M., Schildberg, F. W., Birchmeier, W. & Funke, I. (1993) *Cancer Res.* **53,** 1690–1695.
21. Kinsella, A. R., Lepts, G. C., Hill, C. L. & Jones, M. (1994) *Clin. Exp. Metastasis* **12,** 335–342.
22. Tsuda, H., Zhang, W. D., Shimosato, Y., Yokota, J., Terada, M., Sugimura, T., Miyamura, T. & Hirohashi, S. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6791–6794.
23. Bergerheim, U. S., Kunimi, K., Collins, V. P. & Ekman, P. (1991) *Genes Chromosomes Cancer* **3,** 215–220.
24. Sato, T., Akiyama, F., Sakamoto, G., Kasumi, F. & Nakamura, Y. (1991) *Cancer Res.* **51,** 5794–5799.
25. Berx, G., Becker, K. F., Hofler, H. & van Roy, F. (1998) *Hum. Mutat.* **12,** 226–237.
26. Berx. G, Cleton-Jansen, A. M., Nollet, F., de Leeuw, W. J. F., van de Vijver, M. J., Cornelisse, C. & van Roy, F. (1995) *EMBO J.* **14,** 6107–6115.
27. Becker, K. F., Atkinson, M. J., Reich, U., Becker, I., Nekarda, H., Siewert, J. R. & Hofler, H. (1994) *Cancer Res.* **54,** 3845–3852.
28. Perl, A.-K., Wilgenbus, P, Dahl, U., Semb, H. & Christofori, G. (1998) *Nature (London)* **392,** 190–193.
29. Guilford, P., Hopkins, J., Harraway, J., McLeod, M., McLeod, N., Harawira, P., Taite, H., Scoular, R., Miller, A. & Reeve, A. E. (1998) *Nature (London)* **392,** 402–405.
30. Gayther, S. A., Gorringe, K. L., Ramus, S. J., Hunstman, D., Roviello, F., Grehan, N., Machado, J. C., Pinto, E., Seruca, R., Halling, K., MacLeod, P., Powell, S. M., Jackson, C. E., Ponder, B. A. J. & Caldas, C. (1998) *Cancer Res.* **58,** 4086–4089.
31. Dorudi, S., Hanby, A. M., Poulsom, R., Northover, J. & Hart I. R. (1995) *Br. J. Cancer* **71,** 614–616.
32. Nigam, A. K., Savage, F. J., Boulos, P. B., Stamp, G. W., Liu, D. & Pignatelli, M. (1993) *Br. J. Cancer* **68,** 507–514.
33. Thim, L., Norris, K., Norris, F., Nielson, P. F., Bjorn, S. E., Christensen, M. & Peterson, J. (1993) *FEBS Lett.* **318,** 345–352.
34. Playford, R. J., Marchbank, T., Chinery, R., Evison, R., Pignatelli, M., Boulton, R. A., Thim, L. & Hanby, A. M. (1995) *Gastroenterology* **108,** 108–116.
35. Beck, N. E., Tomlinson, I. P. M., Homfray, T., Frayling, I., Hodgson, S. V., Haracopos, C. & Bodmer, W. F. (1997) *Hum. Genet.* **99,** 219–224.
36. Zhou, X. P., Hoang, J. M., Li, Y. J., Seruca, R., Carneiro, F., Sobrinho-Simoes, M., Lothe, R. A., Gleeson, C. M., Russell, S. E., Muzeau, F., *et al*. (1998) *Genes Chromosomes Cancer* **21,** 101–107.
37. Morgenstern, J. P. & Land, H. (1990) *Nucleic Acids Res*. **18,** 3587–3596.
38. Efstathiou, J. A., Noda, M., Rowan, A., Dixon, C., Chinery, R., Jawhari, A., Hattori, T., Wright, N. A., Bodmer, W. F. & Pignatelli, M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3122–3127.
39. Pignatelli, M., Liu, D., Nasim, M. M., Stamp, G. W. H., Hirano, S. & Takeichi, M. (1992) *Br. J. Cancer* **66,** 629–634.
40. Pignatelli, M. & Bodmer, W. F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5561–5565.
41. Risinger, J. I., Berchuck, A., Kohler, M. F. & Boyd, J. (1994) *Nat. Genet.* **7,** 98–102.
42. Frayling, I. M., Beck, N. E., Ilyas, I. M., Dove-Edwin, I., Goodman, P., Park, K., Bell, J. A., Williams, C. B., Hodgson, S. V., Thomas, H. J. W., *et al*. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 10722–10727.
43. Yoshiura, K., Kanai, Y., Ohiai, A., Shimoyama, Y., Sugimura, T. & Hirohashi, S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7416–7419.
44. Graf, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Isaacs, W. B., Pitha, P. M., Davidson, N. E. & Baylin, S. B. (1995) *Cancer Res.* **55,** 5195–5199.
45. Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S. & Takeichi, M. (1992) *J. Cell. Biol.* **118,** 703–714.
46. Peltomaki, P. (1995) *J. Pathol.* **176,** 329–330.
47. Radman, M. & Wagner, R. (1993) *Nature (London)* **366,** 722.
48. Parsons, R., Myeroff, L., Liu, B., Willson, J., Markowitz, S., Kinzler, K. & Vogelstein, B. (1995) **55,** 5548–5550.
49. Ilyas, M., Efsthathiou, J. A., Straub, J., Kim, H. C. & Bodmer, W. F. (1999) *Proc. Natl. Acad. Sci. USA* **96,** in press.