

Paper

A colorectal cell line with alterations in E-cadherin and epithelial biology may be an in vitro model of colitis

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Abstract

Background—It has been shown previously in ulcerative colitis tissue that E-cadherin can occasionally be mutated in the extracellular domain early in neoplastic progression. E-cadherin is known to maintain differentiation and inhibits invasion in vivo.

Aims—To assess the mechanisms by which such dysfunction occurs.

Methods—Four human colorectal cancer cell lines, HCA-7 colonies 1, 3, 6, and 30, derived from a single heterogeneous colorectal cancer were studied. The HCA-7 cell line has p53 mutations and a random errors of replication “positive” phenotype, as is seen in early colitis associated cancers or hereditary non-polyposis coli cancer (HNPCC).

Results—Cell lines 6 and 30 expressed E-cadherin abundantly and this correlated positively with their degree of differentiation and organisation; however, both cell lines had loss of heterozygosity of E-cadherin. Interestingly, E-cadherin production was downregulated in the poorly differentiated cell line 1, and this was associated with major chromosomal rearrangements of 16q. This cell line also had a mutation in the homophilic binding domain of exon 4, which was associated with disaggregation by low titres of a function blocking antibody, and an invasive phenotype.

Conclusions—These multiple biological alterations further characterise the complex association that E-cadherin has with tumour heterogeneity and suggest that this series of cell lines may be a useful model of colitis associated or HNPCC associated tumorigenesis.

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Keywords: cancer; colorectal; E-cadherin; HCA-7 cell line; mutations; transitions

Ulcerative colitis is a common disease affecting the colon in Western populations, which can cause severe diarrhoea and rectal bleeding, in addition to abdominal pain. At present, there are no entirely satisfactory treatments for this condition, which also increases the accumula-

tion of multiple mutations¹ and the risk of colorectal cancer.² The common epithelial features of ulcerative colitis include denudation of colorectal cells, abnormal mucosal proliferation, depleted goblet cell lineages, and dysplasia, which may lead to adenocarcinoma.³ This differs from the adenoma-carcinoma sequence because the disease is multifocal and arises in the midst of chronic colonic inflammation, with the activation of both regulatory and pro-inflammatory cytokines. Indirect evidence that the main adhesive and occluding junctions (adherens junctions and tight junctions) are perturbed during colitis comes from a report suggesting that mucosal barrier dysfunction and increased permeability is common in ulcerative colitis.⁴

Major developments in animal models of colitis have been reported that might shed some light on damage and repair. In particular, a chimaeric/transgenic murine model expressing a dominant negative N/E-cadherin results in an inflammatory bowel disease resembling Crohn's colitis.^{5,6} In humans, it has also been reported recently that chromosome 16, where both the genes for E-cadherin and P-cadherin are located, is a major susceptibility locus for inflammatory bowel disease.^{7,8} Our group has recently identified germline mutations of E-cadherin in families with a history of gastric cancer.⁹ In addition, we have also identified E-cadherin mutations in cases of early ulcerative colitis and Crohn's colitis.¹⁰ Cadherins are a large family of calcium dependent cell-cell adhesion molecules with functions essential to the maintenance of intercellular connections at the zonula adherens junctions.¹¹ They are characterised by five conserved repeated amino acid sequences (cadherin repeats) in the extracellular domain, which have a conserved α helix loop β barrel structure and a conserved catenin binding domain, by which they are anchored to the cytoskeleton.¹¹⁻¹⁷ Cadherins also act as master morphoregulatory molecules that modulate epithelial polarity, cell plasticity, and cell survival during development and remodelling in disease.^{13,18} In cancer, including sporadic colorectal cancer, the absence of these molecules is associated with a poor prognosis.^{9,12,19} These latter changes in cadherin biology could result from either quantitative and/or functional changes in protein

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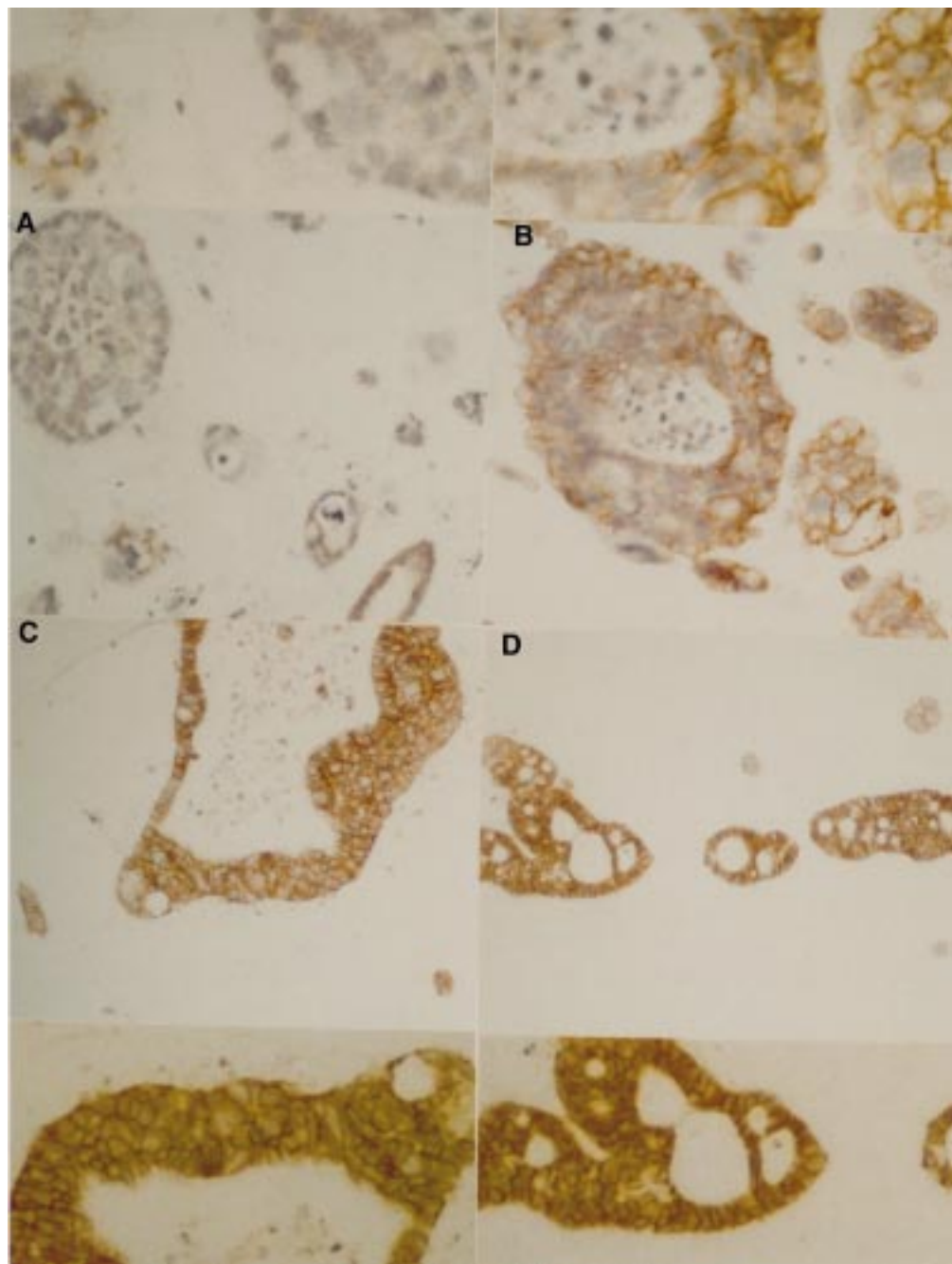


Figure 1 The HCA-7 subcolonies are labelled with E-cadherin antibodies after 15 days growth in collagen gels (magnification, $\times 400$ – 700). (A) Colony 1, large poorly organised structures with occasional smaller hollow structures that express E-cadherin weakly. (B) Colony 3, poor organisation is present and only a few hollow spheroids have weak to moderate expression of E-cadherin. (C) Colony 6, glandular structures with strong membranous immunoreactivity and one area of glandular budding (bottom). (D) Colony 30, highly organised single layered glands with strong E-cadherin expression. (High power inserts are placed above and below the central photographs (magnification, $\times 500$ – 1000 .)

concentrations or more subtle but still biologically important mutations to the gene structure, especially at the 5' end.²⁰

Unfortunately, until recently, there have been few approaches to test the pleiomorphic actions of E-cadherin in a single cell model possessing multiple phenotypes and biology. A series of human colorectal cancer cell lines have previously been isolated from a single cell line (HCA-7).²¹ These subcolonies, however, had similar common mutations associated with a transformed phenotype, namely p53 and random errors of replication (RERs).²² These cell

lines were different in their morphology, differentiation, growth, and invasive biology in agar and xenografts.²¹ In particular, these xenograft tumours showed differentiation ranging from well differentiated villo/tubular/cystic to poorly differentiated villous. Furthermore, some tumours demonstrated a slow expansive growth in vitro, whereas others showed a rapid infiltrative phenomena. Therefore, it seemed possible that additional genetic changes may be the cause of the varied biological characteristics, regardless of the cellular environment, because biological differences were seen with and without extracel-

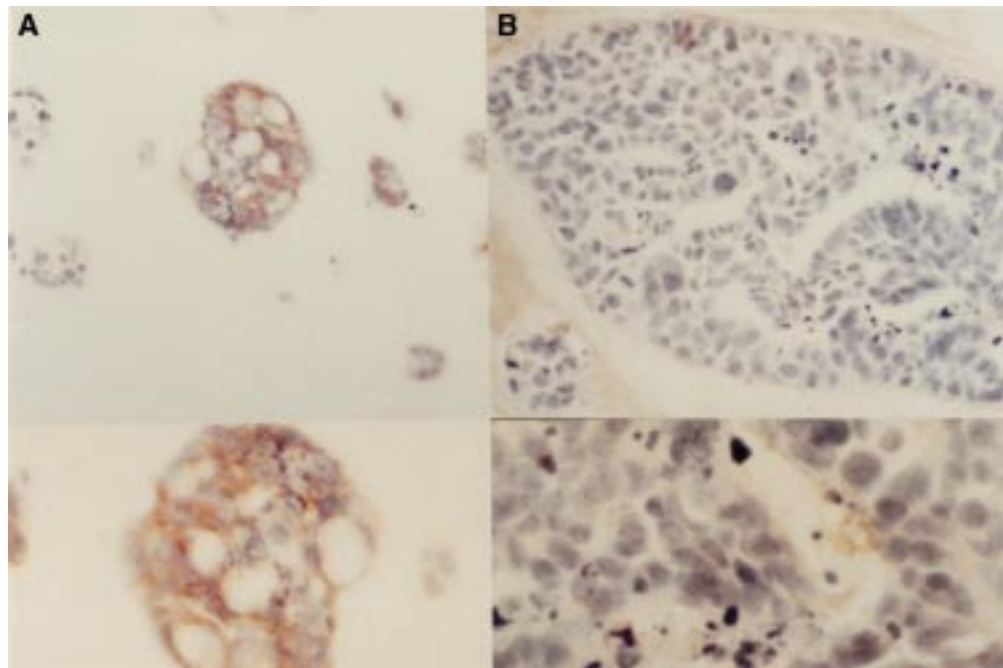


Figure 2 The cell lines are labelled with E-cadherin antibodies after 15 days growth in collagen gels (magnification, $\times 400$ – 700). (A) Caco-2, small cell clumps with mucin production and moderate E-cadherin expression. (B) SW 480, little or no E-cadherin is expressed in the poorly organised cell clumps. (High power inserts are placed above and below the central photographs; magnification, $\times 500$ – 1000 .)

lular matrix. Therefore, we postulated that altered cell–cell adhesion may, in part, explain some of these observations.

Materials and methods

CELL CULTURE

The HCA-7 cells were grown routinely as monolayers in tissue culture treated plastic flasks and were fed daily by a complete change of Dulbecco's modified Eagles medium supplemented with 110 $\mu\text{g}/\text{ml}$ sodium pyruvate, 100 $\mu\text{g}/\text{ml}$ kanamycin, and 10% fetal calf serum. Cells were incubated at 37°C under 7.5% carbon dioxide in a Heraeus gassing incubator. Cell passages were as follows: colony 1 (passages 24–34), colony 3 (passages 28–43), colony 6 (passages 13–22), and colony 30 (passages 22–37).

COLLAGEN GELS

A 5 ml aliquot of cell suspension containing 500 000 cells was placed in a universal container and cells were syringed through a French gauge needle size 20 to obtain a single cell suspension. Collagen solution (1 M NaCl/ 1 M Na_2HPO_4 / 1 M NaOH solution; 8:1:1) was kept on ice and

5 ml was added to 0.5 ml of syringed cells. A 1 ml aliquot of this cell/collagen solution was transferred to an organ culture dish and allowed to set at 37°C for ~ 60 minutes. The collagen gel was covered with growth medium and the remaining 5 ml of cells added to 20 ml of growth medium in a 75 cm^2 flask.

AGAROSE GELS (ANCHORAGE INDEPENDENCE)

A 6% (wt/vol) solution of sea prep agarose (FMC, La Jolla, USA) in double distilled water was prepared by boiling in a water bath for 20–30 minutes. Cells were harvested from flasks by trypsinisation and resuspended in fresh medium. Equal volumes of each cell suspension and agarose solution were mixed, giving a final concentration of 3% agarose. Replicate amounts of each cell agarose suspension were plated out in six well plates, which were then chilled at 4°C for 30 minutes to allow the gels to set. Fresh medium (2 ml) was then added to each well and changed every two days.

IMMUNOHISTOCHEMISTRY

Several anti-E-cadherin antibodies were used to assess protein synthesis; negative controls were similarly stained duplicate sections in which the primary antibody was omitted and replaced by normal isotype matched mouse immunoglobulin.

The specificity of antibody binding was confirmed by competitive incubation of the primary antibody in the presence of excess (50 $\text{mg}/50$ ml) immunising E-cadherin peptide and retention of staining after preabsorption with other non-specific peptides.

The distribution of cellular staining of specific immunoreactivity was classified according to its distribution in the cell membrane or cytoplasm.

Table 1 Phenotype of HCA-7, SW 480, SW 620, and Caco-2 cells in agarose gels

Cell line	Proliferation	Invasion	Colony size	Colony forming units (CFU/ cm^2)
HCA-7				
Colony 1	Moderate	Yes +	Moderate, round	323
Colony 3	Low	No	Small, round	72
Colony 6	Low	No	Small, round	54
Colony 30	Low	No	Small, round	3
Caco-2	Moderate	Yes +	Small, round	130
SW 620	Moderate	Yes +	Small, irregular	369
SW 480	High	Yes ++	Large, irregular	550

Proliferation: low, <10%; moderate, <25%; high, >25% of cells labelled with Ki-67 antibody. Invasion: no, only single cells or small round regular colonies; yes +, small irregular colonies with peripheral aggregates; yes ++ cellular "streaming" into agarose and distortion of agarose gel. Colony size: small, <2 mm; moderate 2–4 mm; large, >5 mm. Colony forming units (CFU): number of colonies with a group of cells $\geq 4/\text{cm}^2$.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOT ANALYSIS

Cells were washed in phosphate buffered saline (PBS) at 0°C, centrifuged at 1000 ×g for five minutes, and the supernatant discarded. Protein was extracted according to standard protocols.²³ One dimensional, fixed percentage resolving gels were prepared, essentially by the method of

Laemmli,²⁴ and the proteins were transferred directly to nitrocellulose using electron transfer buffer (25 mM Tris/HCl, 190 mM glycine, and 20% (vol/vol) methanol). The blot was incubated in a 1/500 dilution of the HECD-1 antibody for two hours at room temperature. Excess unbound antibody was removed by washing the blot several times in TBST (Tris buffer solution containing 0.1% Triton) before

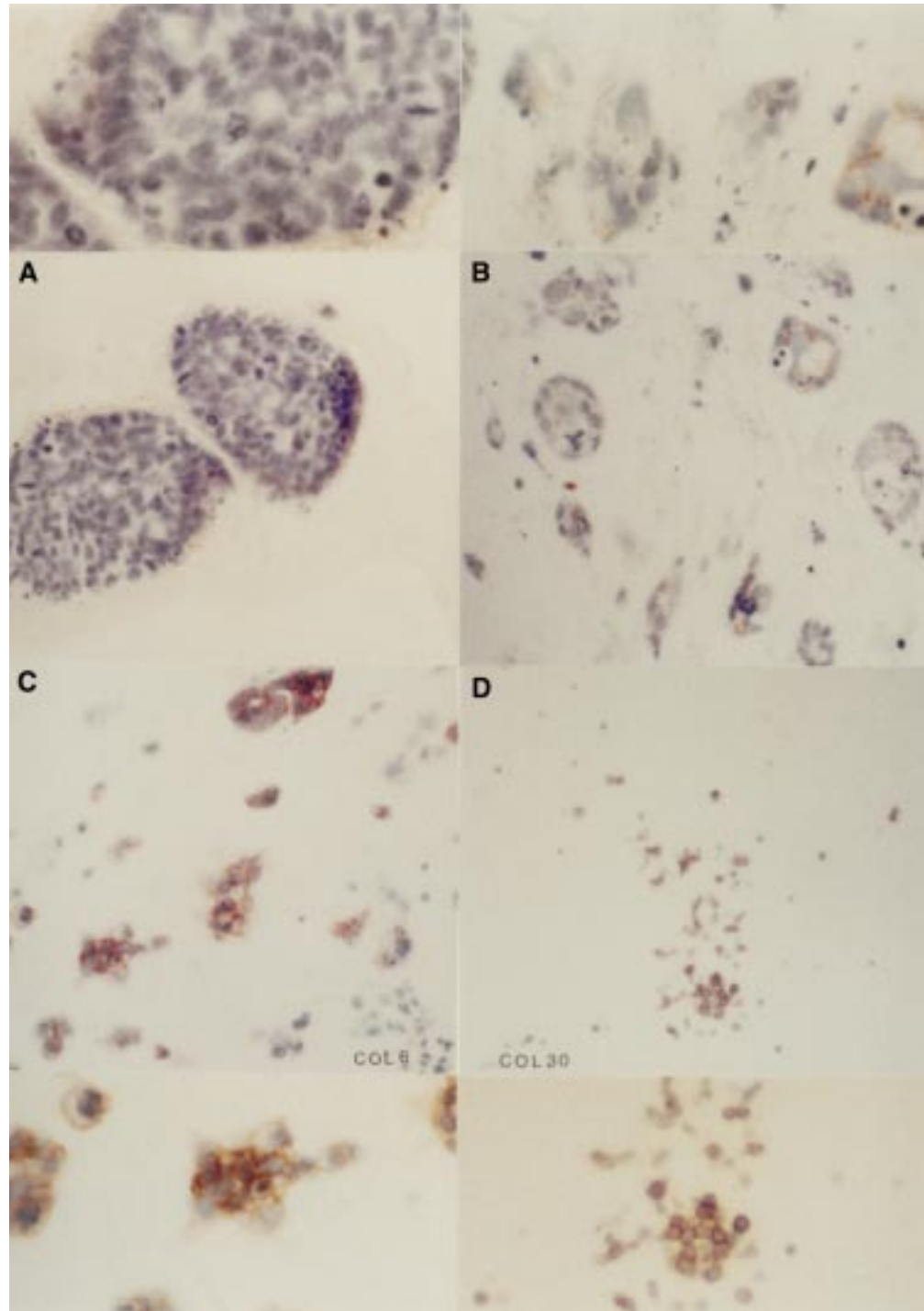


Figure 3 The HCA-7 subcolonies are labelled with E-cadherin antibodies after 15 days growth in agarose gels (magnification, ×400). (A) Colony 1, moderate sized colonies with active proliferation and clumps forming; little or no E-cadherin is demonstrable. (B) Colony 3, only a few cell clumps expressing E-cadherin are apparent. (C) Colony 6, most cells are single cells but where aggregates are present E-cadherin staining is seen. (D) Colony 30, few cells are present; one clump has E-cadherin staining but cells have dubious viability. (High power inserts are placed above and below the central photographs; magnification, ×500–1000.)

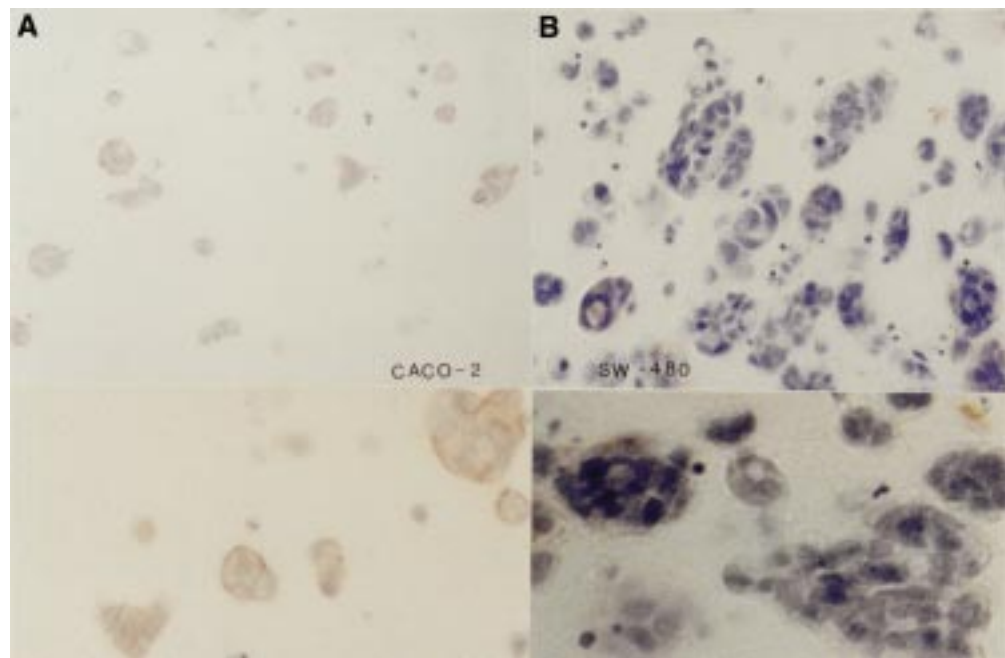


Figure 4 The cell lines are labelled with E-cadherin antibodies after 15 days growth in agarose gels (magnification, $\times 400$). (A) Caco-2, some small cell clumps with weak E-cadherin expression are present but viability is dubious in many other surrounding cells. (B) SW 480, cells are invasive and there is a high degree of proliferation but E-cadherin is not demonstrable. (High power inserts are placed above and below the central photographs; magnification, $\times 500$ – 1000 .)

incubating the blot in a 1/1000 dilution of biotinylated swine antirabbit immunoglobulin (Dako, Copenhagen, Denmark). Incubation was continued for another five minutes with diaminobenzidine tetrahydrochloride in 0.03% (vol/vol) H_2O_2 . Negative controls included similarly processed blots in which the primary antibody had been preadsorbed to the relevant protein, or the total omission of the antibody. Cam 5.2 was used as a positive epithelial loading control.²⁵

EXTRACTION OF RNA FROM CELL LINES AND NORTHERN BLOTTING

Poly A⁺ mRNA was extracted using the polyATtract System 1000 magnetosphere technology (Promega, San Diego, USA), following the manufacturer's instructions.

RNA was fractionated by electrophoresis in a 1% agarose gel with 2.2 M formaldehyde and run in 1 \times MOPs (0.1 M MOPs, 0.01 M EDTA (disodium), 0.5 M sodium acetate, pH 7.0). The RNA sample was prepared by mixing 5.5 μ l of RNA (up to 10 μ g), 1 μ l 10 \times MOPs running buffer, 3.5 μ l formaldehyde, and 10 μ l deionised formamide and heating to 65°C for 10 minutes. UV induced fluorescence of the RNA samples was visualised on a short wavelength transilluminator. The agarose gel was placed under a nitrocellulose/nylon (Hybond-N; Amersham, Middlesex, UK) filter, soaked in $\times 20$ SSC (0.3 M NaCl and 0.03 M sodium citrate), capillary blotted for 18 hours, and fixed by two minutes UV crosslinking at 8 W for nylon filters. The membrane was prehybridised in 20 ml of prehybridisation buffer (10 ml deionised formamide, 4 ml deionised H_2O , 4 ml 50% dextran sulphate, 2 ml 10% SDS, 1.2 g NaCl) and 1 mg/ml sheared salmon sperm DNA. After four hours prehybridisation at 42°C, the radioactive probe containing 50 ng of single stranded DNA labelled by random priming with αP^{32} was added

directly to the membrane and hybridised for a further 16 hours at 55°C. Subsequently, the membrane was washed at high stringency and exposed to autoradiography film.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (PCR) AND GENOMIC PCR

First strand cDNA was generated using 10 U of AMV (avian myeloblastic virus) reverse transcriptase (Pharmacia, London, UK) in the manufacturer's reverse transcription buffer supplemented with 10 U of RNasin (Promega), 0.01 M β -mercaptoethanol, 1 mM of dNTPs, and either 1 μ l of random primers or 1 μ l oligo dT were added to the mixture. The cDNA products were stored at -20°C for up to one week.

Before amplification, the RNA-DNA hybrid or the genomic DNA was denatured at 94°C for five minutes, followed by rapid cooling to 4°C for five minutes.

All oligonucleotide primers were synthesised by the oligonucleotide synthesis unit (Clare Hall Laboratories, Imperial Cancer Research Fund, London, UK). PCR reactions were carried out in a final volume of 50 μ l containing PCR buffer (500 mM KCl, 100 mM Tris/HCl, pH 8.3, 15 mM $MgCl_2$, 0.1% (wt/vol) gelatin), 200 mM dNTPs, 0.5 μ M primers, 1–10 ng template, and 2.5 U Taq polymerase (Pharmacia). The reaction mix was overlaid with light mineral oil (Sigma, Poole, Dorset, UK) to prevent evaporation. Routine amplification reactions were performed using 30 cycles of denaturation at 94°C for one minute, annealing at 55–60°C for one minute, and elongation at 72°C for two minutes using a thermal cycler (Perkin-Elmer Cetus, Norwalk, USA).

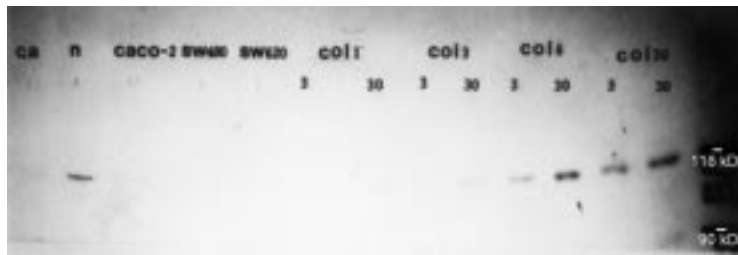


Figure 5 Western blot of E-cadherin production in the HCA-7 cell line. Positive control, normal colonic tissue (n); negative control, SW 480. Concentrations of E-cadherin were highest in colonies 6 and 30 and production was also increased over time. Colonies 1, 3, and Caco-2 have low concentrations of E-cadherin.

CHROMOSOME PREPARATIONS

Each cell line was harvested when the cells reached 70% confluency. To synchronise the cultures, cells were blocked with 0.5 μ M fluorodeoxyuridine and 1.2 μ g/ml uridine for 17 hours and released with 30 μ g/ml bromodeoxyuridine five hours before harvesting. Slides were made and then G-banded by immersion in 2 \times SSC at 60°C for five to 10 minutes and stained 1:3 in 50% Sorenson's buffer (0.06 M Na₂HPO₄, 0.06 M KH₂PO₄). At least 20 metaphase spreads were counted and those with clear banding fully analysed. At least five cells were fully karyotyped for each cell line.

DETECTION OF SINGLE STRANDED CONFORMATIONAL POLYMORPHISMS

The PCR reaction was performed as usual except 2 mM non-radioactive CTP was omitted and replaced by radioactive α P³²-CTP (0.2 mM). A set of primers was used to assess codons 3–5 (E-cadherin 1F: AGG CCT CCG TTT CTG GAA TC; E-cadherin 1R: CTT CAG CCA TCC TGT TTC TC). A neutral polyacrylamide gel was prepared as follows: 25 ml MDE (Quiaex, mutation detection enhancement) solution (AT Biochem, Cambridge, UK), 69 ml H₂O, and 6 ml TBE (Tris buffer/EDTA) were mixed, then 400 μ l ammonium persulphate and 40 μ l TEMED were added. To denature the sample, 2.5 μ l of sample was mixed with 7.5 μ l loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol), heated at 100°C for five minutes, and cooled on ice for five minutes. A 5 μ l aliquot of the denatured solution was loaded and electrophoresed at 6 W for 14 hours. Subsequently, gels were exposed to Hyperfilm at -70°C overnight using intensifying screens.

DOUBLE STRANDED PLASMID DNA SEQUENCING

PCR fragments were cloned into the PCRII cloning vector (Invitrogen, London, UK) and

Table 2 Summary of E-cadherin expression in the colorectal cell lines grown in monolayers, collagen gels, and agarose gels

	Monolayers	Collagen	Agarose
HCA-7			
Colony 1	+	+	-/+
Colony 3	+	++	+
Colony 6	+++	+++	++
Colony 30	++	+++	++
Caco-2	++	++	+
SW 480	-	-	-

E-cadherin expression: -, absent; +/-, minimal (<5–10% cells); +, weak (<25% cells); ++, moderate (<50% cells); +++, strong (>50% cells).

sequenced using α S³⁵-dATP, according to the protocol in the Sequenase™ version 2.0 kit (US Biochemicals, New York, USA), and the sequence was resolved on a standard denaturing sequencing gel. DNA analysis was carried out using the Wisconsin suite of programs on a VAX computer.

RANDOM ERRORS OF REPLICATION

RERs (microsatellite instability) can be identified using short primers containing the repeat of interest and amplifying sequences of CT repeats in tumours and comparing them with an immortalised lymphoblastoid sample derived from the same patient's white blood cells. Two microsatellite markers were chosen to evaluate each sample and primers amplified fragments of between 100 and 400 bp in size. The primers used for each locus were as follows: for D2S123: 5'-ACATTGCTGGA-AGTTCTGGC, 5'-CCTTTCTGACTTG-GATACCA; for D18S58: 5'-GCTCCCGGC-TGGTTTT, 5'-GCAGGAAATCGCAGGA-ACTT; for BAT25: 5'-TCGCCTC-CAAGAAATGTAAGT, 5'-TCTCATTTTA-ACTATGGCTC; for BAT26: 5'-TGACTA-CTTTTGACTTCAGC, 5'-AACCATTCAA-CATTTTTAACCC; and for BAT40: 5'-ATTAACCTCCTACACCACAAC, 5'-GTA-GAGCAAGACCACCTTG. Standard PCR conditions were used except that the annealing temperature was between 45°C and 50°C and 25 cycles were used.²⁴ Tumours were classified as positive if at least two of the markers revealed PCR fragments not found in the normal colon.

Results

EFFECT OF COLLAGEN AND AGAR ON COLORECTAL CELLS IN VITRO

Organisation was determined by the progressive morphological association of individual cells into glandular structures in three dimensional collagen gels. Organisation was most evident in cell line 30 and to a lesser extent cell line 6. Small hollow single layered glandular structures were present in colony 30, but these structures were multilayered in colony 6. In colonies 1 and 3, and the SW 480 cell line, few organised structures were evident (figs 1 and 2).

Alternatively, growth or invasion in agarose has been suggested to be an indicator of tumorigenicity in vitro. In this regard, HCA-7 cell lines 3, 6, and 30 did not infiltrate into the anchorage independent medium of agarose and cell colonies were small (fig 3). HCA-7 cell line 1 expanded slowly and concentrically in agarose gels to form moderate sized colonies, whereas the SW 480 cell line formed large infiltrative multicellular colonies with a characteristic irregular edge (table 1; fig 4).

E-CADHERIN PROTEIN AND mRNA EXPRESSION

Several anti-E-cadherin antibodies were used to assess protein expression in identically prepared preparations. The DECMA-1 (Sigma, London, UK),⁹ 6F9 (Eurodiagnostics, Amsterdam, The Netherlands),²⁶ and HECD-1 (Takara Biomedicals, Tokyo, Japan)²⁷ antibodies specific for E-cadherin gave similar

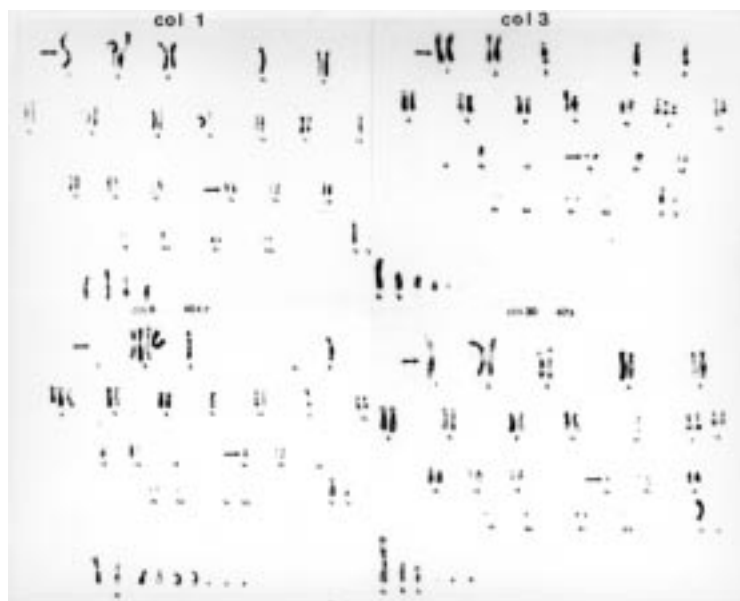


Figure 6 Karyotyping of the HCA-7 cell line colonies. The large numbers refer to the karyotype and the smaller numbers refer to the individual chromosomes 1–46. Colony 6 (col 6), absence of chromosomes 1 and 4 (arrowhead) and single copies of 3, 5, 9, 11, and 16. Colony 30 (col 30), single copies of chromosomes 1, 10, and 16 (arrowhead) in addition to double minute chromosomes at the bottom of the figure.

staining of sections and identified a 120 kDa protein. E-cadherin is upregulated during confluency in colonies 3, 6, and 30, with the highest protein concentrations found in colonies 6 and 30 (fig 5). Colonies 6 and 30 also had greater amounts of E-cadherin mRNA as measured by northern blotting, compared with colonies 1 and 3 (data not shown). Multicellular aggregates in collagen and agar from both the HCA-7 cell lines 6 and 30 expressed abundant E-cadherin (protein and mRNA), whereas single cells expressed little or no E-cadherin (fig 1). Intriguingly, the cellular distribution of E-cadherin is basolateral in cell lines 6 and 30 in collagen, reminiscent of the normal pattern *in vivo*, whereas in cell lines 1 and 3 the distribution is circumferential (fig 1). Table 2 summarises the expression of E-cadherin in the different cell lines. We assessed the expression of other cell–cell adhesion molecules including CEA (carcinoembryonic antigen), CD44, and extracellular matrix (ECM) interacting molecules such as $\alpha 2$ integrin, $\alpha 6$ integrin, and $\beta 1$ integrin and no difference was observed between the colonies (data not shown).

KARYOTYPING

HCA-7 cell lines 1, 3, 6, and 30 were hypodiploid, with a modal number of 45, 43, 42, and 45 chromosomes for each metaphase, respectively. At least 20 cells were analysed for each cell line and at least five were fully karyotyped. All lines were very rearranged, especially HCA-7 colony 1, in which aberrations most frequently involved chromosomes 1, 10, 11, 14, and 16. All four lines had clonal aberrations found in a high percentage of cells, but none were common to all lines. Furthermore, there was loss of heterozygosity of chromosome 16 in colonies 6 and 30 (fig 6).

ALTERED MORPHOLOGY INDUCED BY THE FUNCTION BLOCKING HECD-1 ANTIBODY

The HECD-1 antibody binds to an epitope in the outer two E-cadherin binding domains and has been shown to inhibit cell–cell adhesion from exons 4 to 6.²⁸ In an attempt to assess whether this region had differential effects on the phenotype and biology of the HCA-7 cell lines, this antibody was introduced into live cell cultures. Colony 1, and to a lesser extent colony 30, showed considerable inhibition of cell–cell adhesion when purified 1/1000 HECD-1 antibody (final concentration 40 μ g/ml) was applied to cultures after 24 hours growth (fig 7). However, this effect was greatest at a 1/10 dilution (final concentration, 4 mg/ml). In addition, the effect was also temporal because parallel plates that had an identical HECD-1 concentration of antibody added after 72 hours, as opposed to 24 hours, showed a diminished susceptibility to disaggregation with HECD-1 (fig 7; table 3). The control plates incubated with media alone revealed no disaggregation and plates incubated with the $\alpha 6$ integrin also had no visible disaggregation. Subsequently, the cells were incubated while still viable with a secondary peroxidase antibody and staining was visualised by a chromogen. The staining revealed that all dispersed and rounded up cells had very strong HECD-1 immunoreactivity over the entire surface, whereas adherent cells, with epithelial morphology, had minimal membranous immunoreactivity. Therefore, greater amounts of adherent anti-E-cadherin antibody reacted with the detaching cells than with the other cells (fig 8).

RANDOM ERRORS OF REPLICATION

Multiple DNA repeats were present in the HCA-7 cell line (fig 9). The most common gene mutation that results in RERs, the MSH2 gene mutation, was not found in the HCA-7 cell line.

SINGLE STRANDED CONFORMATIONAL POLYMORPHISMS AND SEQUENCING OF E-CADHERIN MUTATIONS

HCA-7 cell line 1 had polymorphic conformers of E-cadherin in exons 3–5 compared with cell lines 3, 6, 30, the Caco-2 cell line, and normal and neoplastic colorectal tissue (fig 10). Exons 6–7 were also examined and failed to demonstrate any conformational polymers.

Exon 4 spans the last 25 codons of the precursor peptide (155 codons long), including the important proteolytic cleavage site and the first 27 codons of the mature peptide. The sequences revealed heterogeneity of E-cadherin mutations in both colonies 1 and 30 compared with normal colonic mucosa. In particular, HCA-7 cell line 1 had a transition from GTT to GCT (valine to alanine) in codon 158 (exon 4) of the mature peptide; one further mutation was present in the same PCR fragments, CTC to CCC, resulting in a change of leucine to proline (missense mutation) in codon 149 (exon 4) of the precursor peptide. Colony 30 had two abnormalities of the mature peptide, again both present in the same fragments; in particular,

three transitions CCC to CTC (codon 165; proline to leucine), GAA to GGA (codon 168; glutamic acid to glycine), and AAA to GAA (codon 169; lysine to glutamic acid) were identified. No other mutations were identified in the other HCA-7 cell lines or in normal or neoplastic colorectal tissue in vivo. In almost all cases, the point mutations resulted in amino

acids being changed in highly conserved regions (fig 8) with major physicochemical differences (table 4).

Discussion

In many tumours involving the skin, bladder, breast, and stomach dysfunctional E-cadherin is associated with both invasion and

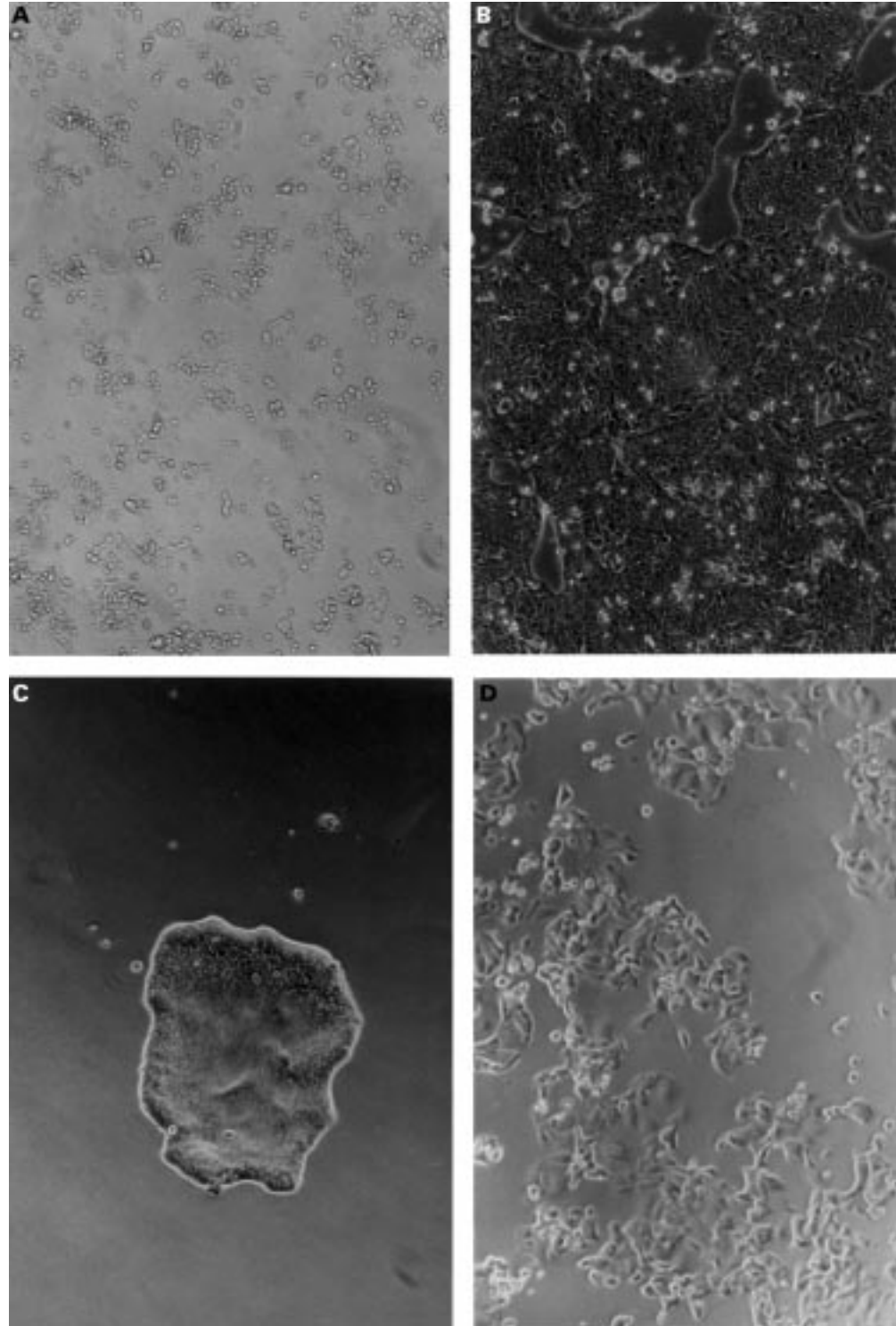


Figure 7 Morphological changes in vitro induced by the E-cadherin HECD-1 antibody after six to 12 hours (magnification, $\times 200$). (A) HCA-7 cell line 1, cells have become spindle shaped or become rounded and effete. (B) Colony 3, a few loose cells are visible but most of the cells are still widely adherent. (C) Colony 6, the original morphology is maintained and only single detached cells have become rounded. (D) Colony 30, the monolayer has become severely disorganised and the epithelial shape has become more fusiform.

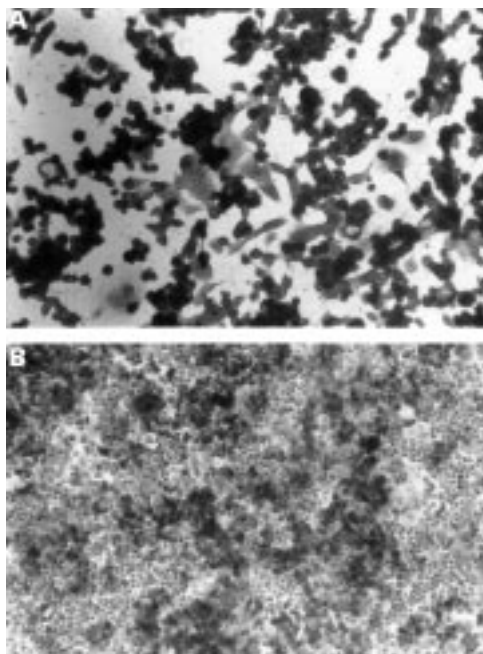


Figure 8 Cellular localisation of E-cadherin antibody on live disaggregated HCA-7 cells (magnification, $\times 400$). (A) HCA-7 cell line 1, E-cadherin antibody is weakly present on the cell membranes of adherent live cells, whereas rounded up cells express abundant amounts of E-cadherin. (B) HCA-7 cell line 6, the anti-E-cadherin antibody is bound to very few live cells, suggesting that the epitope is expressed minimally on the apical cell surface and most is engaged in cell-cell contact in the tight junctions.

Table 3 Disaggregation with the HECD-1 antibody

	HECD-1 antibody (final concentration)		
	1/10 (4 mg/ml)	1/100 (400 μ g/ml)	1/1000 (40 μ g/ml)
Colony 1	++++	++++	+++
Colony 3	+	+	-
Colony 6	+	-	-
Colony 30	++	++	+

Disaggregation: -, none; +, occasional cells become rounded; ++, many cells become rounded (>25%); +++, >50% of cells become rounded or effete; +++++, major change in morphology of the remaining attached cells.

metastasis.²⁹ In the colon, most reports also confirm this association.⁹⁻³⁰ Our results provide further direct evidence of the importance of E-cadherin in colorectal tissue. The various HCA-7 cell lines were shown to produce different amounts of E-cadherin protein by means of immunohistochemistry and western blotting. In particular, E-cadherin positive cells were usually in the better differentiated colonies. The observation of upregulated E-cadherin with differentiation in colonies 3, 6, and 30 also suggests normal gene imprinting of cell adhesion mechanisms. Furthermore, E-cadherin positive cells tended to organise into clumps in monolayer gels and glands in collagen gels, and to exhibit anchorage dependent growth on agarose gels. Conversely the E-cadherin deficient clones had increased numbers of dispersed colonies in collagen gels, which was associated with tissue invasion in agar and xenografted tumours.²¹

These observations are important because we have reported previously that the HCA-7 cell line does not have Ki-ras codon 12

mutations, loss of the deleted in colorectal cancer gene (DCC), or a truncated adenomatous polyposis coli (APC) gene, although all four colonies have both p53 mutations and the mutator phenotype.²² Therefore, it is likely that in this model other genomic factors distinguish the differential morphology and biology of the individual colonies. A precedent has already been made for an involvement of cell-cell adhesion molecules in modulating cancer phenotype in vitro when the other classic mutations are nullified.³¹⁻³³ In this regard, it has been reported that the transfection of wild-type p53, DCC, and APC into colorectal cell line SW 480 leads to suppressed tumorigenicity, but interestingly these lines still retained anchorage independence, and this is consistent with our observation of absent E-cadherin expression in this cell line.³⁴ In our studies, the colonies with detectable E-cadherin mutations were more easily disaggregated with the function blocking HECD-1 antibody, and their morphology was also altered. This latter observation indicates that, regardless of the level of E-cadherin expression, the homotypic binding domains in these cell lines are more easily inhibited by competition with the antibody. This could be interpreted to mean that higher concentrations of cadherin may be required to regulate cellular polarity and differentiation, whereas only minimal amounts of functional E-cadherin may be required for regulating adhesion.³⁵⁻³⁶

The allelic loss of heterozygosity of the E-cadherin gene on chromosome 16q has been reported previously in other cancers, including 40% of cervical and prostate carcinomas, 50% of breast adenocarcinomas,³⁷ and 75% of gastric adenocarcinomas. In a recent study of cytogenetic abnormalities in colorectal tumours, loss of 16q (the E-cadherin locus) was a frequent occurrence.³⁸⁻³⁹ It has been postulated that as a consequence of the loss of heterozygosity (LOH) the decreased E-cadherin transcription may result in major morpho-spatial dysregulation.⁴⁰⁻⁴¹ It is of interest that the 16q allele is lost in ~45% of the HCA-7 cell line 6 cells and 40% of cell line 30 cells, possibly suggesting a role for E-cadherin as an oncosuppressor gene, although our data suggest that the wild-type allele may be preserved.⁴² The rearrangement of 16q that occurs in HCA-7 cell line 1 is a new observation, and it is possible that the E-cadherin promoter may also be structurally changed, explaining in part the downregulation of production of the encoded protein.

Two of the most important functional domains of E-cadherin are the outer two extracellular domains. Decapeptides containing the outer two regions LFGHAVSSNG in E-cadherin inhibit adhesive function, but interestingly E-cadherin without the extracellular domain may still weakly remodel (polarise) the membrane,⁴³ suggesting a diverse and complex transduction pathway. The first 20 codons of E-cadherin are even more highly conserved than the extracellular domains, but little is known about their role in E-cadherin function. It has also been reported that a dysfunctional external domain may act as a

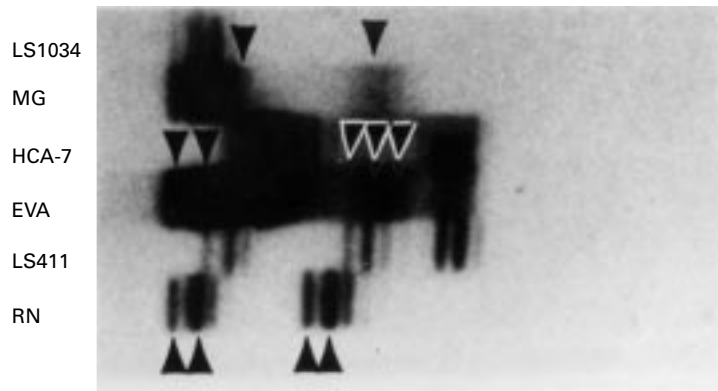


Figure 9 Microsatellite instability of the HCA-7 cell line. The HCA-7 parent cell line exhibited extra fragments of CT dinucleotide repeats not comparable with the patient's paired lymphoblastoid cell line "EVA". Positive control, LS411/RN; negative control, LS1034/MG.

Chicken N-cadherin	DQKKIEDIIFPWQYKDSHLLKRQKR.DWVIPPINLPENSRGP
Mouse P-cadherin	LTRGTVQGGKDMHSPTRILRRRKR.EWVMPPIFVPEKNGKGP
Mouse E-cadherin	DPASESNPELLMFPSVYPGLRRQKR.DWVIPPISCPENEKGP
Human E-cadherin	HQASVSIQAE LL TFPNSPGLRRQKR.DWVIPPISCPENEKGP

Figure 10 Homology map of cadherins across species in exons 3–5. Amino acids underlined are sites of human E-cadherin mutations in the HCA-7 cell lines.

Table 4 Amino acid changes in E-cadherin as a result of point mutations

Colony	Original amino acid	Mutation	Codon site
Colony 1	Leucine (L) (131 Da) pKa 7 (medium size and hydrophobic)	Proline (P) (115 Da) pKa 7 (small and non-hydrophobic)	149 (4/12)
	Valine (V) (117 Da) pKa 7 (small and hydrophobic)	Alanine (A) (89 Da) pKa 7 (tiny and hydrophobic)	158 (5/12)
Colony 3		No mutations detected	(0/12)
Colony 6		No mutations detected	(0/12)
Colony 30	Proline (P) (115 Da) pKa 7 (small and non-hydrophobic)	Serine (L) (131 Da) pKa 7 (medium size and hydrophobic)	165 (3/12)
	Glutamic acid (E) (147 Da) pKa 4 (small and non-polar)	Glycine (G) (75 Da) pKa 7 (positively charged)	168 (3/12)
	Lysine (K) (146 Da) pKa 10 (positively charged)	Glutamic acid (E) (147 Da) pKa 4 (negatively charged)	169 (3/12)

Fractions in brackets under codon sites indicate number of positive bacterial colonies screened. In HCA-7 cell line 1, double mutations were present, whereas, in HCA-7 cell line 30, triple mutations were present.

dominant negative for adhesion,^{44–46} which could be interpreted to mean that some of the mutated molecules could bind wild-type cadherins without achieving either adequate function or decreased intermolecular bonding—"dominant negatives".^{44, 45} Therefore, it is of interest that recent data have indicated that chimaeric/transgenic mice expressing the dominant negative N-cadherin, under the influence of a tissue specific promoter, develop an inflammatory bowel disease resembling Crohn's disease, and to a lesser extent ulcerative colitis.⁴⁷ In humans, these alterations in cadherin biology could result from either changes in the amount of protein produced or more subtle, but still biologically important, mutations to the gene structure. It is also possible that different mutations of E-cadherin could result in a spectrum of biological alterations and, as such, the variety of HCA-7 E-cadherin mutations could explain the different phenotypes and biology. Furthermore, minor anomalies of the N-terminus can also reduce E-cadherin function, such as incorrect

cleavage of the E-cadherin polypeptide.⁴⁸ In this circumstance, E-cadherin is incorporated into the cell membrane but has diminished function. It is therefore possible that the mutation of colony 1 in the precursor peptide could result in diminished adhesive function.

To date, point mutations of E-cadherin have been shown in clinical cases of gynaecological cancer, lung cancer, renal cancer, and two gastric cell lines.^{40–49} These point mutations are also most frequent in the calcium binding domain of the first extracellular domain, but have also been reported at the 5' end, and lead to the loss of adhesive activity. These somatic point mutations of E-cadherin were associated with maintenance of the wild-type allele,⁴⁰ such as in HCA-7 cell line 1, whereas this was not the case in cell line 30. The frequency of point mutations in the extreme 5' region of the E-cadherin gene (exon 4) suggest that this may be a mutation cluster region (MCR) similar to those in codons 428–504 of the APC gene, as well as codons 12 and 13 of the Ki-ras gene.⁵⁰ Alternatively, it could also be possible that our experimental design has a bias to selecting dysfunctional mutations in this region.¹⁹ The altered E-cadherin amino acids themselves had a spectrum of mutability values; however, interestingly all mutations were transitions. Transitions can occur spontaneously because hydrogen atoms on each of the four bases can change their location to produce a tautomer. Specifically, amino (-NH₂) and keto (-C=O) groups tautomerise to form imino (=NH) and enol (=C-OH) groups, respectively.⁵¹ One in every 1000 bases exist as transient tautomers and can form non-standard base pairs that fit into a double helix. For example, the imino tautomer of adenine can pair with cytosine and may result in C being incorporated into new DNA stands where T would be expected, leading to a mutation if left uncorrected. Therefore, it is possible that failure of this strand repair mechanism could explain, in part, the E-cadherin mutations found in the HCA-7 cell line, although it is not clear how this relates to the mutator phenotype present in this cell line. Conversely, if the mutations were the result of the action of common carcinogens, such as alcohol and cigarette smoke, transversions would predominate.⁵² In addition, nitrosamines or alkylating agents frequently cause G to A transitions, which accounted for only 20% of our sequence alterations.⁵³ These mutations may also represent "adaptive mutations" in vitro, whereby cells with no adhesive properties will float and be discarded during media exchanges, leaving other cells with reduced but minimal adhesion and perhaps also a growth advantage over cells with intact adhesion, such as HCA-7 cell line 6.⁵⁴

The E-cadherin palindromic promoter region (E-pal) sequence binds several nuclear binding factors (transacting elements), which are present in well differentiated cancers and normal tissue, but are downregulated in several poorly differentiated cancers.⁵⁵ Nuclear factors that stimulate c-erbB2 transcription may be inhibitory to the E-cadherin gene promoter,^{56–57} and it is of considerable interest therefore that

colon cancer and the HCA-7 cell lines 1 and 3 also produce the c-erbB2 oncoprotein and have low concentrations of E-cadherin.⁵⁸ It is possible also that mutations in the E-pal site of the cadherin promoter may explain in part E-cadherin downregulation in these cell lines, as has recently been shown in breast cell lines.

In this report, several point mutations were detected that resulted in qualitative changes in E-cadherin in vitro. These colonies can be shown to have a temporally related decrease in function using an inhibitory binding assay,^{17 18} confirming the prime importance of intact E-cadherin in maintaining a differentiated phenotype. As a result of this work, it can be said that: "cadherins show a dynamic spatio-temporal pattern of expression associated with morphogenetic events".¹¹

One of the hallmarks of epithelial tumour cells is their phenotypic heterogeneity and the subsequent instability of these phenotypes over time.^{2 59} It is one of the major lessons from the study of the various HCA-7 cell lines that the E-cadherin gene is differentially affected in chromosomal copy number, nucleotide sequence, transcriptional activation, and function and that this is associated with such different phenotypes and biology⁶⁰. In addition, the HCA-7 cell lines have a molecular profile including a "positive" RER phenotype and p53 mutations, which make them an ideal model of colitis or hereditary non-polyposis coli cancer tumours.

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A colorectal cell line with alterations in E-cadherin and epithelial biology may be an in vitro model of colitis.

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