E-Cadherin Deficiency Initiates Gastric Signet-Ring Cell Carcinoma in Mice and Man

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Abstract

The importance of loss of the cell-cell adhesion molecule E-cadherin (encoded by CDH1) to tumor progression is well established. However, CDH1 germ-line mutations predispose to the cancer susceptibility syndrome hereditary diffuse gastric cancer (HDGC), suggesting a role for E-cadherin in tumor initiation. The earliest indications of cancer in the stomachs of CDH1 mutation carriers are microscopic foci of intramucosal signet-ring cell carcinoma (SRCC; designated “eHDGC”). Here, we used N-methyl-N-nitrosourea (MNU) to promote gastric carcinogenesis in wild-type (wt) and cdh1−/− mice. MNU induced a variety of gastric tumors; however, intramucosal SRCC developed with an 11 times higher incidence in cdh1−/− mice compared with wt mice. The murine SRCC resembled the human eHDGCs in that they were hypoproliferative, lacked nuclear β-catenin accumulation, and had reduced membrane localization of E-cadherin and its interacting junctional proteins. The down-regulation of E-cadherin in the murine SRCCs confirmed the importance of the second CDH1 hit to the initiation of diffuse gastric cancer. CDH1 promoter hypermethylation has been proposed to be a major second hit in advanced HDGC; however, its contribution to eHDGC was unknown. We thus examined a series of human eHDGC and detected CDH1 promoter methylation in 50% of foci. Promoter methylation was accompanied by reduced wt CDH1 mRNA levels in the foci and had a monoclonal pattern, consistent with an epigenetic initiation of disease. Together, these findings provide compelling evidence for a deficiency in cell-to-cell adhesion being sufficient to initiate diffuse gastric cancer in the absence of hyperproliferation and β-catenin activation.

Introduction

Perceptions of the initiating events in cancer usually involve a hyperproliferative state caused by mutations in genes implicated in cell cycle control or cell survival. The identification of the gene for the cell-cell adhesion molecule E-cadherin (CDH1) as a cancer susceptibility gene (1), however, has raised the possibility that an event as superficially simple as lost adhesion may be sufficient to initiate cancer.

E-cadherin, the key component of the epithelial adherens junction, is required for the proper formation and maintenance of epithelial sheets (2). E-cadherin enables intercellular adhesion by (a) homophilic interaction of its extracellular domains and (b) indirectly binding to actin via its intracellular portion, thereby providing an anchor that connects the actin cytoskeletons of adjacent cells (3). Additional proteins that participate in the adherens junctions include β-catenin, a structural component and also an essential transducer of Wnt pathway signals, p120 catenin, required for the complex stability, and Lin-7, which is found only in mature adherens junctions (3, 4).

Loss of intercellular adhesion is a hallmark of migratory cells. Given the central role of E-cadherin in this process, the close association between E-cadherin loss and the acquisition of an infiltrating, invasive phenotype of a tumor is not surprising (5). However, the effect of E-cadherin loss on epithelial tissue lacking a preexisting neoplastic phenotype is less well understood.

Gastric cancer is usually classified into intestinal and diffuse types (Lauren classification; see ref. 6). Diffuse gastric cancer can be further subdivided into poorly differentiated carcinoma and signet-ring cell carcinoma (SRCC). Recent evidence suggests early SRCC is an initial, differentiated form of diffuse gastric cancer that may evolve into poorly differentiated carcinoma (7). Although the worldwide incidence of gastric cancer is in decline, the incidence of diffuse gastric cancer, particularly SRCC, is rising in the United States (8).

Carriers of CDH1 germ-line mutations are predisposed to the cancer syndrome hereditary diffuse gastric cancer (HDGC), which is marked by a 70% lifetime risk of advanced diffuse gastric cancer and an elevated risk of lobular breast cancer (9). Almost every CDH1 germ-line mutation carrier first presents with up to several hundred microscopic foci of stage T1a intramucosal SRCC (9, 10). These foci [early HDGC or eHDGC (7); see also tumor definitions in Materials and Methods] are histologically and morphologically similar to sporadic early intramucosal SRCC. Some of these eHDGC foci can acquire poor differentiation and may progress to submucosal invasion (7).

Importantly, eHDGC foci all show reduced E-cadherin expression on immunohistochemistry (7), showing that CDH1 is a classic tumor suppressor gene requiring a second genetic hit before a phenotypic effect is observed. Furthermore, the timing of this second hit at the earliest identifiable stage of HDGC development suggests that the cancer susceptibility caused by CDH1 germ-line mutation is exerted at the time of disease initiation, rather than its role being confined to the enhancement of invasion or metastasis. The large numbers of foci occurring simultaneously in the stomachs of CDH1 mutation carriers argue against other mutational events being required for the initiation of HDGC. For lost adhesion to play a role in cancer initiation without invoking the need for additional mutations, cells with an existing proliferative capacity such as...
stem/progenitor cells would be predicted to be affected. Supporting this possibility, we have recently shown that the apparent origin of eHDGC lies within the proliferative zone of intact gastric epithelium (7).

To provide further evidence supporting the capacity of defective cell-cell adhesion to initiate cancer, we established a cdh1\(^{+/−}\) mouse model of HDGC to show that the induction of SRCC requires E-cadherin deficiency and the associated adhesive loss. In addition, we sought to identify the mechanism of loss of the second CDH1 allele in the earliest observable HDGC stage in human CDH1 mutation carriers. Our identification of promoter hypermethylation in these eHDGC foci provides direct evidence for the role of epigenetic changes in gastric cancer initiation.

**Materials and Methods**

**Mice.** Following ethical approval, an inbred colony was established using a transgenic cdh1\(^{+/−}\) mouse model developed in a C57BL6 background (11). These cdh1\(^{+/−}\) mice are not known to develop gastric or other cancers at rates different to wild-type (wt) mice. Mice were kept at the Animal Research Unit (University of Auckland) under standard conditions: plastic cage with wood shavings, water, and feed ad libitum (Rodent Diet 2018, Harlan Teklad Ltd.). Genotype was determined by PCR on tail-extracted DNA using primers mcad7F (CCCTCTCCTTTGACAGGAACCT) and mcad7R (CAGCCCAGAGGT-GAGCAGACT) or mco8L (GAGATTCGCAATGACAGACCT) for wt or mutant cdh1, respectively. All mice were negative for Helicobacter as assessed on H&E sections and lacked background superficial chronic gastritis with focal activity.

**N-methyl-N-nitrosourea treatment.** At 5 wk of age, groups of 20 to 30 cdh1\(^{+/−}\) or wt mice with similar gender ratios were given drinking water alone or, for five times on every second week, supplemented with 120 ppm N-methyl-N-nitrosourea (N NU; Sigma ref. 12) and killed at 40 wk. A group of cdh1\(^{+/−}\) mice on water was kept to 80 wk to see whether SRC develops spontaneously in older mice. Mice were checked daily and weighed weekly. Sick animals and early deaths were excluded.

**Autopsy.** Each mouse was weighed, as were its kidneys, liver, and spleen. All thoracic (heart, lungs, and thymus) and abdominal (spleen, liver, kidneys, and gastrointestinal tract) organs were inspected and samples were taken. Each stomach was opened along the greater curve, pinned flat, fixed in 10% formalin for 24 h, cut into five to seven longitudinal slices, and embedded in paraffin. The location of macroscopic lesions was recorded on a photographic template. Sections (4 \(\mu\)m) from all slices of each stomach were screened for microscopic lesions. Each lesion and one third of the normal epithelia were dissected from two to three 4-\(\mu\)m paraffin sections (Supplementary Fig. S1). RNA was isolated and DNase treated using the High Pure FFPE RNA Micro kit from Roche followed by transcription into cDNA using the Sensiscrypt RT kit (Qiagen). The CDH1 messenger fragment including the partial CDS (1000-B, exon 7/8 boundary) was amplified with primers RT78F (CAGAGTGCATAGTTGTTGCA) and RT78R (CCAC-CAGGTTATACGTAGGG). Wt or mutant transcripts were detected using MWG probes (Applied Biosystems) WP (TGGACCGAGAGAGT) or MP (TGAGCGCGAGAGAGT) on an ABI 7900HT Fast Real-time PCR system (Applied Biosystems). Primers/probes for the normalization control \(β2\)-microglobulin were purchased as Assay-On-Demand (Applied Biosystems).

**Results**

Elevated frequency of SRC neoplasia in cdh1\(^{+/−}\) mice. To study the effects E-cadherin deficiency has on gastric tumor development, we used mice heterozygous for cdh1 (11). To induce significant numbers of gastric neoplasms, cdh1\(^{+/−}\) mice were treated with the carcinogen MNU, a known promoter of stomach tumors (12). Based on data from a pilot study that examined dose and exposure time, cdh1\(^{+/−}\) mice and wt littermates were exposed for 5 weeks to 120 ppm MNU or water. At 40 weeks, stomachs were examined by counting and characterizing macroscopic and microscopic lesions. Mice not treated with MNU showed few cancers: 1 of 20 cdh1\(^{+/−}\) mice had a SRCC, whereas no tumors were seen in the wt mice. In contrast, MNU-treated mice showed two types of neoplasms (Supplementary Figs. S2 and S3). Both wt and cdh1\(^{+/−}\) mice had tubular adenomas with the same incidence (\(P = 1\), two-sided Fisher’s exact test; Table 1). However, MNU-treated cdh1\(^{+/−}\) mice developed murine SRCs with an 11 times higher incidence than the MNU-treated wt mice (\(P < 0.002\); Table 1). These results show that SRCs in the cdh1\(^{+/−}\) mice developed in addition to the background adenomas induced by MNU in both cdh1\(^{+/−}\) and wt mice.

**Murine SRCs are histologically similar to their human counterpart.** The murine SRCs were intramucosal TNM stage T1a and composed of cords, columns, or nests of cells predominantly found in the antral part of the glandular stomach. All
neoplastic cells in these lesions showed loss of polarity and were mostly SRCs located in the superficial lamina propria. The other neoplastic cells were less differentiated and found in small numbers deep to the SRCs. Unlike small SRCCs, large ones were not flat but polypoid (Fig. 1A and B; Supplementary Fig. S2). One MNU-treated cdh1+/-/C0 mouse showed invasion of the muscularis propria (TNM stage T2). In this mouse, invading cells were poorly differentiated, akin to submucosal disease in HDGC (7), whereas SRCs were concentrated in the superficial mucosa (Fig. 1C; Supplementary Fig. S2). Together, the murine SRCCs parallel eHDGC—they lie underneath an intact surface epithelium, are intramucosal, and composed of predominantly SRCs superficial to small numbers of less differentiated cells (7).

Murine SRCCs share their apparent origin with eHDGC. eHDGCs seem to develop from the mucous neck cell region within the gastric proliferative zone. This has been shown by labeling with the lectin GS-II, which marks both the neck region and the less differentiated eHDGC cells that are invariably found adjacent to the neck region (7). To investigate whether murine SRCCs may also develop from the neck region, they were labeled with GS-II. GS-II marked both the murine mucous neck cell region and the nearby cancer cells (Fig. 1D). Comparable results were obtained using an antibody against mucin 6, a specific neck cell marker (Supplementary Fig. S4). Thus, the GS-II/mucin 6 staining pattern was similar to eHDGC, indicating a developmental origin that mirrors human disease.

E-cadherin down-regulation and adhesive deficiency in murine SRCC. To assess whether the expression of E-cadherin was abnormal in the murine SRCCs, immunocytochemistry was performed. Paraffin-embedded sections of SRCCs from the 11 mice were examined using antibodies against E-cadherin. All lesions displayed decreased E-cadherin expression in the neoplastic cells when examined by immunofluorescence (Fig. 2). When membranous E-cadherin signal was quantified, a significant >50% intensity reduction was observed in SRCs compared with normal epithelial cells (SRCCs from 11 mice; P < 0.0001, Mann-Whitney test; see also Fig. 2C and D). Reduced E-cadherin levels were evident in the vast majority of tumor cells (>90% median F11 SD) and the protein was not detectable in the remainder. Similar results were obtained for the SRCCs of six mice from the pilot study. In contrast, immunofluorescence for cytokeratins revealed no significant difference between SRCs and epithelium (data not shown). These findings indicate the presence of a second cdh1 hit, which has induced down-regulation of the remaining cdh1 wt allele in the murine SRCCs.

An expected consequence of E-cadherin down-regulation is the destabilization of the adherens junction (7). We thus examined the expression of proteins that participate with E-cadherin in the junctional complex (β-catenin, p120, and Lin-7). The junctional

Table 1. Tumor incidence in treated mice

<table>
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<tr>
<th>Treatment</th>
<th>H2O</th>
<th>MNU</th>
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<tr>
<td>cdh1</td>
<td>+/-</td>
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</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Tubular adenoma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SRCC</td>
<td>0</td>
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NOTE: Numbers refer to mice. In general, one tumor of a given type was present per mouse.
* Four of the 18 mice had two tubular adenomas.
† One of the 18 mice had two tubular adenomas, and one had three tubular adenomas.
‡ One of the 11 mice had two SRCCs.

Figure 1. Histopathology and cellular composition of SRCCs in cdh1+/- mice. See Supplementary Fig. S2 for additional examples. A, small murine SRCC TNM stage T1a. B, large polypoid murine SRCC (T1a). C, murine SRCC with submucosal invasion (T2). Bottom, the cellular composition is shown in the magnified squares. D, GS-II stain (green) and H&E stain (bottom) of SRCs and the adjacent proliferative mucous neck region of normal glands (N).
proteins displayed reduced/abnormal expression in the SRCCs of the 11 examined cdh1+/−/C0 mice (Fig. 3), consistent with a functional deficiency affecting E-cadherin and similar to eHDGC (7).

**E-cadherin deficiency induces a hypoproliferative state.** Similarly to eHDGC (17), the murine SRCCs also lacked nuclear β-catenin accumulation (Fig. 3), suggesting absence of Wnt pathway activation. In contrast, tubular adenomas in these mice showed nuclear β-catenin translocation (Fig. 4) and normal E-cadherin expression (Supplementary Fig. S5). Consistent with a growth-promoting role of the Wnt pathway, tubular adenomas displayed strong PCNA expression, unlike murine SRCCs (Fig. 4). Indeed, the proliferation index of murine SRCCs was significantly lower than that of adjacent normal epithelium (16.6% versus 21.1%; n = 11; P < 0.005, Mann-Whitney test), as has been observed in eHDGC (7).

**CDH1 promoter hypermethylation as a second hit in eHDGC.** In human CDH1 mutation carriers, ~50% of advanced HDGCs have a hypermethylated CDH1 promoter (18); however, the CDH1 methylation status of eHDGCs is unknown. To investigate this, we performed methylation analysis on microscopic eHDGCs from prophylactic gastrectomies of patients carrying a CDH1 1008G>T germ-line mutation. DNA isolated from dissected eHDGC foci and matched nonneoplastic mucosa from four gastrectomies (patients 1–4) was bisulfite treated and assayed for CDH1 promoter methylation by methylation-specific PCR. Methylation was detected in 8 of 16 foci of eHDGC and 2 of 12 nonneoplastic samples (Fig. 5A). All investigated foci were methylated in patients 1 (4 of 4) and 4 (3 of 3). Patient 2 showed methylation in one focus (1 of 5), and no methylation was detected in patient 3 (0 of 4). Thus, CDH1 promoter methylation was observed in eHDGC at a level similar to advanced HDGC. To assess whether methylation affects the wt CDH1 allele, RNA was isolated from microdissected SRCs and adjacent epithelium from patients 1 and 4 and examined for expression of wt (1008G) or mutant (1008T) CDH1 message using specific probes and real-time PCR. No mutant message was detected, suggesting nonsense-mediated decay of the truncating CDH1 mRNA. In contrast, wt message was expressed in eHDGC but at much lower levels compared with matched epithelium (5–37%; Fig. 5B). Therefore, expression of wt CDH1 mRNA inversely correlated with the presence of CDH1 promoter methylation.

Sequencing of individual methylated alleles confirmed methylation in the investigated CDH1 promoter region (≥84% of CpGs methylated). Notably, alleles derived from the same focus showed an identical methylation pattern (the position of methylated CpGs), consistent with a clonal evolution of eHDGC (Fig. 5D). In contrast, the methylation pattern differed between individual eHDGCs from the same patient, providing evidence for the independent origin of each eHDGC.

**Discussion**

E-cadherin has a well-documented role in the progression of epithelial cancers. The down-regulation of the protein during...
carcinoma invasion and metastasis has led to a concept of E-cadherin acting as an invasion suppressor during epithelial tumorigenesis (5). In contrast, the view that E-cadherin deficiency may initiate some types of carcinoma is not well established. This study was performed to explore the contribution of E-cadherin to the initiation of diffuse gastric cancer.

Multiple gastric intramucosal foci of SRCC are the first apparent disease stage in humans with CDH1 germ-line mutations (9). Using a murine system, we were able to predictably induce gastric intramucosal SRCC following exposure to MNU. SRCCs developed at a significant frequency only in cdh1+/−/C0 mice, but not wt mice, indicating the dependency of this tumor type on E-cadherin.

![Figure 5. CDH1 promoter methylation in eHDGC. A, methylation-specific PCR for unmethylated (U) and methylated (M) CDH1 promoter alleles in eHDGC and matched mucosa from four CDH1 mutation carriers. Each line represents adjacent tissue samples. Unmethylated alleles were present in all samples studied. B, reduced levels of wt CDH1 mRNA in eHDGC relative to adjacent mucosa in patients with methylated CDH1 promoter. CDH1 message was amplified by quantitative PCR using exon-spanning primers and detected by wt- and mutant-specific probes. Genomic DNA and cDNA samples without reverse transcriptase served as negative controls. The expression level of wt CDH1 message in matched epithelia samples was set to one. Mutant message was undetectable in normal and neoplastic tissue. Columns, mean of two independent RNA preparations; bars, SD. C, immunofluorescence showing presence of reduced E-cadherin levels in an eHDGC from patient 1 surrounded by strongly staining normal mucosa. Right, magnified square. D, the converted sequence of individual cloned alleles from three eHDGCs. M, methylated reference sequence; U, unmethylated reference sequence. Gray, CpG sites. Note the methylation patterns specific to individual eHDGCs.](image)
deficiency. Of note, SRCCs were also detected in 3 of 13 untreated cdh1+/− mice kept for 80 weeks, suggesting that the murine tumors may develop spontaneously when given enough time (data not shown). As in the human SRCCs found in CDH1 mutation carriers, E-cadherin expression was reduced on immunofluorescence in the murine SRCCs relative to normal mucosa, indicating that the remaining allele has been down-regulated. Although insufficient tissue was available to confirm the down-regulation by real-time PCR or Western analysis, additional adherens junction proteins (β-catenin, p120, and Lin-7), but not cytokeratins, also displayed reduced expression, consistent with a functional deficiency in E-cadherin. Intramusosal SRCCs were the first detectable disease signs in cdh1+/− mice, as they are in the human disease (17), indicating that E-cadherin was down-regulated very early in these tumors. In contrast to the SRCCs, E-cadherin expression was normal in tubular adenomas that were induced by MNU irrespective of the cdh1 status of the mice, confirming a specific role for E-cadherin in diffuse-type carcinogenesis. Together, these findings indicate a causal relationship between E-cadherin deficiency and the initiation of gastric SRCC in mice. The murine SRCCs resemble their human counterpart in many respects. In addition to their morphologic parallels, the murine tumors also seem to develop from the gastric mucous neck region and show a transition to a poorly differentiated state when invading beyond the mucosa. The main difference to humans is that murine SRCCs tend to occur in the gastric antrum, whereas eHDGCs are found in all stomach zones. Lesion multifocality as observed in human CDH1 mutation carriers is also not seen in the mice (only 1–2/stomach) but may simply relate to the different stomach size because the lesion density is similar in the two species (0.5–1 lesion/cm2).

Remarkably, both eHDGC and murine SRCC display proliferative activity that is lower than that of normal gastric epithelium. This unique feature implies that E-cadherin deficiency drives carcinogenesis via mechanisms that do not require the selection of a growth advantage and suggests underlying pathways distinct from many other epithelial cancers. This is well illustrated by the comparison with the MNU-induced tubular adenomas, where hyperproliferation is observed along with nuclear β-catenin accumulation, in stark contrast to the neighboring SRCC from the same mouse. Similarly, MNU-treated app−/− mice have a high incidence of stomach tumors with an activated Wnt pathway and of adenomatous origin (19).

The oncogenic consequences of deficient adhesion are not well characterized in vivo. Although no direct evidence exists for mammals, E-cadherin–mediated adhesion is required in Drosophila for the regulation of both the attachment of stem cells to their niches (20) and the correct spindle orientation during asymmetric divisions required to separate progeny with a distinct fate by division out of the epithelial plane (21). Thus, a tempting hypothesis would be that perturbed stem cell control contributes to malignancy initiated by lost adhesion. This hypothesis is consistent with the apparent origin of eHDGC at the upper isthmus, the location of gastric progenitor and stem cells (7).

To further explore the initiating role of E-cadherin in human disease, we sought to show the mechanism of the second hit that precipitates the down-regulation of the protein in eHDGC. CDH1 promoter hypermethylation has been reported in 50% of advanced HDGC (18). We detected CDH1 promoter hypermethylation at the same frequency in eHDGC, the earliest observable disease stage in CDH1 mutation carriers. Promoter methylation coincided with down-regulation of the wt (but not mutant) CDH1 allele, confirming that methylation is the likely second CDH1 hit in eHDGC. This observation is consistent with studies in other hereditary human cancers showing that promoter hypermethylation generally does not affect the mutated susceptibility allele but occurs on the wt copy (22). In support, the uniform methylation pattern specific to each eHDGC indicates the methylation being monoallelic. The methylation patterns further indicate that each eHDGC has an independent monoclonal origin, with methylation being the initiating event. Together with the down-regulation of E-cadherin in all murine and human SRCCs and the large numbers of eHDGC that can develop in a stomach, it is highly improbable that a gene other than CDH1 is consistently mutated in HDGC. It is therefore likely that a second CDH1 hit is sufficient to initiate diffuse gastric cancer in a CDH1 mutation carrier. With promoter methylation as the major mechanism underlying this second hit, our data add to the growing consensus on the significance of methylation in carcinogenesis by providing further direct evidence for the etiologic role of an epigenetic event in cancer.

Promoter methylation leads to down-regulation but not always to complete silencing of CDH1 (23) and thus could explain the residual E-cadherin levels detected by immunofluorescence in eHDGC. Of note, genetic events that lead to complete inactivation of both CDH1 alleles have not been shown for HDGC thus far (18, 24). Close to 50% of the known CDH1 germ-line mutations are missense or within the splice site consensus sequence and therefore may not fully abrogate all allele function, in stark contrast to other familial cancer syndromes such as familial adenomatous polyposis, where >95% of APC mutations are either frameshift or nonsense (25). Minimal CDH1 activity may thus be required for tumors to develop or survive, consistent with the reduced protein levels we observed in SRCs of mice and man. Derksen and colleagues (26) have used conditional cdh1 inactivation to study E-cadherin in murine mammary carcinogenesis. They showed that loss of E-cadherin occurs very early in lobular carcinoma and hence contributes to disease initiation, but only in the absence of wt p53. The authors proposed that complete E-cadherin loss is not tolerated due to apoptotic activation but can initiate tumorigenesis if apoptotic execution is abrogated by deletion of p53. In humans, E-cadherin deficiency is likely to initiate lobular breast cancer (9). However, unlike in the mice described by Derksen and colleagues (26), p53 inactivation seems not to be a requirement for the initiation of either lobular breast cancer or diffuse gastric cancer in humans (6, 27). We propose that the two CDH1 hits are selected in a way that maintains some residual E-cadherin function to prevent apoptotic elimination. Consistent with the reduced protein levels described here, down-regulation of E-cadherin below a threshold value is sufficient to abrogate its adhesive function (28) and to perturb asymmetric division (21).

In conclusion, we established a causal relationship between E-cadherin deficiency and the initiation of diffuse gastric cancer. Our MNU-treated cdh1+/− mice represent the first murine model where gastric SRCC can be induced in a predictable way and should prove valuable for the study of the biology, diagnosis, and therapy of both hereditary and sporadic diffuse gastric cancer. We have shown the presence of a second CDH1 hit in the earliest observable stage of human diffuse gastric cancer, providing evidence for epigenetic down-regulation of E-cadherin as an initiator of malignancy. As gene methylation is reversible, potential exists to interfere at the early stages of gastric carcinogenesis. Our results further suggest that loss of adhesion can initiate cancer evolution.
via pathways that require neither Wnt pathway activation nor hyperproliferation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Acknowledgments

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