Destabilized Adhesion in the Gastric Proliferative Zone and c-Src Kinase Activation Mark the Development of Early Diffuse Gastric Cancer

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Abstract

The initial development of diffuse gastric cancer (DGC) is poorly understood. The study of E-cadherin (*CDH1*) germ line mutation carriers predisposed to DGC provides a rare opportunity to elucidate the genetic and biological events surrounding disease initiation. Samples from various stages of hereditary and sporadic DGC were investigated to determine general mechanisms underlying early DGC development. Paraffin-embedded tissues from 13 *CDH1* mutation carriers and from 10 sporadic early DGC cases were analyzed. Immunofluorescence and immunohistochemistry using differentiation, proliferation, and adhesion markers showed that DGC initiation seems to occur at the proliferative zone (the upper neck) of the gastric epithelium and correlates with absent or reduced expression of junctional proteins (β-actin, p120, Lin-7). Slow proliferation of neoplastic cells at the upper gastric neck leads to the formation of intramucosal signet-ring cell carcinoma (SRCC) displaying differentiated features. As shown by immunolabeling, invasion from SRCC lesions beyond the gastric mucosa is associated with poor differentiation, increased proliferation, activation of the c-Src system, and an epithelial-mesenchymal transition. Our results provide a molecular description of the early development of DGC and explain the relationship between the two main DGC types, poorly differentiated carcinoma and SRCC: both share their origin, but SRCC develops following cancer cell differentiation and seems relatively indolent in its intramucosal stage. [Cancer Res 2007;67(6):2480–9]

Introduction

Gastric cancer, ranking second in terms of global cancer-related mortality, can be classified into two major histologic types, the intestinal and the diffuse forms (1). Diffuse gastric cancer (DGC) can be further subdivided into signet ring cell carcinoma (SRCC) and poorly differentiated carcinoma. While the incidence of intestinal-type stomach cancer is decreasing, a recent epidemiologic study indicates an increase in the diffuse form (2). Intestinal gastric cancer is believed to develop from dysplastic precursors followed by a multistep progression (1). In contrast, the natural history of DGC is poorly understood.

DGC can be discriminated from the intestinal form on the basis of its abnormal expression of the cell-to-cell adhesion molecule E-cadherin (3). Mutations in the E-cadherin gene (*CDH1*) and *CDH1* promoter hypermethylation are found in the majority of sporadic DGCs (4, 5). E-cadherin is a transmembrane protein located at the adherens junctions; its cytoplasmic tail binds either β-catenin or γ-catenin, which in turn mediate interaction with the actin cytoskeleton via α-catenin as a linker (6). By homophilic interaction through its extracellular domain, E-cadherin provides an anchor that connects the actin cytoskeletons of adjacent cells and is essential for the structure and polarity of epithelial planes.

E-cadherin is regarded as an invasion suppressor. Its loss has been correlated with poor differentiation and progression to an invasive phenotype in epithelial cancers (7). In DGC, however, E-cadherin down-regulation may be an early, initiating event in tumorigenesis; *CDH1* germ line mutations cause hereditary DGC (HDGC), a familial form of DGC (8). The earliest known manifestation of HDGC is the accumulation of multiple microscopic foci (up to several hundred) of signet ring cells (SRC) confined to the mucosa of *CDH1* germ line mutation carriers (9). Pathologically, the foci are defined as invasive SRCC tumor-node-metastasis stage T_{1a} and will be referred to as early HDGC (eHDGC). Multiple foci of eHDGC are found in essentially every mutation carrier. This high penetrance is greater than the 70% lifetime risk of advanced HDGC in carriers and suggests that eHDGC represents a relatively indolent stage that precedes progression beyond the mucosa (10).

How E-cadherin insufficiency contributes to tumor initiation is not known. One possibility relies on the E-cadherin/β-catenin interaction and the dual role of β-catenin in adhesion and transcriptional activation. Similar to loss of the colonic tumor suppressor APC, down-regulation of E-cadherin could cause nuclear accumulation of β-catenin and subsequent transformation (11). However, β-catenin is not expressed in eHDGC, indicating that it does not contribute to HDGC initiation (12). Better established is the role of E-cadherin in controlling the Rho family GTPases that regulate actin dynamics and provide a mechanism underlying the influence E-cadherin has on cell shape and motility (13). E-cadherin–mediated adhesion can activate several GTPases including Rac1. Intriguingly, Rac1 has been implicated in regulating self-renewal and differentiation in mammals (14, 15), providing the possibility that down-regulation of E-cadherin in gastric epithelia may initiate disease by interfering with normal stem cell control (16, 17).

Given this background, we studied tumor samples from sporadic DGC cases and one HDGC family to investigate the origins of tumor growth, establish whether destabilization of adherens junctions is an early and consistent event in the initiation of disease, and to determine the relationship between eHDGC and

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advanced HDGC. Based on our findings, we propose a model for the natural history of DGC, in which initiation is characterized by destabilization of adhesion in the gastric proliferative zone and progression is associated with dedifferentiation from the epithelial phenotype.

Materials and Methods

Patients and Tissue Samples

Paraffin-embedded gastric specimens from 13 members of one HDGC kindred were studied (8). All patients were carriers of the *CDH1* 1008G>T germ line mutation. Patients 1 to 4 were diagnosed at annual chromogastroscopic surveillance and had had a total gastrectomy. Upon microscopy, the respective stomachs all had multifocal intramucosal T_{1a} SRCC (eHDGC) despite a normal macroscopic appearance (9). Case 5 also had one T_2 DGC in addition to 17 eHDGCs. Patients 6 to 13 presented with advanced disease (>T_2). Paraffin blocks containing T_{1a} SRCC were available from 10 Korean patients who had been diagnosed with sporadic DGC. In all cases, gastric carcinoma tissue was obtained from a gastroscopic biopsy, open surgical biopsy, or total gastrectomy. Ethical approval was given by the Northern Y Regional Ethics Committee of New Zealand.

Tumor Definitions

eHDGC: hereditary intramucosal SRCC tumor-node-metastasis stage T_{1a}. The earliest neoplasia detected in pathologic specimen of otherwise asymptomatic *CDH1* germ line mutation carriers. Composed of predominantly SRCs below intact surface epithelium. Usually microscopic (often <1 mm in diameter). Larger eHDGC can have a poorly differentiated component.

Sporadic T_{1a} SRCC: intramucosal SRCC in patients without known predisposition. Defined by the presence of >50% SRCs among otherwise usually poorly differentiated cells. Includes some erosion of the surface epithelium as it is endoscopically detected. Similar to eHDGC in its basic structure, but larger in its diameter (>10 mm) and likely more advanced within the stage.

Immunohistochemistry and Immunofluorescence

Four-micrometer sections of formalin-fixed, paraffin-embedded tissue specimens were used for immunostainings. Antigens were retrieved by boiling in citrate buffer. The following primary antibodies were used: rabbit polyclonal E-cadherin (sc7870, dilution 1:100), mouse monoclonal c-Src (sc8056, 1:1,000), rabbit polyclonal Fak (sc932, 1:1,000), all from Santa Cruz Biotechnology (Santa Cruz, CA); sheep polyclonal pepsinogen II (ab9013, 1:1,000), rabbit polyclonal Lin-7 (ab11472, 1:500), and mouse monoclonal β-actin (ab6276, 1:3,000) were from Abcam (Cambridge, United Kingdom); rabbit polyclonal fibronectin (A0245, 1:2000), mouse monoclonal Ki-67 (M7240, 1:50), and mouse monoclonal cytokeratin AE1/AE3 (A0245, 1:50) were from DAKO (Glostrup, Denmark); mouse monoclonal Stat3 (9139, 1:200) and mouse monoclonal pY^{705}-Stat3 (9138, 1:60) were from Cell Signaling; mouse monoclonal Dnmt1 (IMG-261, 1:250; Imgenex, San Diego, CA), rabbit polyclonal pY^{861}-Fak (44-626, 1:250; Biosource), mouse monoclonal H^+/K^+ATPase β subunit (RDI-HKATPabm, 1:500; Research Diagnostics), mouse monoclonal p120 (610133, 1:3,000; BD Transduction Labs, San Jose, CA), mouse monoclonal MUC5A and MUC6 (NCL-MUC-5AC, NCL-MUC-6, both 1:50; Novocastra, Newcastle Upon Tyne, United Kingdom), and mouse monoclonal activated c-Src (clone 28, 1:250, from A-Cube, and a kind gift from Dr. Koji Owada, Kyoto Pharmaceutical University, Japan). The DAKO Envision horseradish peroxidase system (K4001 mouse, K4011 rabbit; DAKO) was used to detect antibodies in histologic sections. For immunofluorescence, the following secondary antibodies were used: Alexa Fluor 350/647 goat anti-rabbit IgG (A11046/A21244), Alexa Fluor 350/647 goat anti-mouse IgG (A11045/A21235), Alexa Fluor 350 donkey anti-sheep IgG (A21097), Alexa Fluor 350 donkey anti-goat IgG (A21081) from Invitrogen (Carlsbad, CA); rhodamine-conjugated sheep anti-rabbit IgG (AQ301R; Chemicon, Temecula, CA), and Texas red–conjugated rabbit anti-goat IgG (TI-5000; Vector Laboratories, Burlingame, CA). GS-II (*Griffonia simplicifolia*) and DBA (*Dolichos biflorus*) lectins were purchased from EY Laboratories (San Mateo, CA), as FITC or Texas Red conjugates and used at 10 or 20 μg/mL, respectively.

Terminal Deoxynucleotide Transferase–Mediated dUTP Nick End Labeling Assay

Terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) staining was done as described in the Apoptosis *In Situ* Cell Death Detection Kit (Roche, Basel, Switzerland) with the following modification: nicked DNA ends were labeled with fluorescein-dUTP for 10 min at 37°C.

Results

eHDGC originates from the proliferative zone of the gastric gland. Gastric mucosa consists of vertical epithelial invaginations called gastric glands or units. To discriminate the main epithelial cell types in gastric units, differentiation markers (pepsinogen II, H^+/K^+ ATPase β subunit, DBA, and GS-II; ref. 18) were applied. In normal epithelium, pepsinogen-producing chief cells are concentrated at the bottom of gastric units, pit cells occupy the surface-oriented part, whereas acid-producing parietal cells are scattered through the lower two-thirds of a unit. In between the pit cells and the chief cells resides the neck region, which includes the proliferative zone or upper neck, with proliferative mucous neck cells as well as gastric progenitor/stem cells, and the lower neck, with mature mucous neck cells in their transition to chief cells. The differentiation markers subdivided the gastric units into the corresponding compartments, with the neck region marked by GS-II (Fig. 1*A*). Because DBA and GS-II are not established lineage markers for the human stomach, their staining pattern was compared with that of MUC5A and MUC6 antibodies, specific markers for human pit and mucous neck cells, respectively. Both DBA/MUC5A and GS-II/MUC6 displayed a similar staining pattern, confirming that the lectins identify the appropriate gastric lineages (data not shown).

To investigate the gastric differentiation pattern in eHDGC, the differentiation markers were applied to 16 eHDGC from four *CDH1* mutation carriers from one HDGC kindred (for tumor definitions see Materials and Methods). All eHDGC showed absent/reduced E-cadherin expression, indicating that a second hit had occurred in the remaining *CDH1* wild-type allele (Fig. 1*B*). All eHDGC were composed of a luminal part displaying typical SRC morphology and the eHDGC base (deeper in the mucosa), where signet ring features are less evident and cells are smaller. The base was always close to the upper neck of a gastric unit (Fig. 1*C*). In all eHDGC, SRCs in the luminal part, but not at the base, stained with DBA (although with varying intensities). Conversely, GS-II staining was absent in the luminal part but was consistently evident at the base close to the GS-II positive neck region of normal mucosa (Fig. 1*D*). Similarly, MUC5A stained luminal SRCs, whereas MUC6 was detected at the lesion base (data not shown).

In order to distinguish between the lower neck and the proliferative upper neck, the proliferation marker Ki-67 was applied. Ki-67 expression was observed in the upper neck of normal epithelium and, similarly to GS-II, at the eHDGC base (Fig. 2*A* and *B*). On average, ~5% of all eHDGC cells were Ki-67 positive (16 lesions; geometric mean, 5.3 ± 8.8 SD). However, the vast majority of positive cells were found at the eHDGC base (17% ±14 SD as opposed to only 0.8% ±5 SD in the luminal eHDGC part; *t* test unequal variance, $P < 0.00005$). The eHDGC base and the proliferative neck region were shown by GS-II/Ki-67 double staining to overlap (Fig. 2*C*). Morphologically, Ki-67 expression was predominantly observed in the small SRCs and less differentiated cells typically found in the eHDGC base. Together,

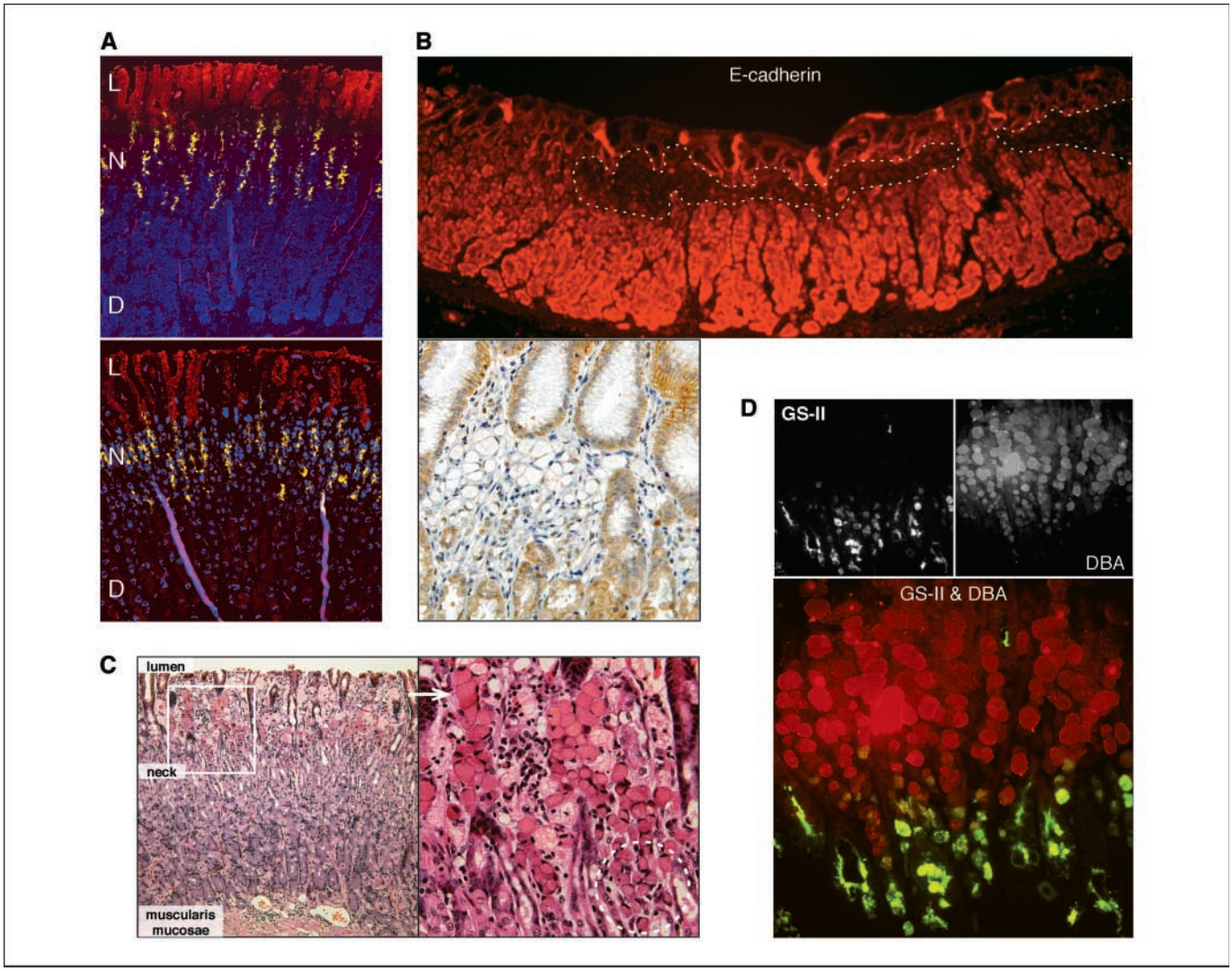

Figure 1. Lineage-specific labeling of normal mucosa and eHDGC. *A,* characterization of the lineage markers GS-II and DBA in human gastric mucosa. *Top,* vertical gastric units horizontally divided into the luminal pit cell compartment (*L, red*, DBA), the neck region including the upper, proliferative neck (*N, green*, GS-II), and the deep mucosa part (*D*) with chief cells (*blue*, pepsinogen II). *Bottom,* H^+/K^+ATPase β subunit antibody instead of pepsinogen antibody was used to stain parietal cells (*blue*), which are found in the lower two-thirds of gastric units. *B,* E-cadherin immunofluorescence (*top*) and immunohistochemistry (*bottom*) of eHDGC from two *CDH1* mutation carriers. Dotted lines mark the lesion. *C,* HE section of mucosa containing eHDGC. *Right,* magnification of inset showing eHDGC with typical SRCs (*arrow*) in the luminal part and small SRCs or less differentiated cells at the eHDGC base (*circle*). *D,* eHDGC stained with GS-II (*green*) and DBA (*red*). Note the overlap of the GS-II positive eHDGC base and the neck region.

these data show that the upper neck region of normal gastric units is spatially and phenotypically related to the eHDGC base and is therefore the likely origin of disease.

In contrast to eHDGC, 30% to 40% of cancer cells in a stage T_2 HDGC and in an additional six advanced (>T_2) HDGCs were Ki-67 positive (data not shown). Therefore, although its base is proliferative, eHDGC is slow-growing compared with advanced HDGC. This was corroborated by the absence of histologically detectable mitoses in typical SRCs of eHDGC.

Dysfunctional adhesion is common to all eHDGC cells. An expected consequence of E-cadherin down-regulation in the proliferative neck is the destabilization of adherens junctions in the earliest tumor stage. In order to examine the integrity of adherens junctions, the expression of junctional proteins was assessed by immunohistochemistry. β-actin, p120 catenin, and Lin-7 were examined, because they represent markers of adherens junction structure, stability, and maturity, respectively (6, 19, 20). All three proteins displayed reduced or absent expression in eHDGC cells compared with adjacent mucosa (Fig. 2*D*). Dysfunctional adhesion was evident in all eHDGC cells, suggesting that it is central to disease initiation.

eHDGC shows a change in cellular morphology with increasing depth of invasion. Small eHDGCs (<3 mm diameter; Fig. 1*B*) were predominantly composed of SRCs. In larger eHDGCs (>3 mm diameter), an increased proportion of poorly differentiated carcinoma cells (cells without SRC features) could be present in the deep layer below the typical SRCs, i.e., extending from the eHDGC base/upper neck level downwards (Fig. 3*A*). Similarly, poorly differentiated cells in the T_2 HDGC were located deep to SRCs downwards from the neck (Fig. 3*A*) and invaded into the muscularis mucosae (Fig. 3*B*). Poor differentiation was also predominant in all advanced (>T_2) HDGCs (data not shown), indicating that it correlates with invasion beyond the mucosa. Poorly differentiated cells from all stages stained positive with GS-II, but not with markers

of terminally differentiated gastric cells (Fig. 3*C*), suggesting that they originated from cells of the eHDGC base and not from the more differentiated SRCs in the luminal eHDGC part.

Some of the poorly differentiated cells had a morphology reminiscent of mesenchymal cells, but were carcinoma cells as evidenced by positive staining with the epithelial marker cytokeratin AE1/AE3 (Fig. 3*A* and *B*) and the absence of E-cadherin (data not shown). This observation suggests that an epithelial-mesenchymal transition (EMT), a process associated with carcinoma invasion and progression, occurs during the progression of HDGC.

c-Src kinase is activated in mesenchyme-like cancer cells and correlates with progression. To further investigate whether an EMT is involved in HDGC invasion beyond the mucosa, the expression of proteins with an established role in EMT was assessed. The kinase c-Src is a well-characterized EMT inducer (21, 22). Immunohistochemistry with an antibody against the noncatalytic domain of the kinase was done and revealed that c-Src was not expressed in the SRCs of small eHDGCs (data not shown). In larger eHDGCs with poorly differentiated cells, however, c-Src expression gradually appeared in small SRCs at the neck level and was strong in poorly differentiated and dedifferentiated cancer cells deeper in the mucosa (Fig. 4*A*). In the stage T_2 HDGC, c-Src was strongly expressed in poorly differentiated and dedifferentiated cells in the mucosal layer and the cells invading the muscularis mucosae (Fig. 4*A*), but not in intramucosal SRCs (Fig. 4*C*). Furthermore, c-Src staining was strong in the majority of cells in six out of the six advanced (>T_2) cancers examined (Fig. 4*A*). Similar results were obtained with an antibody directed against the active form of c-Src, indicating that the kinase is activated during progression of HDGC (Fig. 4*A* and *C*).

To further establish a correlation between c-Src activation and EMT induction, downstream targets of c-Src kinase were studied. c-Src–mediated EMT results in the production of the mesenchymal marker fibronectin (22). In contrast to intramucosal SRCs, carcinoma cells invading beyond the mucosa displayed strong fibronectin expression, consistent with an EMT-associated dedifferentiation (Fig. 4*B*). Furthermore, Fak and Stat3 were examined, as both are thought to

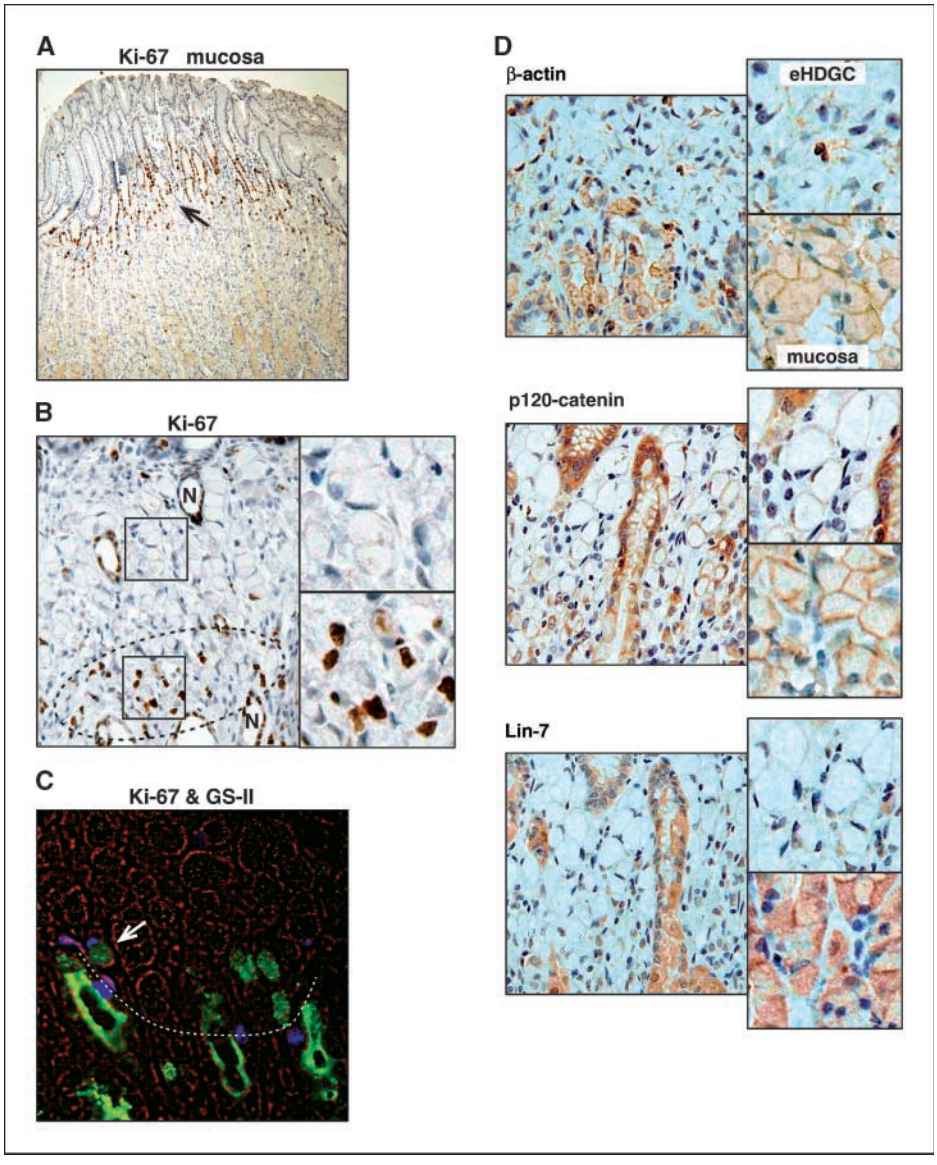

Figure 2. Expression of Ki-67 and junctional proteins in eHDGC. *A,* Ki-67 expression in the proliferative neck region (*arrow*) of normal mucosa of a mutation carrier. *B,* Ki-67 staining is present at the eHDGC base (*circle*) and in the neck of normal glands (*N*). *Right,* magnification of insets. *C,* small eHDGC base stained with Ki-67 (*blue*) and the neck marker GS-II (*green*). A few normal proliferative cells and a Ki-67/GS-II positive SRC (*arrow*) are seen at the eHDGC base (*dotted line*). All sections were also examined for apoptosis by TUNEL staining. Apoptotic cells were detected only in the normal surface epithelium, in which cells are shed into the lumen. *D,* junctional markers (β-actin, p120, and Lin-7) indicate dysfunctional adhesion in eHDGC.

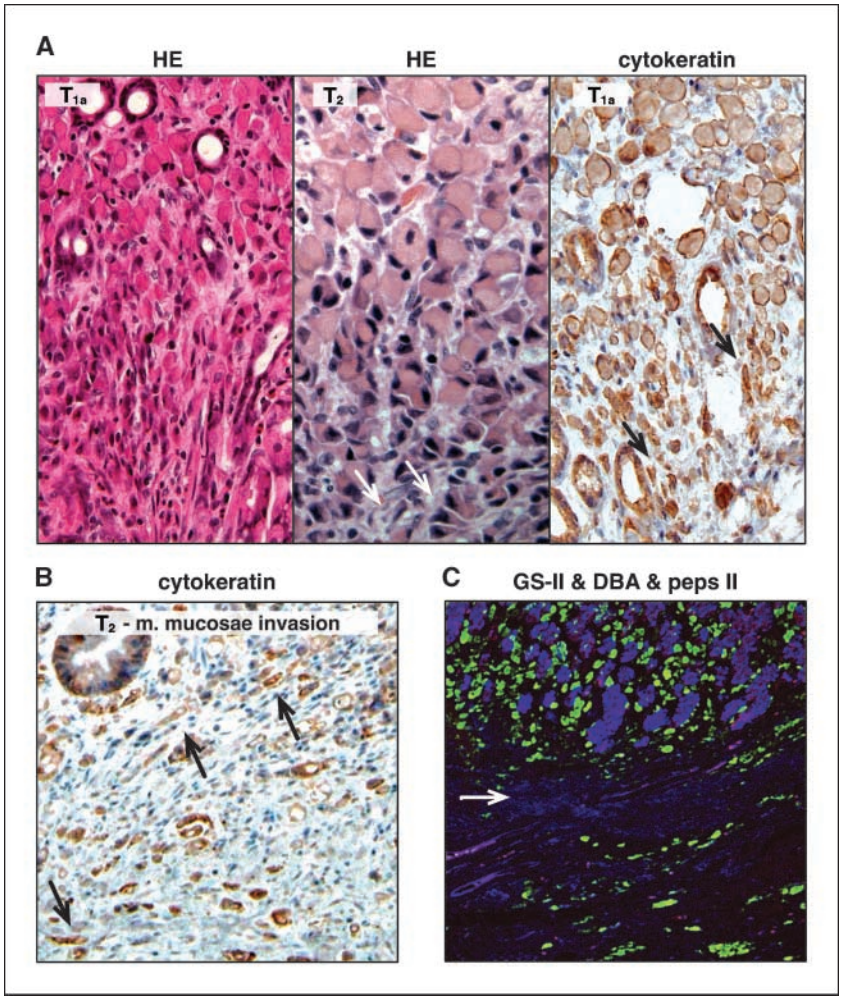

Figure 3. Correlation between dedifferentiation and deeper invasion. *A,* HE stain of an eHDGC, of a T_2 HDGC, and a cytokeratin AE1/AE3 stain of an eHDGC. Note the transition from typical SRCs to poorly differentiated cells with increasing depth. *Arrows,* examples of dedifferentiated cells with mesenchymal-like appearance. *B,* cytokeratin stain of a T_2 HDGC with poorly differentiated and dedifferentiated cells (*arrows*). *C,* lineage marker stain of a T_2 HDGC at the mucosa/muscularis mucosae border (*arrow*). Only GS-II consistently stained poorly differentiated cells in stages $\geq T_{1a}$.

be activated by c-Src via phosphorylation of specific Tyr residues and to contribute to c-Src–mediated invasion (21, 23). Using immunohistochemistry with antibodies specific for the respective phosphorylated (P-) forms, both P-Fak and P-Stat3 were largely undetectable in intramucosal SRCs. Similar to c-Src, expression of P-Fak and P-Stat3 became apparent in poorly differentiated and dedifferentiated cells of eHDGC (data not shown). In the stage T_2 HDGC, P-Fak and P-Stat3 were expressed in poorly differentiated cells located deep to the intramucosal SRCs and correlated with the expression of active c-Src (Fig. 4*C*). Multichannel immunofluorescence of stage $\geq T_2$ HDGC showed active c-Src to colocalize with P-Fak in dedifferentiated cancer cells, further supporting a functional relationship between c-Src activation and Fak phosphorylation (Fig. 4*D*).

Together, the results show that activation of c-Src and its downstream targets correlates with the acquisition of the dedifferentiated cancer cell phenotype and is associated with progression.

Early sporadic DGC is similar to eHDGC with respect to differentiation, adhesive dysfunction, and activation of c-Src kinase. The data presented above imply two central phases in the development of HDGC: (*a*) an initiation phase, which is characterized by destabilization of adherens junctions in the proliferation zone and concomitant formation of SRCs; and (*b*) a progression phase, which is characterized by poor differentiation, activation of c-Src kinase and induction of an EMT.

To determine whether these two phases could be generalized to sporadic DGC, paraffin blocks from 10 individuals diagnosed with nonfamilial stage T_{1a} SRCC were analyzed. These tumors were confined to the mucosa, but comprised large mucosal areas and were macroscopically visible, unlike eHDGC. Histologically, the sporadic T_{1a} SRCC included both SRCs (usually in the luminal mucosa) and a significant proportion of poorly differentiated cells. Samples of three patients looked similar to eHDGC, as they contained large areas with predominantly SRCs covered by intact surface epithelium. The samples from the remaining seven patients had severely eroded mucosal architecture.

The 10 sporadic T_{1a} SRCC were examined for the expression of gastric differentiation markers. In all cases, typical SRCs stained positive for the pit cell marker DBA. The intramucosal SRCC base and poorly differentiated cancer cells were positive for the neck region marker GS-II (Fig. 5*A* and *D*). Thus, the staining pattern with differentiation markers was similar to that observed in HDGC.

Eight of the 10 patients displayed absent/reduced or diffuse cytoplasmic expression of E-cadherin upon immunofluorescence, indicating biallelic *CDH1* down-regulation (Fig. 5*A*). Abnormal expression was also evident at the GS-II positive SRCC base, suggesting that E-cadherin down-regulation may be an early event in these patients.

In all patients, cancer cells displayed a heterogeneous degree of nonmembranous, reduced or absent p120, β-actin, and Lin-7

expression (example in Fig. 5*B*). Abnormal expression of at least one of the junctional markers was seen in all cells from the 10 tumors, suggesting that destabilization of adherens junctions is a common feature of SRCC. In the two patients without consistent E-cadherin down-regulation, the adhesion molecule was absent/reduced in typical SRCs, but could be detected in some of the small SRCs and less differentiated cells (Fig. 5*C*). Interestingly, these cells also expressed active c-Src and had reduced/absent staining of β-actin and Lin-7, consistent with the observation that c-Src can disrupt cadherin-mediated adhesion (21). Alternatively, defective adhesion may be the result of E-cadherin inactivation by mechanisms such as missense mutations.

In all 10 cases, active c-Src was present in poorly differentiated and dedifferentiated cells and increased in expression with the depth of mucosal invasion (Fig. 5*D*). Whereas the overall expression of c-Src was comparable to eHDGC, the observation of c-Src expression in small SRCs positive for E-cadherin in two patients suggests that more than one mechanism for the disruption of adhesion junctions can occur in sporadic disease. However, as in HDGC, decreasing differentiation was associated with the simultaneous presence of activated c-Src and loss of membranous E-cadherin.

Together, SRCs in sporadic early DGC are associated with destabilization of adherens junctions. In addition, poorly differentiated and dedifferentiated cells display activation of c-Src that increases with the depth of invasion.

Discussion

HDGC develops from the gastric proliferative zone. DGC, the dominant cancer in *CDH1* germ line mutation carriers, is believed to originate from gastric mucosa without premalignant changes such as intestinal metaplasia. However, no data have thus far been available to directly support this hypothesis. In this study, we provide strong evidence for HDGC developing from the upper proliferative neck region of the gastric epithelium. GS-II, which specifically marked the neck of gastric units in our study, consistently stained the eHDGC base. The proliferative activity of eHDGC is concentrated at its base, which physically overlaps with the proliferative upper neck region. Because no significant proliferation was observed in the luminal eHDGC part containing typical SRCs, eHDGC must evolve from its base. Therefore, the likely origin of the earliest detectable HDGC stage seems to lie within the upper gastric neck region that contains the proliferative mucous neck cells, committed progenitors and the stem cells. In mice, GS-II is being used to specifically mark mucous neck cells and their progenitors (18). In our study, GS-II stained the region that includes the mucous neck cells and was also positive for MUC6. However, genetic marking would be required to show that GS-II does not label other lineage progenitors or stem cells found in this region.

We propose that following inactivation of the second *CDH1* allele within the proliferative zone, cells detach from, or divide out of, the epithelial plane at the upper neck region to form the eHDGC base (16). From there, further division would lead to the intramucosal SRCCs typically found between the epithelial planes

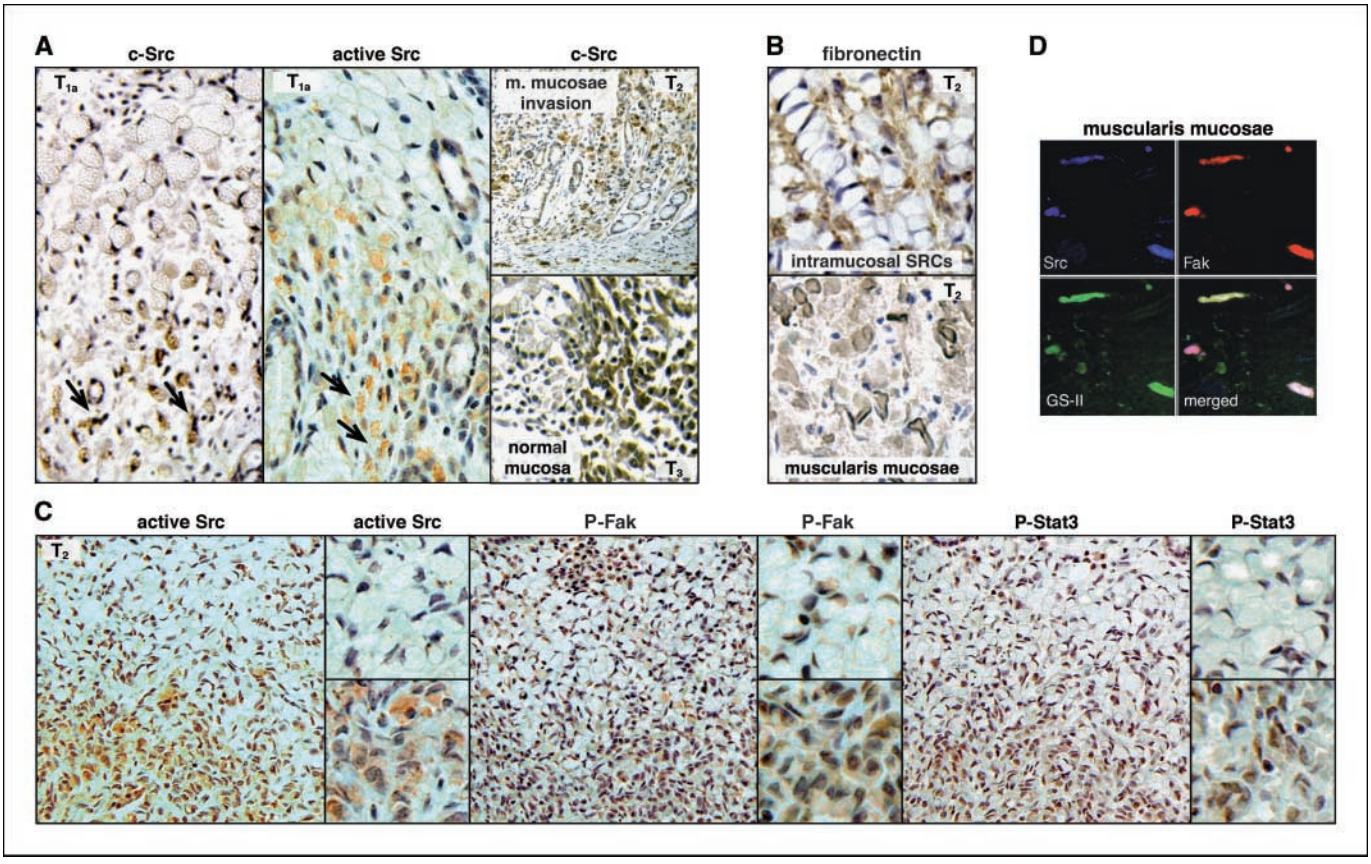

Figure 4. Correlation between c-Src activation and dedifferentiation. *A,* expression of c-Src and its activated form correlates with the transition to a dedifferentiated and mesenchyme-like phenotype (*arrow*) in eHDGC. c-Src is consistently expressed in stages >T_{1a} (*right*). *B,* fibronectin stain of a T_2 HDGC with intramucosal SRCs and dedifferentiated cells. Note the absence of fibronectin in SRCs. *C,* serial sections with SRCs (*top inset,* magnified) and poorly differentiated and dedifferentiated cells (*bottom inset,* magnified) in the mucosa stained for active c-Src, P-Fak, and P-Stat3. Note the nuclear localization of P-Stat3. Antibodies against total Fak and Stat3 were used as positive controls. *D,* colocalization of active c-Src and P-Fak in dedifferentiated cancer cells marked with GS-II.

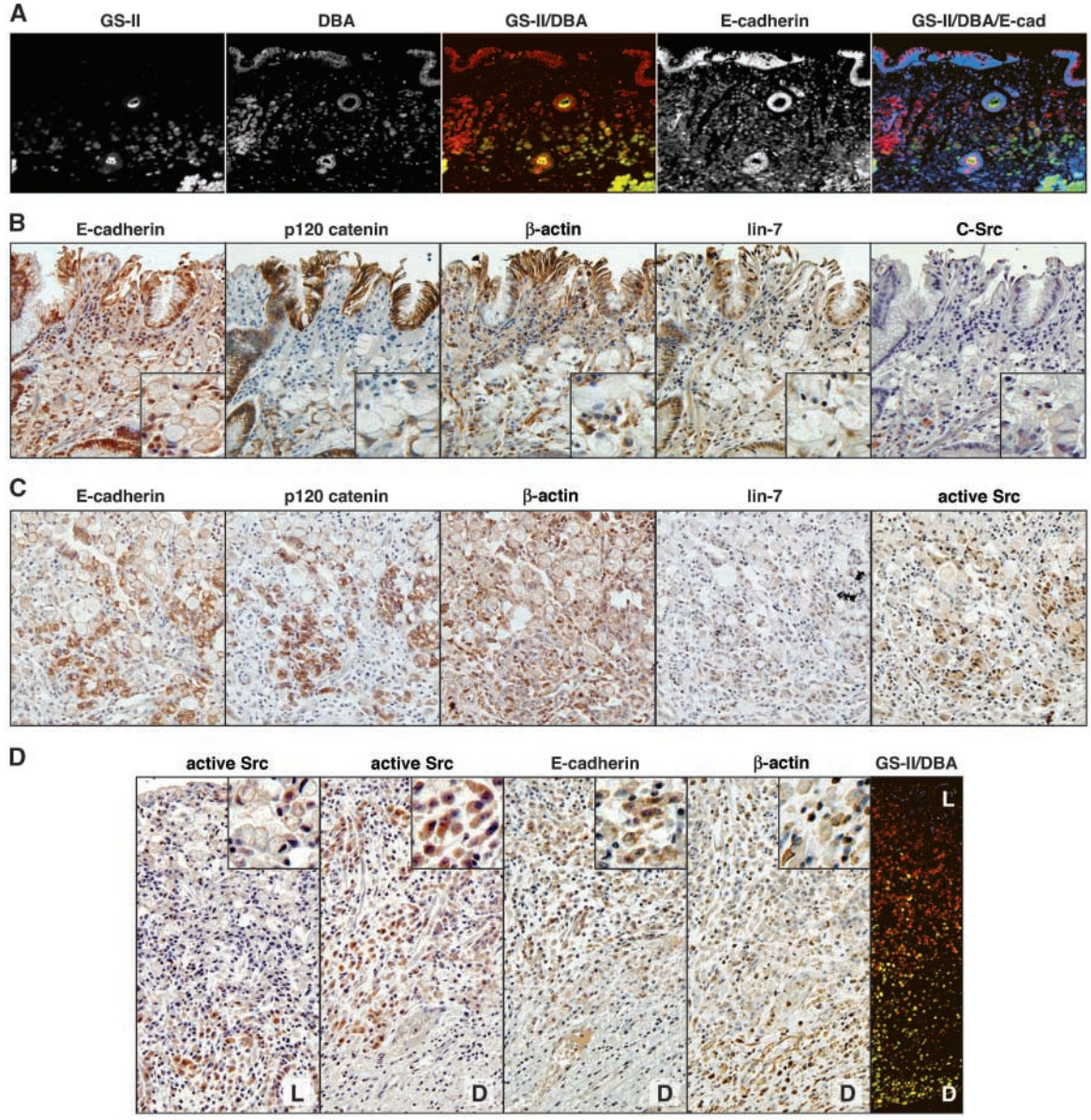

Figure 5. Junctional proteins, c-Src kinase, and gastric lineage markers in early sporadic DGC. *A,* sporadic intramucosal SRCC stained with GS-II (*green*), DBA (*red*), and E-cadherin (*blue*). Note the reduced/absent expression of E-cadherin in the GS-II/DBA–marked cancer cells. *B,* serial sections of an intramucosal SRCC stained for junctional proteins and c-Src. *Insets,* magnifications of the central lesion. *C,* serial sections of an intramucosal SRCC from a patient with partially preserved E-cadherin expression. Note the similar staining pattern of E-cadherin, p120, and active c-Src. *D,* serial sections of a sporadic intramucosal SRCC. The luminal section part (*L*) is shown only for c-Src. The deep part (*D*) is also shown for E-cadherin and β-actin (note their reduced/cytoplasmic expressions). SRCs are found predominantly in the luminal part. *Right,* the complete section stained with GS-II (*green*), DBA (*red*), and pepsinogen II (*blue*).

of gastric units in *CDH1* germ line mutation carriers (Fig. 6*A*). The *in situ* SRCs that are occasionally observed within intact epithelium (9, 12) may occur when detachment is incomplete or directed towards the lumen.

E-cadherin down-regulation leads to dysfunctional adhesion, but not to a hyperproliferative state. A likely cause of the typical spheroidal shape of SRCs is the loss of proper adhesive junctions. Reduced or absent expression of the junctional markers β-actin, p120, and Lin-7 was uniformly evident in all eHDGC examined. Dysfunctional adhesion is therefore present in very early tumor stages and likely required for disease initiation.

Interestingly, E-cadherin down-regulation does not directly result in increased proliferation, with only 5% of eHDGC cells being Ki-67 positive. Indeed, the proliferation rate in eHDGC is lower than that of normal gastric mucosa (A. Charlton, unpublished observation), indicating that additional events are required for eHDGC cells to progress to a rapidly proliferating state.

Intramucosal SRCC is differentiated and relatively indolent. According to the WHO classification, SRCC is defined as a poorly differentiated cancer (24). However, the consistent absence of Ki-67, combined with the labeling by pit cell markers, suggests that typical

SRCs in the luminal part of eHDGC have entered a state of terminal differentiation. Therefore, although their structural features may not be well developed, SRCs seem to be functionally arrested.

This advanced state of differentiation is reflected in their expression levels of invasion-associated proteins. Unlike cells of HDGC stages $>T_{1a}$ (invasion beyond mucosa), typical intramucosal SRCs were consistently negative for c-Src, Fak, and Stat3, indicating a relatively low potential for SRCs to progress. Accordingly, poor differentiation and staining with GS-II (which does not stain differentiated SRCs) are dominant in stages $>T_{1a}$.

The lack of expression of Ki-67 and the invasion-associated proteins, together with the positive staining with markers of terminally differentiated cells, strongly argues that intramucosal SRCs—the predominant cell type in eHDGC—represent a relatively indolent, differentiated state with a low progression potential. Clinical data are in line with our conclusions: in the 11 asymptomatic family A mutation carriers (including the patients of this study), an average of 146 eHDGC per stomach were observed (9). Assuming a life time penetrance of 70% for developing an advanced HDGC for these patients (10), as few as 0.5% of individual foci will eventually progress to advanced stages, illustrating their indolent nature.

Dedifferentiation and c-Src kinase activation are central events required for the progression of disease. Poorly differentiated and dedifferentiated cancer cells can be observed in larger eHDGC and are the dominant cell type in all later ($>T_{1a}$) stages. This apparent progressive change is strongly associated with activation of c-Src, its downstream targets, and the production of mesenchymal fibronectin. c-Src kinase therefore seems crucial in HDGC progression beyond the mucosa, consistent with its proposed role in the induction of a dedifferentiated mesenchyme-like state during epithelial cancer progression (21). In the developing avian stomach, viral delivery of c-Src into the epithelial layer induces epithelial cells to migrate away from the lumen, resulting in the formation of a mesenchymal layer underneath (25). In HDGC, other proteins that regulate EMT (e.g., p120, Twist and Snail, LOXL2) may independently contribute to a malignant phenotype (19, 26, 27). However, E-cadherin down-regulation (a marker for all HDGC cells) did not correlate with a mesenchymal phenotype, and alone does not seem to be sufficient to induce EMT, consistent with the large proportion of eHDGC that never progress. We thus propose that E-cadherin insufficiency together with the activation of an EMT inducer is required for invasion beyond the mucosa.

c-Src activation became apparent in small SRCs located at the level of the upper neck region and increased with the depth of invasion. Together with GS-II marking both the eHDGC base and the poorly differentiated cells, this observation is consistent with a model, in which c-Src activation at the eHDGC base leads to the transition from an indolent SRCC phase to a more aggressive behavior preluding the next stage of tissue invasion (Fig. 6*B*).

It is not clear what genetic events initiate the transition to poor differentiation and an invasive phenotype. In sporadic DGC, chromosomal analysis has shown that poorly differentiated carcinoma have frequent gain at Xp, Xq, and 20q in addition to the chromosomal aberrations observed in SRCC (28). The gain at 20q is of particular interest, as this chromosomal region harbors the c-Src gene, suggesting that gene amplification may be one mechanism underlying c-Src activation. However, we did not detect c-Src amplification in advanced HDGC using gene dosage QPCR (B. Humar, unpublished observations). Epigenetic events may provide an alternative mechanism for the transition. Increased DNA methylation has been correlated with poorer differentiation in gastric cancer (29) and could lead to down-regulation of physiologic c-Src inhibitors such as caveolin (30), as has been shown for colorectal cancer (31).

HDGC and sporadic disease share a similar natural history. As E-cadherin is a central tumor suppressor in the majority of DGCs (3–5), HDGC should serve as a model for the sporadic disease. However, only one third of families with a high DGC incidence carry identifiable *CDH1* mutations, implying that other genes can be involved in disease initiation (9). Yet familial DGCs are histologically indistinguishable, suggesting that all DGCs should be similar regarding basic developmental aspects irrespective of the initiating event. The study of prophylactic gastrectomies from *CDH1* mutation carriers has provided the first opportunity to systematically investigate the development of early DGC stages that usually remain undetected in sporadic disease. We have evaluated sporadic T_{1a} SRCC, pathobiologically the DGC form closest to eHDGC, in order to identify features common to the development of both hereditary and sporadic disease. This comparison showed that eHDGC and sporadic intramucosal SRCC share (*a*) their apparent developmental path starting at the upper neck region, (*b*)

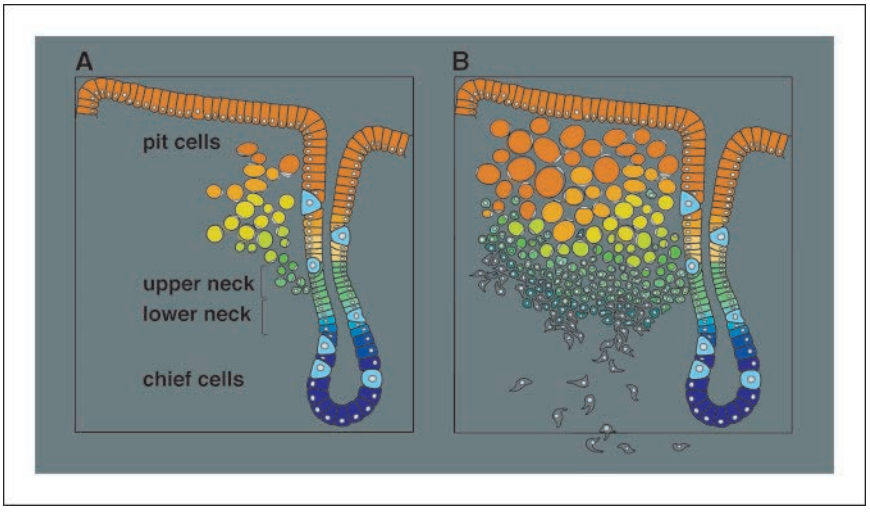

Figure 6. A model for the development of DGC. *A,* early intramucosal eHDGC with the proliferative base at the upper neck region and differentiating SRCs towards the lumen. *B,* an expansion of poorly differentiated cells below the eHDGC base and concomitant activation of c-Src is associated with an EMT and the invasion beyond the gastric mucosa.

destabilized adherens junctions, (*c*) the differentiated pit cell features of typical SRCs, and (*d*)—along with loss of membranous E-cadherin—activation of c-Src kinase that correlates with acquisition of dedifferentiation, invasion, and progression. c-Src activity seems to be elevated in advanced gastric cancer (32, 33), further supporting a role for the kinase in the progression of sporadic disease.

Other similarities to eHDGC include the SRCC structure and location, with typical SRCs in the luminal part and the proliferative activity concentrated in small cells close to the neck region (34, 35). Our view is in agreement with that of Sugihara et al., who almost 20 years ago proposed that luminal SRCs may be terminally differentiated, and arise from the neck region (35).

The data presented here have implications for the clinical features, risk factors, and treatment of DGC. Firstly, our results indicate that early sporadic intramucosal SRCC is relatively indolent. The majority of clinical studies on sporadic SRCC have reported a significantly better clinical outcome of early SRCC (36–42) and less lymph node metastasis (36–38, 41–42) compared with other gastric cancer types, whereas advanced SRCC does not seem to differ from non-SRC gastric cancer with respect to survival (37, 39–44). In addition, these studies have found that the proportion of SRCs was significantly higher in early DGC compared with advanced DGC, in line with our notion that intramucosal SRCs are an initial, differentiated phenotype of DGC, and are unlikely to progress. Therefore, we propose that the proportion of poorly differentiated cells within an SRCC will provide an indication of the likelihood of disease recurrence. Our results further imply that any factor causing destabilization of adherens junctions is a potential DGC risk factor. For example, upon infection of gastric cells with *Helicobacter pylori*, E-cadherin and other junctional proteins translocate away from the membrane (45), providing one explanation for the increased gastric cancer risk. The role of the adherens junction in gastric cancer initiation raises the possibility that compounds which are able to stabilize the adhesive complex may constitute a novel class of chemopreventive agents. Furthermore, it seems probable that a successful therapy for DGC would target the poorly differentiated cells, not the SRCs. An alternate therapeutic goal would be the prevention of the EMT. In this context, c-Src antagonists may be suitable for the treatment of early stage cancer, particularly because c-Src is overexpressed in the proliferating, poorly differentiated DGC cell population.

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