Methylation Patterns of the E-cadherin 5′ CpG Island Are Unstable and Reflect the Dynamic, Heterogeneous Loss of E-cadherin Expression during Metastatic Progression*

(Received for publication, September 23, 1999, and in revised form, November 15, 1999)

Jeremy R. Graff‡§, Edward Gabrielson¶, Hiraoki Fujii‡, Stephen B. Baylin‡, and James G. Herman‡

From the ‡Comprehensive Cancer Center and the ¶Department of Pathology, Johns Hopkins University, Baltimore, Maryland 21231

Metastatic progression of most common epithelial tumors involves a heterogeneous, transient loss of expression of the homotypic cell adhesion protein, E-cadherin, rather than the uniform loss of a functional protein resulting from coding region mutation. Indeed, whereas E-cadherin loss may promote invasion, reexpression may facilitate cell survival within metastatic deposits. The mechanisms underlying such plasticity are unclear. We now show that the heterogeneous loss of E-cadherin expression in primary human breast cancers reflects a heterogeneous pattern of promoter region methylation, which begins early prior to invasion. In cultured human tumor cells, such heterogeneous methylation is dynamic, varying from allele to allele and shifting in relation to the tumor microenvironment. Following invasion in vitro, which favors diminished E-cadherin expression, the density of promoter methylation markedly increased. When these cells were cultured as spheroids, which requires homotypic cell adhesion, promoter methylation decreased dramatically, and E-cadherin was reexpressed. These data show that the methylation associated with E-cadherin loss in human breast cancer is heterogeneous and unstable and suggest that such epigenetic plasticity may contribute to the dynamic, phenotypic heterogeneity that drives metastatic progression.

Tumor progression involves the accumulation of multiple, irreversible genetic “hits” (1). However, the evolution of metastases is characterized by an unstable, phenotypic heterogeneity that fluctuates too rapidly to be mediated exclusively by mutation, suggesting that epigenetic and biochemical alterations drive many of the processes that elicit the metastatic phenotype (2–5). The metastatic phenotype itself is unstable (2–5), further suggesting that many of the molecular alterations governing this phenotype are potentially reversible. Indeed, the acquisition of invasiveness often accompanies diminished homotypic cell-cell adhesion, whereas the successful reestablishment of such adhesion may be necessary for the survival and subsequent growth of metastases in distal, secondary organ sites (6–8).

Homotypic cell-cell adhesion of differentiated epithelial tissues is largely governed by E-cadherin (E-cad) (6, 7). Diminished E-cad expression has been related to the acquisition of invasiveness within experimental tumors (9, 10), and many advanced stage human carcinomas, including ductal carcinoma of the breast (7, 11, 12). Mutational inactivation resulting in uniform loss of E-cad expression has been detected only in gynecologic cancers, lobular carcinomas of the breast, and gastic carcinomas (13–17). Recent reports that E-cad is inactivated by mutation in early stage lobular breast carcinomas (16) and in the germline of patients with familial gastric carcinoma (17) suggest a tumor suppressor role for E-cad in these tumor types.

However, in most common carcinomas, including ductal carcinomas of the breast, the loss of E-cad expression is heterogeneous (7, 11, 12) and may be modulated by the tumor microenvironment (7, 18–22), suggesting that the mechanism for E-cad loss in these tumors does not involve irreversible, genetic alterations. Indeed, recent reports have indicated that aberrant hypermethylation of the E-cad promoter region CpG island (23–26), coupled with alterations in chromatin structure and transcription factor activity (23, 24, 27–30), may conspire to extinguish E-cad expression. We therefore examined preinvasive, invasive, and metastatic lesions to determine when the methylation associated with E-cad loss occurs during malignant progression of ductal breast carcinoma. Because E-cad loss in tumors can be modulated by microenvironmental pressures (7, 18–22) and because methylation patterns in tumors are unstable (31, 32), we further examined whether the dynamics of E-cad loss could involve unstable methylation patterns within the promoter region. Our data indicate that the methylation associated with loss of E-cad expression begins early, in the preinvasive ductal carcinoma in situ (DCIS) lesions, and displays a striking heterogeneity that persists throughout all stages of malignant progression. Most importantly, our data in experimental cell culture models reveal that these heterogeneous methylation patterns exhibit a remarkably unstable, allele-to-allele variability that, like the dynamic expression of E-cadherin during metastatic progression, can be modulated or selected for in relation to the tumor microenvironment.

EXPERIMENTAL PROCEDURES

Primary Tissue Collection and Cell Culture—Primary tissues were collected as described (33). Tumor cell lines were maintained as described (23). For spheroid growth, 5 × 10^5 cells were cultured 5 days on a 0.65% base agar as described (34). For invasion in vitro, 2 × 10^5 cells were plated on a Matrigel®-coated invasion chamber (Collaborative Biosciences, Cambridge, MA) and allowed to invade over the course of 3

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‡ To whom correspondence should be addressed: Lilly Research Labs, Eli Lilly and Company, Drop Code 0546, Lilly Corporate Center, Indianapolis, IN 46285. Tel.: 317-277-0220; Fax: 317-277-3652; E-mail: graffJeremy@lilly.com.

1 The abbreviations used are: E-cad, E-cadherin; DCIS, ductal carcinoma in situ; PCR, polymerase chain reaction; MSP, methylation-specific PCR.
Dynamic E-cadherin CpG Island Methylation during Tumor Progression

RESULTS

E-cadherin CpG Island Methylation during Metastatic Progression—To assess when methylation-associated loss of E-cad occurs during malignant progression of ductal breast carcinoma, we examined preinvasive DCIS, invasive ductal carcinoma, and metastatic ductal carcinoma by methylation-specific PCR (36). Aberrant promoter methylation was not evident in normal breast epithelia (37) but was detected in 11 of 35 preinvasive DCIS lesions, 19 of 37 invasive lesions, and 7 of 16 metastatic ductal carcinomas (Fig. 1). These data indicate that aberrant E-cad promoter methylation begins early in tumor progression, prior to invasion (Fig. 1A), and persists in invasive and metastatic tumors (Fig. 1, B and C), reflecting the loss of E-cad expression in such lesions (12).

Throughout all stages of tumor progression, this methylation exhibited a striking heterogeneity within individual lesions, as unmethylated copies of the E-cad CpG island were always evident in the samples with hypermethylation, even in the carefully microdissected DCIS lesions (Fig. 1A). The patterns of E-cad promoter methylation also manifested distinct heterogeneity between lesions, as multiple preinvasive and invasive tumor foci from the same patient displayed distinct patterns of methylation (Fig. 1D). Furthermore, the heterogeneity of promoter methylation reflected the heterogeneous loss of E-cad reported for ductal carcinoma of the breast (7, 11, 12). Samples with minimal evidence of aberrant E-cad CpG island methylation (951 and 6456, Fig. 1C) displayed generally strong E-cad immunostaining (Fig. 2, A and B), whereas those with prominent methylation (for example, 6849; Fig. 1C) showed distinct, heterogeneous loss of E-cad immunoreactivity (Fig. 2C). Together, these data indicate that the tumor-specific methylation of the E-cad 5' CpG island is remarkably heterogeneous, is associated with loss of E-cad in primary tumors, and begins early during malignant progression.

Heterogeneous Patterns of Methylation Reflect Allele-to-Allele Variability—To evaluate the heterogeneity of E-cad CpG island methylation in more detail, we sequenced individual alleles for 21 CpG sites within the E-cad proximal promoter in two human breast cancer cell lines, MCF7 and MDA-231, and the human prostate cancer cell line TSUPr1. We chose to sequence this region because it lies within the area of highest CpG density for the E-cad 5' CpG island, the region for which methylation is most tightly associated with loss of E-cad expression (37). MCF7 cells, which express high levels of E-cad (Fig. 2E) and are not invasive in vitro (38), were unmethylated at every site examined in 13 independent alleles (Fig. 3A). By contrast, MDA-231 cells, which lack E-cad expression (Fig. 2F) and are invasive in vitro (38), showed methylation of nearly every site in 49 independent alleles (Fig. 3A). Like MDA-231 cells, TSUPr1 cells are also invasive in vitro (35). However, TSUPr1 cells showed a heterogeneous pattern of methylation by MSP (36), similar to that of the primary breast cancer tissues examined (Fig. 1). Coincidentally, TSUPr1 cells do express minimal basal levels of E-cad protein, which can be readily up-regulated following azacitidine-induced demethylation within the E-cad promoter (23). Genomic bisulfite sequencing of 16 independent alleles from TSUPr1 cells revealed that the heterogeneous patterns of methylation revealed by MSP (36) reflects a striking variability between the methylation of individual CpG sites in each allele, as well as a difference in the overall density of methylation between individual alleles (Fig. 3, A and B).

E-cad Methylation Patterns Can Be Selected for and/or Modulated by the Cellular Microenvironment—Like the heterogeneous loss of E-cad expression, the heterogeneous patterns of E-cad methylation may be unstable, particularly because methylation patterns may not be faithfully maintained in tumor cells (31, 32). As such, 5' CpG island methylation may reflect an attractive epigenetic mechanism involved in the transient nature of E-cad loss during metastatic progression. To evaluate the possibility that E-cad promoter methylation could be influenced in relation to tumor cell behavior and E-cad expression, we used in vitro models of tumor invasion and of three-dimensional tumor growth.

We first evaluated the relationship between E-cad promoter methylation and tumor cell invasion, because E-cad-mediated cell-cell adhesion has been inversely related to invasion in vitro (7, 35, 38). Because the pattern of E-cad CpG island methylation in TSUPr1 cells most closely resembled that of primary breast cancers, we evaluated the E-cad CpG island methylation status by genomic bisulfite sequencing in TSUPr1 cells following invasion through an artificial basement membrane. These invasive cells (TSU 1+) showed an increase in the overall density of methylation relative to TSUPr1 cells grown in monolayer culture (p = 0.036; Fig. 3, B and C), particularly in CpG sites 1–5 (p = 0.0001). Interestingly, this region had the lowest basal density of methylation in the parental TSUPr1 cells grown in monolayer culture (Fig. 3, A–C) and encompasses the critical transcription elements of the E-cad proximal promoter (29, 30). However, when these invasive cells were returned to monolayer culture for 2 weeks (Fig. 3B, TSU I+Cr), the overall pattern of methylation shifted, displaying an intermediate density that was not statistically different from either the parental TSUPr1 cells nor the invasive TSUPr1 cells (Fig. 3B; overall density, 50.9% in parental TSUPr1, 62.3% in TSU 1+, and 55.9% in TSU I+Cr). These data suggest that the heterogeneous patterns of E-cad promoter methylation in TSUPr1 are not
faithfully maintained and can fluctuate in relation to tumor invasiveness.

We next challenged the cells to grow in contact with each other and without substratum attachment as tumor spheroids, a three-dimensional model for tumor growth (39) that can influence by spheroid culture. TSUPr1 cells are shown in D and H, MCF 7 cells are shown in E and I, and MDA-231 cells are shown in F and J. SK-BR3 cells, for which the E-cad gene is homozygously deleted (41), were included as a negative control for E-cad immunostaining in G.

DISCUSSION

Diminished E-cad expression has been related to tumor cell invasiveness in most common human and experimental carcinomas (6, 7). However, except in rare carcinomas (13–17), E-cad loss is heterogeneous and can be transient, as the diminished expression of E-cad in invasive tumors can be partially restored in metastases (7, 8). Therefore, the mechanism underlying the heterogeneous, transient loss of E-cad expression during metastatic progression must be potentially reversible. Our results indicate that epigenetic promoter alterations may provide such plasticity to the loss of E-cad during metastatic progression.

Methylation associated with loss of E-cad expression was evident early during malignant progression, prior to invasion, and persisted in invasive and metastatic lesions. Throughout all stages of malignant progression, these patterns of promoter methylation exhibited a striking heterogeneity, mimicking the heterogeneous loss of E-cad expression typical in ductal carcinomas of the breast (7, 12). Such heterogeneous methylation patterns were also evident in established human tumor cell lines, such as TSUPr1, and reflected remarkable allele-to-allele variability. Moreover, in these cells, the methylation patterns and the expression of E-cad changed dynamically according to the cellular microenvironment. Invasion of an artificial basement membrane elicited increased E-cad promoter region methylation, whereas three-dimensional modeling of in vivo tumor growth, which favors E-cad expression (8, 40), resulted in decreased promoter methylation and renewed E-cad expression. Together, these data demonstrate that the aberrant methylation of the E-cad 5′ CpG island displays a remarkable heterogeneity throughout all stages of malignant progression, which, in cultured human tumor cells, reflects a dynamic, allele-to-allele variability that can be modulated in relation to different microenvironmental conditions.

The processes that elicit these dynamics are unclear. It is plausible that the cellular microenvironment selects for cells that have the “appropriate” methylation pattern. Hence, cells with the highest density of E-cad CpG island methylation may be the most proficient for invasion and are therefore selected. Likewise, the formation of spheroids in our experimental model could select for the few cells that express at least minimal E-cad and, accordingly, harbor the lowest density of E-cad CpG island methylation. However, our spheroids were cultured only 5 days, which may not be sufficient time to allow for the outgrowth of a minority population of preexisting E-cad-positive cells. Therefore, it seems likely that modulation of E-cad methylation patterns and E-cad expression reflects not only a selection process, but also a cellular response to microenvironmental pressure. For instance, spheroid formation, which is facilitated by E-cad-mediated cell-cell adhesion (8), may increase the transcriptional pressure for E-cad expression (40), which in turn may drive regional hypomethylation of the densely methylated CpG island. In support of this notion, a recent report has indicated that the formation of active transcriptional complexes, coupled with DNA replication, can drive regional hypomethylation of a densely methylated promoter, subsequently eliciting renewed gene expression (42).
islands, including that of E-cad, are unmethylated and main-
tain a transcriptionally favorable local chromatin configuration
(31, 32). Aberrant methylation of these normally unmethylated
CpG islands is associated with underacetylated histones and a
shift to a transcriptionally repressive chromatin structure (43,
44). Transcription from densely methylated promoters can be
partially restored by azacytidine treatment alone but can be
markedly enhanced by co-treatment with an inhibitor of his-
tone deacetylase (45). Furthermore, transcription from a
densely methylated promoter construct is not precluded until a
repressive chromatin structure is assembled (46). Together,
these data clearly show that methylation-associated transcrip-
tional silencing involves an intimate cooperation between the
density of methylation within a CpG island and the associated
local chromatin structure changes.

The model depicted in Fig. 4 represents how our current data
show heterogeneous E-cad expression (7, 48), suggesting that
the loss of E-cad during the evolution of the invasive cell may
be reversible. Indeed, in experimental cancer models, E-cad
loss is dynamic and can be modulated by the tumor microen-
vironment (7, 8, 18–22).

We propose that as tumors evolve, individual cells with vary-
ing degrees of E-cad CpG island hypermethylation may be
generated. Those harboring the most densely methylated E-cad
alleles, and presumably the least transcriptionally favorable
chromatin configuration, may have the lowest E-cad expression and therefore the greatest potential for dissociating from the
primary tumor mass, invading surrounding tissue, and extrav-
asating within a distal organ site (Fig. 4). To survive success-
fully and establish metastases within this foreign microenvi-
nronment, tumor cells may need to “circle the wagons,” by
partially restoring E-cad expression and cadherin-mediated
intercellular adhesion (7, 8). Indeed, recent reports have dem-
onstrated that expression of functional E-cad protein can pro-
mote both tumor cell survival (8, 49–51) and proliferation (49),
in part by activating intracellular survival kinases (51). Hy-
pomethylation of entire alleles, or of subregions within the
densely methylated E-cad 5' CpG island, may be involved in a
remodeling of local chromatin structure to that more favorable

**FIG. 3. Methylation analyses of the E-cadherin promoter region.** A shows a graphical comparison of the average density of methylation in 15 independent MCF7 alleles (6 from monolayer-grown cells and 7 from spheroid cultures), 49 MDA-231 alleles, and 16 TSUPr1 alleles from cells grown in monolayer culture. Genomic bisulfite sequence analysis of individual alleles from parental TSUPr1 cells in monolayer culture, invasive TSUPr1 cells (TSU I+), TSU I+ cells returned to culture for 2 weeks (TSU I+Cx), or TSUPr1 cells grown as spheroids (TSU spheroids) are shown with a schematic of the E-cad proximal promoter region (B). Each circle depicts an individual CpG site. Closed circles represent methylated CpG sites, whereas open circles denote unmethylated CpG sites. Data for TSU, TSU I+, and TSU spheroid are also represented graphically (C). D shows a graphical comparison of the average density of methylation through this region for MDA-231 cells in monolayer or spheroid culture.
for renewed E-cad expression. Subsequently, renewed E-cad expression may promote the survival and outgrowth of these tumor cells within the foreign microenvironment of the metastatic site, thereby reestablishing the heterogeneous patterns of E-cad 5′ CpG island methylation that existed in the primary tumor (Fig. 4).

Our data may also provide clues regarding the differing patterns of metastasis for tumor types with epigenetic inactivation of E-cad versus those with complete, irreversible loss of functional E-cad protein due to mutation. This may be most vividly illustrated by the distinct patterns of metastasis for the two major histotypes of breast carcinoma, ductal and lobular carcinoma. Lobular carcinomas, unlike ductal breast carcinomas, frequently harbor inactivating coding region mutations of E-cad, which yield uniform loss of E-cad expression throughout the tumor (13). Although these tumors invade very efficiently, they form poorly cohesive, loosely dispersed linear strands on distant organ surfaces rather than the organized nests or ductal type structures formed within secondary organ sites by ductal carcinoma metastases.

In summary, our analysis of primary tumors from different stages of malignant progression coupled with manipulation of tumor cells in culture implicates a dynamic, heterogeneous promoter region methylation pattern as an integral component of the processes underlying the changing patterns of E-cad expression during metastatic progression. It is plausible that the epigenetic inactivation of other tumor suppressor genes, particularly the tissue inhibitors of metalloproteinase (52), the estrogen receptor (53), and the DNA repair proteins MLH-1 (54) and O6-MGMT (55), may be similarly dynamic and subject to microenvironmental influences during malignant progression. As such, epigenetic plasticity, like that shown in this report for E-cad promoter region CpG island methylation, may provide a dynamic mechanism for modulating gene expression and may therefore facilitate the dynamic, phenotypic heterogeneity that drives metastatic progression (32).

Acknowledgments—We are grateful to Dr. Bert Vogelstein and to Dr. Steven Belinsky for critical review of the manuscript. We are also grateful to Philip Iversen for expert statistical analysis. Finally, we thank Tammy Means for helping to assemble the manuscript.

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