

E-Cadherin Promoter Hypermethylation in Preneoplastic and Neoplastic Skin Lesions

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E-cadherin is a calcium-dependent, intercellular adhesion molecule that is specifically expressed in epithelial tissues and plays an important role in maintaining epithelial stability. E-cadherin is widely regarded as a prognostic marker in many types of human cancers. The inactivation of the *E-cadherin* gene is linked to increased potential for tumor invasiveness and distant metastasis. We previously demonstrated reduced expression of E-cadherin protein immunohistochemically in invasive squamous cell carcinomas of the skin as compared with adjacent normal skin. An epigenetic alteration in association with promoter hypermethylation is one important mechanism of gene silencing. In the present study, we analyze the *E-cadherin* gene promoter hypermethylation in preneoplastic and neoplastic skin lesions to determine whether epigenetic alteration of the *E-cadherin* gene also plays an important role in cutaneous squamous carcinogenesis. A total of 33 cases was examined for evidence of *E-cadherin* promoter hypermethylation, and these consist of nine cases of spongiotic dermatitis as nonneoplastic skin control, nine cases of actinic keratosis, eight cases of squamous cell carcinoma *in situ*, and seven cases of invasive squamous cell carcinoma. Promoter hypermethylation of the *E-cadherin* gene was detected in 6 of 7 cases (85%) of invasive squamous cell carcinoma, 4 of 8 cases (50%) of squamous cell carcinoma *in situ*, 4 of

9 cases (44%) of actinic keratosis, and 2 of 9 cases (22%) of nonneoplastic skin. We conclude that *E-cadherin* promoter hypermethylation occurs frequently and may represent an important mechanism of *E-cadherin* inactivation in cutaneous preneoplastic and neoplastic lesions. The frequencies of *E-cadherin* promoter hypermethylation appear to be correlated with more advanced stage of squamous carcinogenesis in skin.

KEY WORDS: Cutaneous squamous cell carcinoma, E-cadherin, Promoter hypermethylation.

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E-cadherin is a calcium-dependent cell adhesion molecule, which is expressed primarily on the surface of epithelial cells. E-cadherin plays a major role in cell–cell interactions in epithelium and is critical in maintaining the structure and integrity of the epithelial sheet (1). The *E-cadherin* gene is localized at 16q24.3 (2), a chromosomal region demonstrating frequent allelic loss in many types of human cancer, including breast and lung carcinomas (2, 3). It has also been demonstrated that *E-cadherin* expression is greatly reduced in many types of human cancer, such as cancer in the breast, prostate, colon, stomach, esophagus, thyroid, head and neck, and bladder, as compared with normal epithelial tissues (4, 5). Thus, the *E-cadherin* gene is widely regarded as a tumor suppressor gene, and inactivation of this gene is critically involved in tumor formation, invasiveness, and metastatic potential (6).

According to Knudson's (7) "two-hit" hypothesis, both alleles of a tumor-suppressor gene have to be inactivated for complete loss of function. Allelic loss of one allele is often associated by gene mutation or other forms of aberrations, such as promoter methylation in the second allele (8, 9).

Promoter CpG island hypermethylation (epigenetic alteration) is one important mechanism for gene inactivation in human cancer (10). The epige-

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netic silencing of the *E-cadherin* gene has been reported in a wide variety of human tumors including gastric (11, 12), prostatic (13), cervical (14), colorectal (15), bladder (16, 17), breast (18), esophageal (19), and head and neck squamous cell carcinoma (20–22).

Extensive studies on cutaneous malignancies also establish that E-cadherin protein expression is reduced in both basal cell carcinoma (23, 24) and squamous cell carcinoma (25–29) by immunohistochemistry. It appears that loss of E-cadherin protein expression is associated with high mitotic index (29) and increased metastatic potential (25) in cutaneous squamous cell carcinoma. Work in our laboratory and others has also demonstrated that loss of E-cadherin protein expression is associated with poorer tumor differentiation (27, 28). Promoter hypermethylation of the *E-cadherin* gene in cutaneous malignant lesions has not been analyzed previously.

MATERIALS AND METHODS

Tissue Collection

A total of 33 cases of nonneoplastic skin, actinic keratosis, and skin cancers were gathered from the paraffin block archives in the Department of Pathology, University of Arkansas for Medical Sciences, in 2002. These cases consisted of eight cases of nonneoplastic skin control, nine cases of actinic keratosis, eight cases of squamous cell carcinoma *in situ*, and seven cases of invasive squamous cell carcinoma (Table 1).

DNA Extraction and Bisulfite Modification

DNA samples were collected using the EX-WAX DNA Extraction Kit (Intergen Co., New York, NY) from five deparaffinized 5- μ m-thick tissue sections from each tissue block, followed by bisulfite modification before methylation-specific PCR using CpGenome DNA modification Kit (Intergen Co.).

PCR Amplification and Primers

Amplification of the promoter region of the *E-cadherin* gene was carried out in a Touchgene Gradient Thermal Cycler (Techne Inc., Princeton, NJ) in a 50- μ L PCR reaction mixture containing 2 μ L of bisulfite-treated genomic DNA as described previously (30). The primers used for the unmethylated reaction were as follows: 5'-TAA TTT TAG GTT AGA GGG TTA TTG T-3' (sense); and 5'-CAC AAC CAA TCA ACA ACA CA-3' (antisense); the primers used for the methylated reaction were as follows: 5'-TTA GGT TAG AGG GTT ATC GCG T-3' (sense) and 5'-TAA CTA AAA ATT CAC CTA CCG AC-3' (antisense). All primers were purchased from

Operon Technologies Inc. (Alameda, CA). The PCR conditions were as follows: initial denaturation and hot start at 95° C for 15 minutes, then 40 cycles consisting of 30 seconds at 95° C, 30 seconds at 53° C (unmethylated reactions) or 57° C (methylated reactions), and 1 minute at 72° C. Positive- and negative-control DNA samples and controls without DNA were used for each set of PCR reactions.

RESULTS

We used a highly specific and sensitive methylation-specific PCR to analyze the status of promoter methylation of the *E-cadherin* gene in 33 cases of nonneoplastic skin, actinic keratosis, squamous cell carcinoma *in situ*, and invasive squamous cell carcinoma using gene specific primer sets as described previously (30). Spongiotic dermatitis was used as the nonneoplastic skin control. The distribution of cases and analysis results are presented in Table 1.

Promoter hypermethylation of the *E-cadherin* gene was detected in 6 of 7 cases (85%) of invasive squamous cell carcinoma, 4 of 8 cases (50%) of squamous cell carcinoma *in situ*, 4 of 9 cases (44%) of actinic keratosis, and 2 of 9 cases (22%) of non-

Table 1. E-Cadherin Promotor Hypermethylation in Skin Lesions

Sample#	Diagnosis	E-Cadherin MSP*
4	Nonneoplastic skin	Negative
14	Nonneoplastic skin	Negative
6	Nonneoplastic skin	Negative
25	Nonneoplastic skin	Negative
8	Nonneoplastic skin	Negative
29	Nonneoplastic skin	Negative
16	Nonneoplastic skin	Negative
3	Nonneoplastic skin	Positive
30	Nonneoplastic skin	Positive
15	Actinic keratosis	Negative
7	Actinic keratosis	Negative
28	Actinic keratosis	Negative
26	Actinic keratosis	Negative
22	Actinic keratosis	Negative
33	Actinic keratosis	Positive
32	Actinic keratosis	Positive
18	Actinic keratosis	Positive
11	Actinic keratosis	Positive
10	SCC§ <i>in situ</i>	Negative
35	SCC <i>in situ</i>	Negative
24	SCC <i>in situ</i>	Negative
34	SCC <i>in situ</i>	Negative
13	SCC <i>in situ</i>	Positive
19	SCC <i>in situ</i>	Positive
36	SCC <i>in situ</i>	Positive
12	SCC <i>in situ</i>	Positive
2	Invasive SCC	Negative
9	Invasive SCC	Positive
5	Invasive SCC	Positive
1	Invasive SCC	Positive
21	Invasive SCC	Positive
17	Invasive SCC	Positive
31	Invasive SCC	Positive

* MSP; methylation-specific PCR

§ SCC; squamous cell carcinoma

neoplastic skin control. Figure 1 shows representative methylation-specific PCR results obtained from five skin samples. Samples 5 (invasive squamous cell carcinoma), 19 (squamous cell carcinoma *in situ*), and 33 (actinic keratosis) show amplification of both unmethylated and methylated-specific amplicons, indicating the presence of *E-cadherin* promoter hypermethylation (Samples 5, 19, and 33 in Fig. 1). In Samples 10 (squamous cell carcinoma *in situ*) and 15 (actinic keratosis), only the amplification of unmethylated-specific amplicons is seen, indicative of a lack of *E-cadherin* promoter hypermethylation in these samples (Samples 10 and 15 in Fig. 1). Positive and negative controls worked appropriately in each round of PCR reaction.

The overall prevalence of *E-cadherin* promoter hypermethylation in dysplastic (actinic keratosis), carcinoma *in situ*, and invasive carcinoma is estimated at 14/24 (58%), underlining that *E-cadherin* is a common target for epigenetic silencing in cutaneous squamous carcinogenesis.

DISCUSSION

Previous immunohistochemical studies have suggested that decreased or lost protein expression of the *E-cadherin* gene in primary skin squamous cell carcinomas is frequent (25–29) and is associated with high mitotic activity (29), poorer tumor differentiation (27, 28), and increased tendency for regional lymph node metastases (25). Similarly, decreased protein expression of the *E-cadherin* gene has been shown to correlate with advancing T and N stages of oral squamous cell carcinoma and a poor tumor cell differentiation (31). It has been well established that the *E-cadherin* gene is a tumor suppressor gene and is a prime target for epigenetic silencing in association with CpG island hypermethylation within the promoter region in a wide variety of human cancer (11–22).

A CpG island is a small DNA stretch (500 to 200 base pairs in length), located around or within the promoter region of many housekeeping genes, and typically consisting of $\geq 50\%$ cytosine (C) and guanosine (G) content with CpG/GpC ratio of ≥ 1 (32). The CpG island is normally free of methylation to permit normal gene function and is suppressive

for gene expression when methylated (32). The CpG islands are typically unmethylated in normal tissues but are methylated to varying degrees in many human cancer types (33, 34). The epigenetic silencing in association with promoter CpG island hypermethylation often cooperates with genetic changes (somatic mutation or allelic loss) in inactivating a number of tumor suppressor genes such as *p16^{Ink4a}*, *E-cadherin*, *pVHL*, and *death associated protein kinase* (35).

Promoter hypermethylation of one allele is often preceded by loss of the opposite allele (8, 9), simulating the type of loss of heterozygosity that is usually seen with loss of tumor suppressor function due to point mutation.

Because *E-cadherin* has been established as a prime target for epigenetic silencing in many human tumor types, we attempt to determine the prevalence of *E-cadherin* promoter hypermethylation in the continuum from nonneoplastic skin to actinic keratosis, squamous cell carcinoma *in situ*, and invasive squamous cell carcinoma. In the present study, we found that the overall frequency of *E-cadherin* promoter hypermethylation was very high (14/24; 58%) in both preneoplastic (actinic keratosis) and malignant (carcinoma *in situ* and invasive carcinoma) cutaneous lesions.

The highest frequency of *E-cadherin* promoter hypermethylation was detected in invasive squamous cell carcinoma (6/7; 85%), followed by that seen in squamous cell carcinoma *in situ* (4/8; 50%) and in actinic keratosis (4/9; 44%). Interestingly, promoter hypermethylation was also present in 2 of 9 (22%) nonneoplastic skin controls (see Results and Table 1).

Our data clearly indicate that *E-cadherin* gene is a prime target for epigenetic silencing in ultraviolet-induced squamous carcinogenesis of the skin, as has been established in many other forms of human cancer (11–22) and that increased frequency of *E-cadherin* promoter hypermethylation appears to be associated with more advanced stages of squamous carcinogenesis of the skin.

Because we did not perform immunohistochemical studies on these 33 cases, we did not know the functional status of the *E-cadherin* gene in those 10 cases of dysplastic or malignant skin lesions (5 actinic keratosis, 4 carcinoma *in situ*, and 1 invasive carcinoma) that were negative for *E-cadherin* promoter hypermethylation (Table 1). In the future, studies should include both protein expression and promoter methylation analyses in a larger series of dysplastic or malignant skin lesions to further clarify the significance of *E-cadherin* promoter hypermethylation and the relationship between the epigenetic alterations of the *E-cadherin* gene and protein expression in cutaneous squamous carcinogenesis.

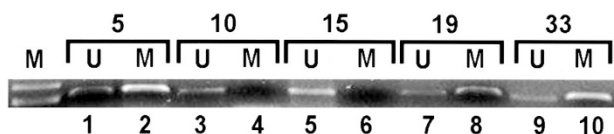


FIGURE 1. Bisulfite-modified DNA harvested from skin samples numbered as Samples 5 (Lanes 1–2), 10 (Lanes 3–4), 15 (Lanes 5–6), 19 (Lanes 7–8), and 33 (Lanes 9–10) was subjected to methylation-specific PCR using unmethylated (U) or methylated-specific (M) MSP primer sets for *E-cadherin*.

It is noteworthy that two of nine cases of non-neoplastic skin control were also positive for *E-cadherin* promoter hypermethylation (see Results and Table 1). It is well recognized that *E-cadherin* promoter hypermethylation can be seen in normal nonneoplastic tissues, particularly in elderly individuals (11, 17). In a study by Bornman and his colleagues (17), *E-cadherin* promoter hypermethylation was present in 20 of 47 (43%) cases of bladder cancer as well as in 3 of 9 (33%) samples of histologically normal bladder tissues. All these three samples were from individuals older than 70 years of age. In another study on neoplastic and nonneoplastic gastric epithelia obtained at autopsy by Waki *et al.* (11), the investigators identified *E-cadherin* promoter hypermethylation in stomach cancer (44/94, 47%), nonneoplastic gastric mucosa adjacent to the cancer (63/94; 67%), normal gastric mucosa from healthy individuals aged >45 years (12/14; 86%) but not in the normal gastric mucosa from healthy individuals aged <22 years (0/6). It is hypothesized that *E-cadherin* promoter hypermethylation during aging may signal the beginning of gastric cancer in these older, but otherwise healthy individuals (11).

Two nonneoplastic skin controls positive for *E-cadherin* promoter hypermethylation were from patients of 50 and 43 years of age, respectively, in this study, and this finding may indicate the earliest stage of squamous carcinogenesis in these two individuals.

In summary, we have used a highly specific and sensitive methylation-specific PCR to determine the prevalence of promoter hypermethylation in the *E-cadherin* gene in a series of nonneoplastic, dysplastic, and malignant skin lesions. We found that *E-cadherin* promoter hypermethylation was frequent in dysplastic and malignant skin lesions (14/24; 58%) and that the frequency is increased with more advanced diseases. We therefore conclude that the *E-cadherin* gene, as has been established in many other types of human cancer, is also a prime target for epigenetic silencing in primary squamous carcinoma of the skin.

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