Analysis of Promoter Hypermethylation of Death-Associated Protein Kinase and *p16* Tumor Suppressor Genes in Actinic Keratoses and Squamous Cell Carcinomas of the Skin

Lisa N. Tyler, M.D., Lingbao Ai, M.D., Chunlai Zuo, M.D., Chun-Yang Fan, M.D., Ph.D., Bruce R. Smoller, M.D.

Deparents of Pathology (LNT, LA, CL, CYF, BRS) and Dermatology (BRS), University of Arkansas for Medical Sciences; and John L. McClellan Memorial Veterans Administration Hospital (CYF, BRS), Little Rock, Arkansas

Death-associated protein kinase is a serine/threonine protein kinase implicated in promoting apoptosis and tumor suppression, whereas *p16* is a tumor suppressor gene that inhibits cyclin-dependent kinase 4 and 6 activity and arrests the cell cycle in the G1 phase. Hypermethylation of death-associated protein kinase or *p16* gene with resultant gene inactivation has been described in a wide variety of human cancers. Promoter methylation of the death-associated protein kinase and *p16* gene has been found in about 55% and 30% cases of head and neck squamous cell carcinoma respectively but has not yet been analyzed in cutaneous premalignant and malignant lesions. A total of 33 cases were examined for evidence of death-associated protein kinase and *p16* hypermethylation and these consist of 9 cases of spongiotic dermatitis as nonneoplastic skin control, 9 cases of actinic keratosis, 8 cases of squamous cell carcinoma *in situ,* and 7 cases of invasive squamous cell carcinoma. Death-associated protein kinase promoter methylation was detected in 1 case of squamous cell carcinoma *in situ* and 1 case of nonneoplastic skin control but none of the cases of invasive squamous cell carcinoma or actinic keratosis. *P16* promoter methylation was detected in 1 case of invasive squamous cell carcinoma and 1 case of nonneoplastic skin control but none of the cases of squamous cell carcinoma *in situ* or actinic keratosis. Promoter hypermethylation of the death-associated protein kinase and *p16* genes does not appear to play an important role in the development of cutaneous squamous cell carcinoma. The data thus suggest that the mechanisms of ultraviolet-induced cutaneous carcinomas differ from those involved in the development of head and neck squamous cell carcinoma, a malignant disease induced by tobacco and alcohol exposure.

KEY WORDS: Cutaneous squamous cell carcinoma, Death-associated protein kinase, *p16* tumor suppressor gene, Promoter hypermethylation.

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Control of gene expression is a complex process that is involved in the development of many cancers. Death-associated protein kinase is a multidomain calmodulin regulated serine/threonine protein kinase implicated in control of apoptosis, tumor suppression and susceptibility to disease (1). Death-associated protein kinase is involved in apoptosis induced by *Fas* ligand, *TNF-α*, and detachment–induced cell death. Death-associated protein kinase was recently characterized as an upstream regulator of *p53* (2, 3). Inactivation of the death-associated protein kinase promoter contributes to immortalization of cells and development of cancer.

The tumor suppressor function of *p16* is due to its inhibition of the catalytic activity of the cyclin-dependent kinase 4–6/cyclin D complex that is required for phosphorylation of retinoblastoma protein (*pRb*) (4, 5). The unphosphorylated *pRb* is specifically bound to the E2F family of transcription factors, thus inhibiting their activities, essential for cell cycle progression (6, 7). The *pRb*/E2F complex releases E2F transcriptional factors upon phosphorylation of pRb by cyclin-dependent kinase

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Address reprint requests to: Bruce R. Smoller, M.D., Deparent of Pathology, University of Arkansas for Medical Sciences, 4301 West Markham, Slot 517, Little Rock, AR 72205; fax: 501-603-1479; e-mail: smollerbruce@exchange.uams.edu.

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4–6/cyclin D complex and promotes cell cycle progression past the G_1 checkpoint (6–8). By binding to cyclin-dependent kinase 4/6 and inhibiting *pRb* phosphorylation, *p16* promotes the formation of *pRb*/E2F repressive complex and cell cycle arrest (8).

Gene inactivation can occur via a number of mechanisms, including homozygous deletion, point mutation in the coding sequence, and CpG hypermethylation in the promoter region. Promoter hypermethylation of death-associated protein kinase has been reported in B-cell lymphomas and multiple myeloma (9), urinary bladder carcinoma (10), gastric carcinoma (11), prostate cancer (12), non-small cell carcinoma of the lung (13), head and neck squamous cell carcinoma (14, 15), and nasopharyngeal carcinoma (3).

Hypermethylation of *p16* promoter has also been identified in head and neck squamous cell carcinoma (14–16), lung cancer (13), colorectal cancer (17), B-cell lymphoma (18), glioblastoma multiforme (19), and gastric carcinoma (20, 21).

Recent work in our laboratory identified increased *p16* expression in actinic keratosis, squamous cell carcinoma *in situ,* and invasive squamous cell carcinoma of the skin using immunohistochemistry (22). Promoter hypermethylation of death-associated protein kinase and *p16* in cutaneous malignant lesions has not been analyzed previously.

TABLE 1. Death Associated Protein Kinase and *p16* Promoter Hypermethylation in Skin Lesions

Sample#	Diagnosis	DAPK* MSP	*p16* MSP¶
4	Nonneoplastic Skin	−†	−
14	Nonneoplastic Skin	−	−
6	Nonneoplastic Skin	−	−
25	Nonneoplastic Skin	−	−
8	Nonneoplastic Skin	−	−
29	Nonneoplastic Skin	+‡	−
16	Nonneoplastic Skin	−	−
3	Nonneoplastic Skin	−	+
30	Nonneoplastic Skin	−	−
15	Actinic Keratosis	−	−
7	Actinic Keratosis	−	−
28	Actinic Keratosis	−	−
26	Actinic Keratosis	−	−
22	Actinic Keratosis	−	−
33	Actinic Keratosis	−	−
32	Actinic Keratosis	−	−
18	Actinic Keratosis	−	−
11	Actinic Keratosis	−	−
10	SCC§ in situ	−	−
35	SCC in situ	−	−
24	SCC in situ	−	−
34	SCC in situ	−	−
13	SCC in situ	+	−
19	SCC in situ	−	−
36	SCC in situ	−	−
12	SCC in situ	−	−
2	Invasive SCC	−	−
9	Invasive SCC	−	−
5	Invasive SCC	−	−
1	Invasive SCC	−	+
21	Invasive SCC	−	−
17	Invasive SCC	−	−
31	Invasive SCC	−	−

* DAPK; death associated protein kinase.
¶ MSP; methylation-specific PCR.
† −; negative MSP or negative for promoter hypermethylation.
‡ +; positive MSP or positive for promoter hypermethylation.
§ SCC; squamous cell carcinoma.

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 Deparent of Pathology, University of Arkansas for Medical Sciences, Little Rock, in 2002. These cases consisted of 8 cases of nonneoplastic skin, 9 cases of actinic keratosis, 8 cases of squamous cell carcinoma *in situ,* and 7 cases of invasive squamous cell carcinoma (Table 1). The histology of each case was reviewed, and representative tissue sections containing the pertinent epithelial component were selected for extraction of DNA for promoter methylation analysis.

Sample Collection and DNA Extraction

DNA samples were collected using the EX-WAX DNA Extraction Kit (Intergen Co., New York, NY) from five deparaffinized 5-μm-thick tissue sections from each tissue block. Human placental DNA (Sigma) was used as a negative control, and CpGenome universal methylated human DNA (Intergen) served as a positive control.

Bisulfite modification of DNA for methylation-specific PCR

In DNA samples from cutaneous lesions, negative and positive controls were subjected to bisulfite modification before methylation-specific PCR using CpGenome DNA modification Kit (Intergen).

PCR amplification and primers

Amplification of the promoter region of the *p16* and death-associated protein kinase genes was carried out in a Touchgene Gradient Thermal Cycler (Techne Inc., Princeton, NJ) in a 50-μL PCR reaction mixture containing 2 μL of bisulfite-treated genomic DNA, dNTPs (each at 200 μM), primers (50 pmol each per reaction), 2.5 mM $MgCl_2$, and 1.25 U Hotstar *Taq* (Qiagen, Valencia, CA) in 1× PCR buffer. All reagents were supplied with the Qiagen Hotstar *Taq* Kit (Qiagen). The only exception was the dNTP mix (Roche Molecular Biochemicals, Indianapolis, IN).

The primers used for the unmethylated reaction were as follows: for *p16* gene are designed as follows: 5'-TTA TTA GAG GGT GGG GTG GAT TGT-3' (sense); and 5'-CAA CCC CAA ACC ACA ACC ATA-3'

(antisense); for death-associated protein kinase, 5'-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3' (sense) and 5'-CAA ATC CCT CCC AAA CAC CAA-3' (antisense). The primers used for the methylated reaction were as follows: for *p16*, 5'-TTA TTA GAG GGT GGG GCG GAT CGC-3' (sense) and 5'-GAC CCC CGA ACC GCG ACC GTA A-3'(antisense); for death-associated protein kinase, 5'-GGA TAG TCG GAT CGA GTT AAC GTC-3' (sense) and 5'-CCC TCC CAA ACG CGC A-3' (antisense), as described previously (14). 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 60° C (unmethylated reactions) or 65° 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 *p16* and death-associated protein kinase genes in 33 cases of nonneoplastic skin, actinic keratosis, carcinoma *in situ,* and invasive carcinomas using gene specific primer sets as described previously (14). The distribution of cases and analysis results are presented in Table 1. Spongiotic dermatitis was used as a nonneoplastic skin control. Briefly, death-associated protein kinase promoter hypermethylation was detected only in one of eight cases of squamous cell carcinoma *in situ* and one of eight cases of nonneoplastic skin. None of the cases of invasive squamous cell carcinoma or actinic keratosis displayed death-associated protein kinase promoter hypermethylation. The *p16* promoter hypermethylation was detected only in one of seven cases of invasive squamous cell carcinoma and one of eight cases of nonneoplastic skin. None of the cases of squamous cell carcinoma *in situ* or actinic keratosis showed *p16* promoter hypermethylation. Figure 1 shows representative methylation-specific PCR data obtained from five skin samples. Samples 13 (squamous cell carcinoma *in situ*) and 29 (nonneoplastic skin) show amplification of both unmethylated and methylated-specific amplicons for death-associated protein kinase, indicating the presence of death-associated protein kinase promoter hypermethylation (Cases 13 and 29 in Fig. 1; upper panel). In Samples 1 (invasive squamous cell carcinoma), 3 (nonneoplastic skin), and 19 (squamous cell carcinoma *in situ*), only the amplification of unmethylated-specific amplicons is seen, indicative of a lack of death-associated protein kinase promoter hypermethylation in these samples (Cases 1, 3, and 19 in Fig. 1; upper panel). In Samples 1 (invasive squamous cell carcinoma) and 3 (nonneoplastic skin), we observed amplification with both unmethylated and methylated-specific amplicons for *p16* gene, indicating the presence of *p16* promoter hypermethylation in both samples (Cases 1 and 3 in Fig. 1; lower panel). Samples 13 (squamous cell carcinoma *in situ*), 19 (squamous cell carcinoma *in situ*), and 29 (nonneoplastic skin) show amplification with p16 unmethylated-specific primers only, indicating a lack of p16 promoter hypermethylation in these samples (Cases 13, 19, and 29 in Fig. 1; lower panel). Positive and negative controls worked appropriately in each round of PCR reaction.

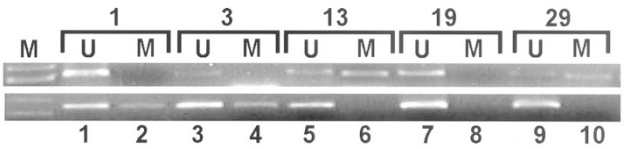

FIGURE 1. Bisulfite-modified DNA harvested from skin samples designated 1 (**Lanes 1 and 2**), 3 (**Lanes 3 and 4**), 13 (**Lanes 5 and 6**), 19 (**Lanes 7 and 8**), and 29 (**Lanes 9 and 10**) was subjected to methylation-specific PCR using unmethylated (**U**) or methylated-specific (**M**) MSP primer sets for death-associated protein kinase (**upper panel**) and *p16* (**lower panel**) promoters.

DISCUSSION

Gene expression is controlled by a number of mechanisms including genetic factors, such as gene deletion, point mutation, and epigenetic factors such as promoter hypermethylation. Promoter hypermethylation is perhaps one of the most common molecular changes in human cancer (23, 24). DNA methylation typically occurs at cytosines located 5' to a guanosine (CpG) (25). The number of CpG nucleotides is markedly decreased to about 20% of the predicted frequency in the mammalian genome except for the CpG islands where CpG nucleotides occur in the expected frequency. These islands are 500 to 2000 base-pairs in length and located most frequently around or within the transcription start sites of about half of human genes (25). Although CpG islands are unmethylated in normal tissues, they are methylated to varying degrees in many human cancer types (23, 26). These epigenetic silencing often cooperates with genetic changes (gene mutation and deletion) in silencing a number of tumor suppressor genes such as $p16^{Ink4a}$, *E-cadherin, pVHL,* death-associated protein kinase, and *MLH1* (27). Promoter hypermethylation of one allele is frequently accompanied by deletion of the opposite allele (28, 29), simulating the type of loss of heterozygosity usually seen with loss of tumor suppressor function due to genetic alterations (*e.g.,* point mutation).

Because both *p16* and death-associated protein kinase have been established as targets for epigenetic alterations in multiple tumor types, we attempted to determine the prevalence of *p16* and death-associated protein kinase promoter hypermethylation in the continuum from nonneoplastic skin to actinic keratosis, squamous cell carcinoma *in situ,* and invasive squamous cell carcinoma. In this study, we found that the frequency of *p16* and death-associated protein kinase promoter hypermethylation was very low (6% for both genes) in nonneoplastic and malignant skin lesions, indicating that the *p16* and death-associated protein kinase genes are not the targets for epigenetic silencing in cutaneous squamous carcinogenesis.

There is no apparent consensus regarding the roles of *p16* tumor suppressor gene in ultraviolet-induced cutaneous squamous carcinogenesis. Although Mortier *et al.* (30) reported increased loss of heterozygosity and loss of protein expression of the *p16* gene during progression of actinic keratosis to invasive squamous cell carcinoma, we found that *p16* gene expression is greatly induced from actinic keratosis to invasive squamous cell carcinoma (22). Our results are consistent with those obtained by others who showed that ultraviolet-irradiation to skin keratinocytes could induce *p16* expression (31–34) as an adaptive response, causing G2 phase cell cycle arrest (33). Alternatively, high levels of *p16* protein in invasive squamous cell carcinoma in our previous study may result from overexpression of mutant *p16* protein (analogous to that described with mutant *p53*).

Although epigenetic alterations and gene expression of the death-associated protein kinase gene have been well studied in many different human tumors, similar studies on cutaneous squamous cell carcinoma are not found in the English literature. Thus, the roles of this gene in ultraviolet-induced skin cancer remain largely unknown.

Promoter hypermethylation of *p16* and death-associated protein kinase genes, by contrast, has been characterized in head and neck squamous cell carcinoma, an etiologically distinct (tobacco-induced) but morphologically similar disease. The prevalence of promoter hypermethylation is estimated at about 30% for *p16* (27) and 55% for death-associated protein kinase (3, 14) in head and neck squamous cell carcinoma. The p16 protein expression analyzed by immunohistochemical staining is frequently lost in head and neck squamous cell carcinoma, ranging from 55% to 90% in three studies involving 332 cases with an average frequency of 74% (35–37). Thus, low prevalence of *p16* and death-associated protein kinase promoter hypermethylation in this study and frequent expression of *p16* protein in our previous study (22) on skin lesions suggest that the mechanisms of ultraviolet-induced cutaneous squamous carcinomas differ from those involved in the development of head and neck squamous cell carcinoma, a neoplasm induced by exposure to tobacco and alcohol.

In summary, we have used a highly specific and sensitive methylation-specific PCR to determine the prevalence of promoter hypermethylation in two well-known tumor-causing genes, *p16* and death-associated protein kinase. Death-associated protein kinase and *p16* hypermethylation does not appear to be involved in the development of ultraviolet-induced cutaneous squamous carcinomas. These findings indicate that the mechanisms for the development of malignancy differ for cutaneous squamous cell carcinoma from those arising in the head and neck region.

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