PGP9.5 Promoter Methylation Is an Independent Prognostic Factor for Esophageal Squamous Cell Carcinoma

Diana L. Mandelker,[1] Keishi Yamashita,[1] Yutaka Tokumaru,[1] Koshi Mimori,[2] David L. Howard,[1] Yoichi Tanaka,[3] Andre Lopes Carvalho,[1] Wei-Wen Jiang,[1] Hannah Lui Park,[1] Myoung Sook Kim,[1] Motonobu Osada,[1] Masaki Mori,[2] and David Sidransky[1]

[1]Department of Otolaryngology, Head and Neck Cancer Research, Johns Hopkins University, Baltimore, Maryland; [2]Department of Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, Tsurumibaru, Beppu; and [3]Department of Surgery, Saitama Prefectural Cancer Center Hospital, Kitaadachi-gun, Japan

Abstract

PGP9.5/UCHL1 is a member of the carboxyl-terminal ubiquitin hydrolase family with a potential role in carcinogenesis. We previously identified PGP9.5 as a putative tumor-suppressor gene and methylation of the promoter as a cancer-specific event in primary cancer tissues. In this current study, we analyzed PGP9.5 methylation in 50 esophageal squamous cell carcinoma (ESCC) primary tumors with well characterized clinicopathologic variables including patient outcome. Two independent modalities for methylation analysis (TaqMan methylation-specific PCR and combined bisulfite restriction analysis) were used to analyze these samples. The two data sets were consistent with each other, as the 21 patients (42%) with highest methylation levels by TaqMan analysis all showed visible combined bisulfite restriction analysis bands on acrylamide gels. Using an optimized cutoff value by TaqMan quantitation, we found that patients with higher PGP9.5 methylation ratios in the primary tumor showed poorer 5-year survival rates than those without PGP9.5 methylation (P = 0.01). A significant correlation was also seen between PGP9.5 promoter methylation and the presence of regional lymph node metastases (P = 0.03). Multivariate analysis subsequently revealed that PGP9.5 methylation was an independent prognostic factor for ESCC survival (P = 0.03). These results suggest that PGP9.5 promoter methylation could be a clinically applicable marker for ESCC progression. (Cancer Res 2005; 65(11): 4963-8)

Introduction

Esophageal cancer is the eighth most common cancer (1), and sixth leading cause of cancer death worldwide (2). The two predominant forms of esophageal cancer, which differ both epidemiologically and pathologically, are squamous cell carcinoma and adenocarcinoma. Globally, squamous cell carcinoma accounts for >90% of esophageal cancers (3). Most esophageal squamous cell carcinoma (ESCC) is diagnosed at an advanced stage, and even superficial ESCC, which seems to extend no further than the submucosa, metastasizes to the lymph nodes in 50% of cases (4). For localized ESCC, surgery is the primary therapeutic option. The ability to predict ESCC outcomes after surgical resection is critical for clinicians, as it would impact the use of adjuvant chemotherapy and radiation therapies. An independent prognostic indicator of ESCC survival would therefore be invaluable to physicians and patients in selecting treatment options.

Hypermethylation of gene promoters has been explored as both a mechanism and marker of tumorigenesis (5, 6). We previously identified novel cancer-specific methylated genes by pharmacologic unmasking and subsequent microarray analysis for ESCC and head and neck squamous cell carcinoma (7, 8). Among the methylated genes identified in the screen was PGP9.5. PGP9.5 is a neuron-specific protein with opposing functions as both a ubiquitin carboxyl-terminal hydrolase and ligase (9, 10). Currently, there is conflicting evidence regarding the role of PGP9.5 in carcinogenesis. PGP9.5 overexpression has been implicated as a marker for pancreatic (11) and ESCC (12). Conversely, PGP9.5 has been identified as a methylated gene in a cancer-specific manner in pancreatic (13) and head and neck squamous cell carcinoma (8). In this study, we tested PGP9.5 methylation status in primary ESCC. We found that PGP9.5 promoter methylation correlated strongly with lymph node metastases and poor prognosis. By multivariate analysis, we show that PGP9.5 promoter methylation is an independent prognostic indicator of ESCC survival.

Materials and Methods

Esophageal squamous cell carcinoma tissues and cell lines. ESCC and paired normal mucosa specimens were obtained from 50 patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University and the Saitama Cancer Center. All the patients had undergone a potentially curative resection of the primary carcinoma. The patients included 43 males and 7 females, and informed consent was obtained. The tumor was located in the upper esophagus (n = 3), the middle esophagus (n = 28), or the lower esophagus (n = 19). Eleven tumors were well-differentiated squamous cell carcinomas, 27 were moderately differentiated and 12 were poorly differentiated. Three were submucosal carcinomas and 47 were more advanced lesions with invasion in the muscularis propria (n = 7) or adventitia (n = 40). The presence (n = 41) or absence (n = 9) of lymph node metastasis were noted. Among the 50 cases, 12 patients survived for more than 5 years after surgery, whereas 26 patients died within 5 years after surgery. In the remaining 12 cases, patients were alive at the last follow-up or died from causes unrelated to ESCC. Specimens were obtained from tumors, avoiding necrotic centers, immediately after resection. Corresponding normal mucosa specimens, which were at least 5 cm away from the tumor edge, were also obtained by sharply dissecting the mucosa off the muscularis propria. All specimens were quick-frozen in liquid nitrogen and stored at −80°C until processing.

Seven ESCC cell lines (TE3, TE4, KYSE30, KYSE70, KYSE140, KYSE410, and KYSE520) were obtained from the Cell Response Center for Biomedical Research Institute of the Department of Aging and Cancer, Tohoku

Note: D.L. Mandelker and K. Yamashita contributed equally to this work.

Requests for reprints: David Sidransky, Department of Otolaryngology, Division of Head and Neck Cancer Research, Johns Hopkins University, 818 Ross Building, 720 Rutland Avenue, Baltimore, MD 21205-2196. Phone: 410-502-5153; Fax: 410-614-1411; E-mail: dsidrans@jhmi.edu.

©2005 American Association for Cancer Research.

Downloaded from cancerres.aacrjournals.org on November 13, 2019. © 2005 American Association for Cancer Research.

University (Sendai, Japan), or kindly provided by Dr. Shimada of the Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University (Kyoto, Japan). These cell lines were maintained in RPMI1640 supplemented with 10% fetal bovine serum.

Re-expression of PGP9.5 by 5′-aza-2′-deoxycytidine treatment of esophageal squamous cell carcinoma cell lines. Cells were split to low density (1×10^6 per T-75 flask) 12–24 hours before treatment. Cells were then treated for 5 days with 1 or 5 μmol/L 5-aza-2′-deoxycytidine (Sigma, Inc., St. Louis, MO), and 300 nmol/L trichostatin A (Sigma) was added to the medium (final 24 hours) as described previously (7).

RT-PCR of esophageal squamous cell carcinoma cell line DNA. RNA extraction was done from cell lines using Trizol (Invitrogen Inc., Carlsbad, CA) as previously described (7). Eight micrograms of each total RNA were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen), and 1:100 of the reaction mixture was amplified by PCR and analyzed as reported previously (7). PCR was done for 1 minute at 95°C (denaturation), 1 minute at 61°C (annealing), and 1 minute at 72°C (elongation) for 28 cycles. Oligonucleotide primer pairs for PGP9.5 were purchased from Invitrogen. The forward primer sequence was 5′-TGATGGACGAATGCCTTTTCC-3′ and was located at position +590 relative to the PGP9.5 transcription start site. The reverse primer sequence was 5′-ACTGGGGAGAATGCTTCACCA-3′ and located at position +947. Glyceraldehyde-3-phosphate dehydrogenase primers were as previously described (7).

Bisulfite treatment for DNA. For DNA denaturing, 2 μg of genomic DNA was incubated with 5 μg of salmon sperm DNA in 0.3 mol/L NaOH for 20 minutes at 50°C. The DNA sample was then diluted with 500 μL of a 2.5 mol/L sodium metabisulfite/125 mmol/L hydroquinone/0.4 mol/L sodium hydroxide solution, and placed at 70°C for 1 hour. After 1 hour, the sample was applied to a column (Wizard DNA Clean Up System, Promega Inc., Madison, WI), incubated with 0.3 mol/L NaOH for 10 minutes, and then treated with 3 mol/L ammonium acetate for 5 minutes. ETOH was added (2.5-fold volume of 100%), and DNA was allowed to precipitate for 1 hour at room temperature. DNA was resuspended in 100 μL water and stored at −80°C.

PCR amplification of bisulfite-treated DNA for combined bisulfite restriction analysis and sequencing. We extracted genomic DNA from Trizol (Invitrogen)-treated samples and performed bisulfite modification of genomic DNA as described (7). The primers were designed to recognize the DNA alterations caused by the bisulfite treatment. The primer sequences were 5′-ACTCAAAAAACACCCACCAACAAAT-3′ (PGP9.5 F1-sense sequence from positions −223 to −198 relative to PGP9.5 transcription start site) and 5′-GTAGAAATAGTTTAGGGAAGA-3′ (PGP9.5 R-antisense sequence from positions +50 to +29 relative to PGP9.5 transcription start site). PCR amplifications were done as follows: a 5-minute 95°C incubation step was followed by 45 cycles of 1 minute at 95°C, 1 minute at 54°C, and 2 minutes at 72°C. A 7-minute elongation step at 72°C completed the PCR amplification program.

Combined bisulfite restriction analysis and sequencing analysis. PCR amplification products were run on a 1% agarose gel stained with ethidium bromide and visualized under UV light. Amplified bands were excised, purified (spin column, Denville Scientific, Inc., Metuchen, NJ), and resuspended in 30 μL water. Fifteen microliters of DNA was digested in a mix containing 1 × NEB buffer 2 and *Bst*UI (New England BioLabs, Inc., Beverly, MA). The mix was covered with mineral oil and placed at 60°C overnight. Following digestion, the samples were loaded on a 6% acrylamide gel, stained with ethidium bromide and visualized under UV light. Sequencing was done using F1 primers as previously described (7).

Real-time quantitative PCR. For quantitative methylation analysis, PCR primers were designed to hybridize to regions of the PGP9.5 that we previously determined to be methylated in the ESCC cell line (KYSE30) by sequencing. In addition, a fluorescent probe was designed to hybridize to the amplified region of DNA. The PGP9.5 primers had the following sequences: 5′-CGGCGAGTGAGATTGTAAGGTT-3′ (PGP9.5 TAQF) and 5′-GAACGATCGCGACCAAATAAATAC-3′ (PGP9.5 TAQR). The probe design was 6FAM 5′-TTCGGTCGTATTATTTCGCGTTGCGTAC-3′ TAMRA. The β-actin primer sequences were previously described (8). For all reactions, 3 μL of bisulfite-treated DNA was added to a final volume of 20 μL. Amplifications were carried out in a 384-well Clear Optional Reaction Plate (Applied Biosystems, Inc., Foster City, CA) with Optical Adhesive Cover (Applied Biosystems) as described previously (8). Serial dilutions of human leukocyte DNA treated as *in vitro* methylated were used to construct a calibration curve, and all reactions were done in triplicate. The methylation ratio was defined as quantity of fluorescence intensity derived from PGP9.5 promoter amplification divided by fluorescence intensity from β-actin amplification, multiplied by 100 (we designated this value as TaqMan methylation value: TaqMeth V).

Multivariate analysis for patient prognosis. We first computed bivariate associations between each putative confounder variable and survival, and between each putative confounder and PGP9.5 methylation status. The only variable that was independently associated with survival, other than PGP9.5 methylation status, was tumor location (Table 1). Consequently, we constructed a Cox proportional hazards model with PGP9.5 methylation status and tumor location as the covariates. The significance level used was 0.05 and all statistical analyses were conducted using STATA Version 8 (STATA, Inc., College Station, TX). In our search for independent clinicopathologic factors, we did not include tumor-node-metastasis (TNM) staging as a variable factor from our multivariate analysis because TNM staging itself depends upon depth of tumor invasion, lymph node metastasis, and the presence of distant metastasis.

Results

Methylation of the promoter DNA of PGP9.5 in esophageal squamous cell carcinoma cell lines and their consistency with gene silencing. We first examined PGP9.5 promoter methylation in seven ESCC cell lines by combined bisulfite restriction analysis (COBRA) analysis (Fig. 1*A*). Methylated alleles were cleaved by the enzyme *Bst*UI, which cleaves the sequence CGCG. The size shift due to this cleavage can be visualized on a gel after separation. Of the seven cell lines examined by this method, five ESCC cell lines were found to be methylated. Among these, KYSE30 was completely methylated by direct sequencing (Fig. 1*B*), suggesting that PCR products were only partially digested by *Bst*UI. KYSE140 did not harbor any methylated alleles by direct sequencing (Fig. 1*B*).

We then sought to determine the RNA expression of PGP9.5 in the ESCC cell lines. RT-PCR analysis of ESCC cell line cDNA indicated that in cell lines where PGP9.5 was methylated (Fig. 1*C*), PGP9.5 was absent or expressed at low levels. Upon administration of 5-aza-2′-deoxycytidine and/or trichostatin A, PGP9.5 expression increased robustly. Conversely, cell lines in which the PGP9.5 promoter was unmethylated showed a consistently high level of

Table 1. Relationship between variables and prognosis

Variables	Hazard ratio	*P*
PGP9.5		
High methylation versus low methylation	2.5	0.01
Location		
Lower versus upper	2	0.04
Lymph node metastasis		
Metastasis versus nonmetastasis	1.98	0.05
TNM staging		
Stages III and IV versus stage I and II	1.98	0.05
Depth of invasion		
Without wall versus within wall	1.53	0.13

Downloaded from cancerres.aacrjournals.org on November 13, 2019. © 2005 American Association for Cancer Research.

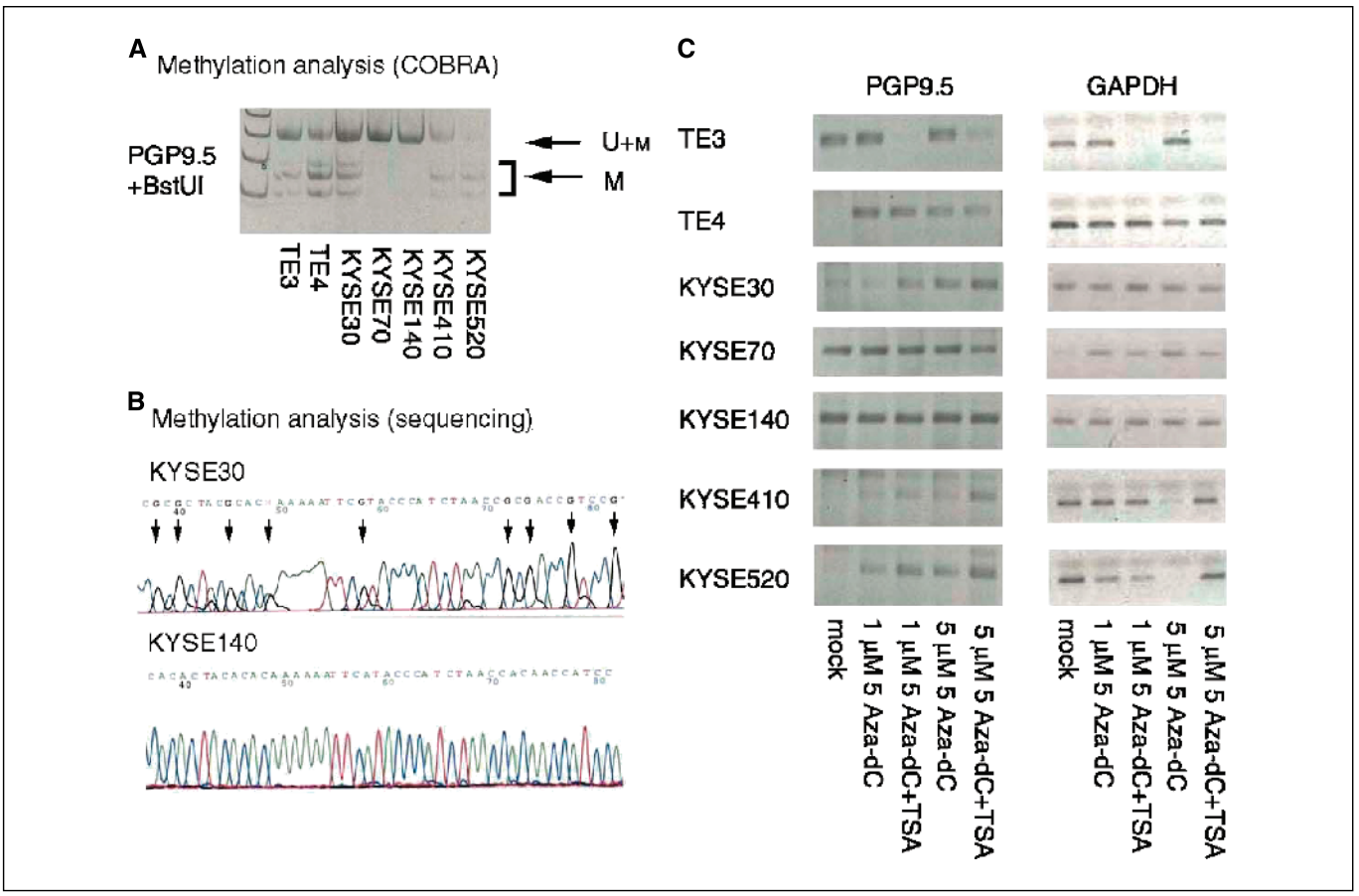

Figure 1. Methylation of PGP9.5 promoter in ESCC cell lines and gene silencing. *A,* COBRA analysis was done for seven ESCC cell lines (TE3, TE4, KYSE30, KSYE70, KYSE140, KYSE410, and KYSE520). With the exception of KYSE70 and KYSE140, the PGP9.5 promoter was methylated in the remaining cell lines. M, methylated band; U, unmethylated band. COBRA does suffer from partial digestion of *Bst*UI. KYSE30, which was confirmed to be completely methylated by direct sequencing, was not digested completely by *Bst*UI. *B,* direct sequencing in representative ESCC cell lines. KYSE30 showed complete methylation in the tested region, whereas KYSE140 showed no methylation. All guanines (*arrows*) present after sequencing represent methylated cytosines (protected) on the complementary strand. *C,* RT-PCR for ESCC cell lines. One or 5 μmol/L 5-aza-2′-deoxycytidine treatment and/or trichostatin A reactivated PGP9.5 at the mRNA level. Glyceraldehyde-3-phosphate dehydrogenase expression was used as a loading control of each lane. Note that, in cell lines where PGP9.5 was methylated, PGP9.5 was absent or expressed at low levels. Upon administration of 5-aza-2′-deoxycytidine and/or trichostatin A, PGP9.5 expression increased robustly. Conversely, in cell lines in which the PGP9.5 promoter was not methylated, PGP9.5 expression was recognized both before and after treatment. Of the five ESCC cell lines that harbored PGP9.5 promoter methylation, only TE3 cells expressed PGP9.5 at the mRNA level. However, the methylation level in this cell line was quite low, suggesting that these cells contain only a small methylated population.

PGP9.5 expression both before and after pharmacologic treatment. Thus, in all ESCC cell lines, PGP9.5 promoter methylation was consistent with PGP9.5 expression.

Combined bisulfite restriction analysis of PGP9.5 promoter methylation in esophageal squamous cell carcinoma primary tumors. ESCC primary tumor DNA was then bisulfite-treated, PCR-amplified, and digested with the restriction enzyme *Bst*UI. Of the 50 primary ESCC tumors, 21 (42%) were found to have PGP9.5 promoter methylation (Fig. 2*A* and *B*). The tumors with detectable PGP9.5 promoter methylation had a significantly worse prognosis and a higher frequency of lymph node metastasis than ESCC primary tumors without methylation (P = 0.01, data not shown).

TaqMan methylation-specific PCR analysis of PGP9.5 promoter methylation in esophageal squamous cell carcinoma primary tumors. We also evaluated the 50 tumor samples using TaqMan methylation-specific PCR analysis, and quantified them in order of increasing methylation ratios (TaqMeth V; Fig. 2*B*). Interestingly, all the top 21 patients (42%) with higher levels of methylation by TaqMan quantitation showed visible COBRA bands in an acrylamide gel. This result suggests that TaqMan quantitative analysis is consistent with actual methylation status, and reflects the true quantity of PGP9.5 methylated alleles in primary cancers.

Clinicopathologic correlation with methylation status by TaqMan methylation-specific PCR in primary esophageal squamous cell carcinoma. We quantified tumor methylation ratios by using TaqMeth V as described in Materials and Methods. Using this value, methylation values ranged from 0 to 228.3 (mean value 41.8, and median value was 10.0). Cases with higher PGP9.5 TaqMeth V showed poorer prognosis than those with lower PGP9.5 TaqMeth V. We investigated several cutoff lines for TaqMeth V using either P value or hazard ratio for patient prognosis to determine the best optimized cutoff line (Fig. 3*A*). The most accurate cutoff line for predicting patient prognosis was 20.0, but values from 10 to 40 were also statistically significant (Fig. 3*A*). Drawing a cutoff line at 20.0, there were 22 cases with higher methylation ratios (PGP9.5 TaqMeth V >20.0) and 28 cases with lower methylation ratios (PGP9.5 TaqMeth V ≤20.0; see Fig. 2*B*). The higher PGP9.5 methylation group included 1 (11%) of the

Downloaded from cancerres.aacrjournals.org on November 13, 2019. © 2005 American Association for Cancer Research.

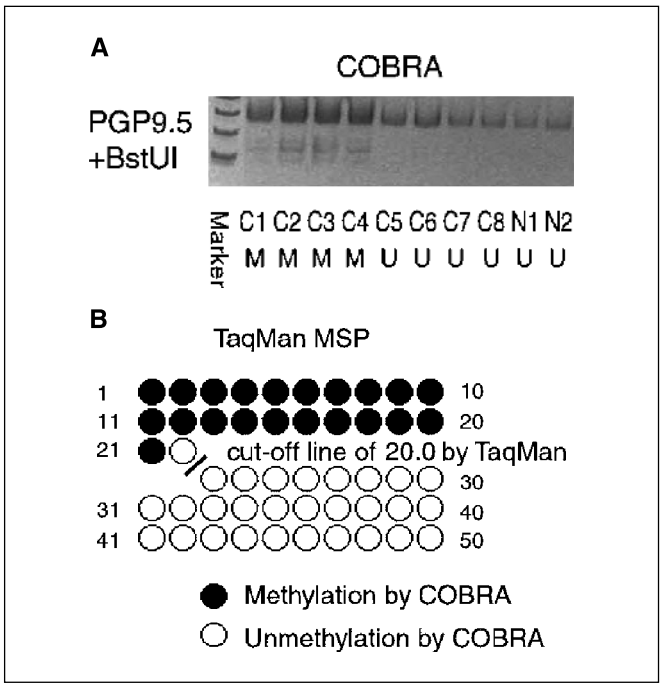

Figure 2. Demonstration of methylation in primary ESCC tissues by two independent modalities (COBRA and TaqMan PCR analysis). *A,* COBRA analysis was done for primary ESCC tissue specimens and the corresponding normal mucosa specimens. M, methylated band; U, unmethylated band; C1-C4, primary ESCC tissues with methylation of the PGP9.5 promoter; C5-C8, primary ESCC tissues without any methylation of PGP9.5. Corresponding normal mucosa samples (N1 and N2) were not methylated. *B,* TaqMan quantitative analysis was consistent with COBRA analysis. The figure is in descending order of TaqMeth V as described in Materials and Methods. (●) methylated or (○) unmethylated by COBRA. Note that 21 patients with the highest levels of methylation by TaqMan quantitation showed visible COBRA bands on acrylamide gels.

9 patients with no lymph node metastasis, and 21 (47%) of the 44 patients with lymph node metastasis. This difference for lymph node metastasis was statistically significant (P = 0.03). No other clinicopathologic factors showed a significant correlation with PGP9.5 methylation status (Table 2). Student's t test analysis of TaqMeth V and lymph node metastasis also showed a significant difference (P = 0.05; Fig. 3*B*). Finally a Kaplan-Meier survival curve for the 50 patients with ESCC was constructed to analyze survival discrepancies between patients with PGP9.5 methylation levels above or below the 20.0 cutoff line. The statistical difference was determined to be 0.01 by a Cox-Mantel model (hazard ratio, 2.5). Kaplan-Meier curves, by stage, lymph node status, and/or PGP9.5 methylation are shown in Fig. 4.

Multivariate analysis of PGP9.5 methylation for patient prognosis. Results of multivariate Cox-proportional hazards modeling indicated that PGP9.5 methylation was associated with a 147% increase in the hazard for death (hazard ratio, 2.47; 95% confidence interval, 1.09-5.58), with a P value of 0.03. The only variable that was independently associated with survival, other than PGP9.5 methylation status, was tumor location, however, it was not significant (P = 0.06).

Discussion

In this study, we found that PGP9.5 methylation in primary cancers was significantly correlated with lymph node metastasis and patient prognosis. We showed such relevance using quantitative TaqMan methylation-specific PCR. This suggests that within the ESCC primary tumor, cancer cells with PGP9.5 promoter methylation comprise a dominant clone with highly malignant potential. Our selected patients were successive surgical archival samples, resected with curative intent. Currently, the presence or absence of lymph node metastases is the strongest independent prognostic factor for ESCC survival (14). Of the 50 cases analyzed in this study,

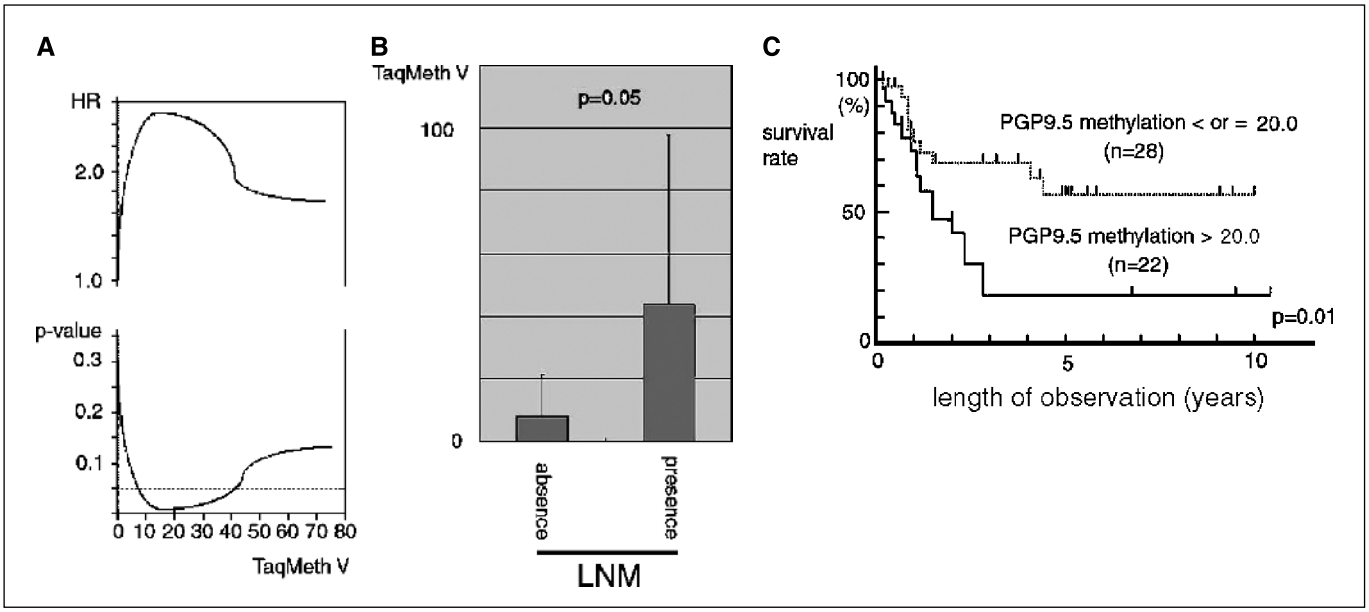

Figure 3. Clinical relevance of PGP9.5 methylation in primary ESCC tissues. *A,* identification of the best optimized cutoff point using the Cox-Mantel prognostic analysis. The best cutoff point for TaqMeth V is 20.0, whereas statistical significance (P < 0.05) remained between 10 and 40. *B,* Student's t test analysis for lymph node metastasis using TaqMeth V. The methylation ratio level of PGP9.5 significantly correlated with lymph node metastasis (P = 0.05). *C,* Kaplan-Meier curves in primary ESCC comparing cases with higher TaqMeth V (≥20.0; n = 22) and those with lower TaqMeth V (<20.0; n = 28). The Cox-Mantel analysis comparing the two groups revealed a significant difference (P = 0.01), namely, the group with higher methylation yielded a poorer prognosis than the group with lower methylation of PGP9.5.

Downloaded from cancerres.aacrjournals.org on November 13, 2019. © 2005 American Association for Cancer Research.

only 9 cases had no lymph node metastases. As a consequence of this patient distribution, any marker found to be significantly correlated with ESCC prognosis in our selected patients must be relatively independent of lymph node metastases status. In accordance with our hypothesis, PGP9.5 was confirmed to be an independent prognostic factor by multivariate analysis ($P = 0.03$).

An independent prognostic factor's clinical utility is its ability to more accurately predict patient survival when used in combination with other known prognostic factors. Such a marker would be invaluable to surgeons and patients selecting treatment options. Our data showed that PGP9.5 promoter methylation, in combination with lymph node metastases, more accurately predicted patient prognosis (Fig. 4*B*) than lymph node metastases alone. From our present data, we propose several designs to study the feasibility of PGP9.5 methylation as a clinical tool. We must increase the sample size of node-negative ESCC patients, to determine if PGP9.5 methylation can accurately predict curability (>5 year survival) in this patient population (Fig. 4*B*). Interestingly, the one case with negative-lymph node metastasis and higher PGP9.5 methylation died of ESCC 34 months after surgical resection. Although such cases were small in number ($n = 8$), in this study, the 5-year survival for patients who are both lymph node- and PGP9.5 methylation-negative is >85% (expected results are around 60%: 85 × 0.33—stage I + 50 × 0.67—stage II according to the previous reports; ref. 15). Conversely, for node-positive cases (mostly stage III and IV cancers), PGP9.5 methylation and lymph node metastases did not show an additive effect in predicting patient prognosis (Fig. 4*B* and *C*). For such cases, perhaps other yet to be identified prognostic factors could be combined with PGP9.5 methylation status to better predict patient prognosis.

Table 2. Clinicopathologic variables and PGP9.5 methylation in primary ESCC

	PGP9.5 TaqMan methylation analysis		*P*
	Methylation (+), *n* = 22	Methylation (+), *n* = 28	
Age (years)	60 ± 8.5	61 ± 9.5	NS
Sex			NS
Male	20 (47%)	23 (53%)	
Female	2 (29%)	5 (71%)	
Location			NS
Upper	2 (67%)	1 (33%)	
Middle	9 (32%)	19 (68%)	
Lower	11 (58%)	8 (42%)	
Differentiation			NS
Well	6 (55%)	5 (45%)	
Moderately	12 (44%)	15 (56%)	
Poorly	4 (33%)	8 (67%)	
Depth of invasion			NS
Submucosa	1 (33%)	2 (67%)	
Muscularis propria	1 (14%)	6 (86%)	
Adventitia	20 (50%)	20 (40%)	
Lymph node metastasis			$P = 0.03$
Absent	1 (11%)	8 (89%)	
Present	21 (51%)	20 (49%)	
Lymphatic penetration			
Absent	1 (17%)	5 (83%)	NS
Present	21 (47%)	23 (53%)	
Vascular penetration			NS
Absent	5 (45%)	6 (55%)	
Present	17 (44%)	22 (56%)	
TNM staging			
Stages I and II	1 (11%)	8 (89%)	$P = 0.03$
Stages III and IV	21 (51%)	20 (49%)	

Abbreviation: NS, not significant.

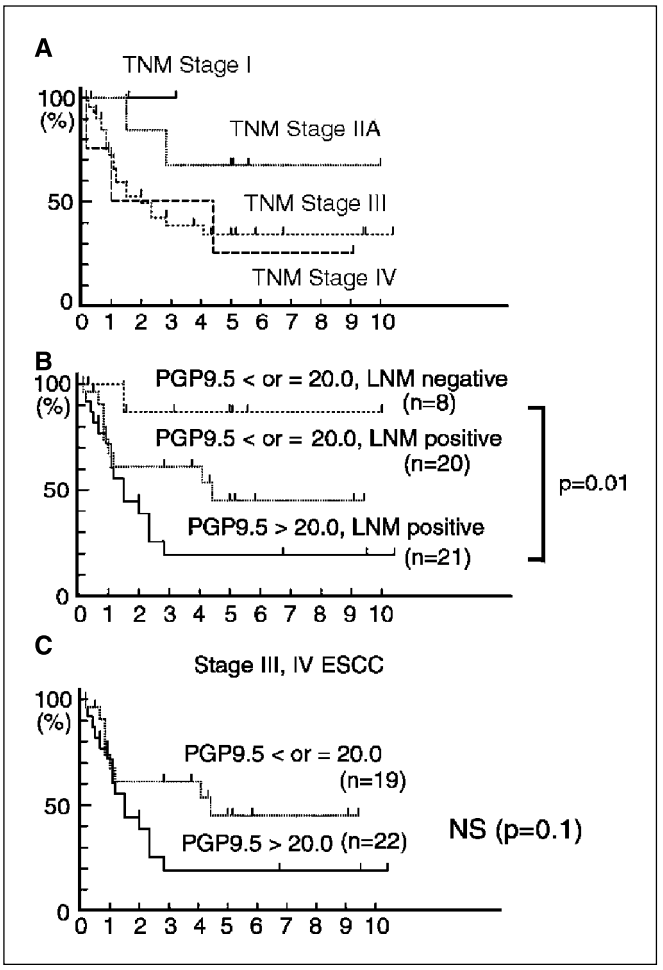

Figure 4. PGP9.5 methylation as an independent prognostic factors in primary ESCC. *A,* clinical staging of selected patients according to TNM classification. Five-year survival of each stage is 75% (stage IIA), 35% (stage III), and 25% (stage IV). For cases with stage I ($n = 3$), follow-up was within 5 years. *B,* a combination of PGP9.5 and lymph node metastasis is an excellent predictor of ESCC patient survival. Patients with higher PGP9.5 methylation showed dismal prognosis (<20% 5-year survival), whereas those with lower PGP9.5 showed excellent prognosis (>85% 5-year survival; $P = 0.01$). *C,* for the patients with advanced cancers (stages III and IV), PGP9.5 showed a trend towards, but not a significant correlation with patient prognosis ($P = 0.1$).

Recently, we have investigated promoter DNA methylation as a tumor marker in primary tumors and body fluids (16–20). A combination of several methylation markers was reported to be significantly correlated with patient prognosis in esophageal adenocarcinoma (21). However, in this study, PGP9.5 was found to be an independent prognostic factor as a single DNA marker.

Determining the functional relevance of PGP9.5 with respect to a tumor's metastatic ability is crucial to legitimizing its use as a prognostic marker. Little evidence currently exists regarding

Downloaded from cancerres.aacrjournals.org on November 13, 2019. © 2005 American Association for Cancer Research.

PGP9.5's potential role as a tumor suppressor. Moreover, there are numerous reports that PGP9.5 is overexpressed in a subset of primary cancers (11, 12). Overexpression in primary cancer tissues could be the cause of or the result of transformation. If it is the cause of transformation, PGP9.5 would be an oncogenic molecule, but the clinical profile reported here does not support this notion. We recently established four squamous carcinoma cell lines with stably transfected PGP9.5, and none of them showed increased tumorigenesis in soft agar.[4] This result suggests that PGP9.5 overexpression in primary cancers may merely be a result of transformation. Among the four cell lines tested, the tumorigenic ability of two lines was dramatically suppressed for tumorigenesis by PGP9.5 expression (data not shown). These findings suggest that PGP9.5 may actually act as a tumor suppressor gene, a function that is supported by its cancer-specific methylation pattern (8).

One mechanism by which PGP9.5 could act as a tumor suppressor is through the degradation of oncogenes. The PGP9.5 gene encodes two opposing activities in the ubiquitin pathway, one functioning as a ubiquitin COOH terminus hydrolase to generate single ubiquitin and another demonstrating ligase activity leading to multiple ubiquitinations (10). PGP9.5 ligase activity results in the accumulation of α-synuclein, a possible mediator of the Parkinson's phenotype (10). PGP9.5 knockout mice (gad mice) have been used to show that PGP9.5 induction by stress stimuli induced ubiquitin expression (22). In gad mice, ubiquitin expression was not induced, and after apoptotic stimulation, Bcl-2 and XIAP accumulated, resulting in an apoptosis-resistant phenotype (22). Moreover, ubiquitin expression is up-regulated in various types of primary cancers (23–25), and PGP9.5 may be involved in this alteration of ubiquitin regulation. These results suggest that PGP9.5 is more likely to be a tumor suppressor molecule than an oncogene.

In conclusion, this study represents the first accurate clinical profile of PGP9.5 promoter methylation in primary ESCC. We conclude that a clone harboring PGP9.5 promoter methylation may have greater malignant potential in human cancers. Detecting PGP9.5 promoter methylation in primary tumors could be applied clinically, as this marker is an independent predictor of the 5-year survival in patients with ESCC.

[4] Data in preparation for publication.

Acknowledgments

Received 11/1/2004; revised 1/14/2005; accepted 2/24/2005.

Grant support: Supported by NCI grants CA 98-028 and R01 DE13561.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. WHO. The World Health Report. 1997.
2. Pisani P, Parkin D, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 1999;83:18–29.
3. Stoner G, Rustgi A. Biology of esophageal squamous cell carcinoma. Gastrointest Cancer Biol Diagn Ther 1995;8:141–6.
4. Goseki N, Koike M, Yoshida M. Histolopathologic characterization of early stage esophageal carcinoma. Cancer 1992;69:1088–93.
5. Jones P. DNA methylation and cancer. Oncogene 2002; 21:5358–60.
6. Sidransky D. Emerging molecular makers of cancer. Nat Rev Cancer 2002;2:210–9.
7. Yamashita K, Upadhyay S, Osada M, et al. Pharmacological unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. Cancer Cell 2002;2:485–95.
8. Tokumaru Y, Yamashita K, Osada M, et al. Inverse correlation between cyclin A1 hypermethylation and p53 mutation in head and neck cancer identified by reversal of epigenetic silencing. Cancer Res 2004;64: 5982–7.
9. Wilkinson K, Lee K, Deshpande S, Duerksen-Hughes P, Boss J, Pohl J. The neuron-specific protein PGP9.5 is a ubiquitin carboxy-terminal hydrolase. Science 1989;246: 670–3.
10. Liu Y, Fallon L, Lashuel H, Liu Z, Lansbury P Jr. The UCH-L1 gene encodes two opposing enzymatic activities that affect α-synuclein degradation and Parkinson's disease susceptibility. Cell 2002;111:209–18.
11. Tezel E, Hibi K, Nagasaka T, Nakao A. PGP9.5 as a prognostic factor in pancreatic cancer. Clin Cancer Res 2000;6:4764–7.
12. Takase T, Hibi K, Yamazaki T, et al. PGP9.5 overexpression in esophageal squamous cell carcinoma. Hepatogastroenterol 2003;50:1278–80.
13. Sato N, Fukushima N, Maitra A, et al. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. Cancer Res 2003;63:3735–42.
14. Sugimachi K. Prognostic factors of esophageal carcinoma: univariate and multivariate analysis. J Surg Oncol 1986;31:108–12.
15. Nozoe T, Sugimachi K. Simple staging criteria for esophageal carcinoma. Am J Clin Oncol 2003;26:307–11.
16. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res 1999;59:67–70.
17. Kawakami K, Brabender J, Lord RV, et al. Hypermethylated APC DNA in plasma and prognosis of patients with esophageal adenocarcinoma. J Natl Cancer Inst 2000;92:1805–11.
18. Sanchez-Cespedes M, Esteller M, Wu L, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res 2000;60:892–5.
19. Jeronimo C, Usadel H, Henrique R, et al. Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. J Natl Cancer Inst 2001;93:1747–52.
20. Usadel H, Brabender J, Danenberg KD, et al. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma DNA of patients with lung cancer. Cancer Res 2002;62: 371–5.
21. Brock M, Gou M, Akiyama Y, et al. Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. Clin Cancer Res 2003;9:2912–9.
22. Harada T, Harada C, Wang Y, et al. Role of ubiquitin carboxy terminal hydrolase-L1 in neural cell apoptosis induced by ischemic retinal injury *in vivo*. Am J Pathol 2004;164:59–64.
23. Ishibashi Y, Takada K, Joh K, Ohkawa K, Aoki T, Matsuda M. Ubiquitin immunoreactivity in human malignant tumors. Br J Cancer 1991;63:320–2.
24. Osada T, Sakamoto M, Mishibori H, et al. Increased ubiquitin immunoreactivity in hepatocellular carcinomas and precancerous lesions of the liver. J Hepatol 1997;26:1266–73.
25. Ishibashi T, Hanyu N, Suzuki Y, et al. Quantitative analysis of free ubiquitin and multi-ubiquitin chain in colorectal cancer. Cancer Lett 2004;211:111–7.

Downloaded from cancerres.aacrjournals.org on November 13, 2019. © 2005 American Association for Cancer Research.

AACR American Association for Cancer Research

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

PGP9.5 Promoter Methylation Is an Independent Prognostic Factor for Esophageal Squamous Cell Carcinoma

Diana L. Mandelker, Keishi Yamashita, Yutaka Tokumaru, et al.

Cancer Res 2005;65:4963-4968.

Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/11/4963

Cited articles This article cites 23 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/11/4963.full#ref-list-1

Citing articles This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/11/4963.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/65/11/4963.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC)
Rightslink site.

Downloaded from cancerres.aacrjournals.org on November 13, 2019. © 2005 American Association for Cancer Research.