O^6-Methylguanine-DNA Methyltransferase Gene: Epigenetic Silencing and Prognostic Value in Head and Neck Squamous Cell Carcinoma

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Abstract

Background: Alkylating *N*-nitroso compounds can interact directly with DNA, forming O^6-alkylguanine, a DNA adduct proved to be mutagenic and carcinogenic if not sufficiently repaired. A specific DNA repair enzyme, *O^6-methylguanine-DNA methyltransferase (MGMT)*, can remove the alkyl group from the O^6-position of the guanine, thereby preventing its mutagenic and carcinogenic effects. Inactivation of the *MGMT* gene in association with promoter hypermethylation results in persistence of O^6-alkylguanine in DNA, leading to G:C to A:T transition mutation and these G:C to A:T transition mutations can inactivate *p53* tumor suppressor gene or activate *ras* proto-oncogene. Methods: We analyzed *MGMT* promoter hypermethylation and protein expression patterns in 94 cases of primary head and neck squamous cell carcinoma (HNSCC) by methylation-specific PCR (MSP) and immunohistochemical staining. The results were then correlated with clinical follow-up data. Results: *MGMT* promoter hypermethylation was present in 17 of 94 patients (18.1%) and apparent loss of protein expression was seen in 19 of 93 HNSCC patients (20.4%). The presence of *MGMT* promoter hypermethylation was significantly correlated with loss of MGMT protein expression in HNSCC. Both *MGMT* promoter hypermethylation and loss of protein expression were significantly correlated to increased tumor recurrences and decreased patient survival, independent of other risk factors, such as tumor site, tumor size, nodal status, age, and chemoradiation therapy. Conclusions: *MGMT* promoter hypermethylation and apparent loss of protein expression are reliable and independent prognostic factors in HNSCC. The above study may also provide guideline or basis for applying alkylating antitumor agents to patients with HNSCC that display *MGMT* promoter hypermethylation and/or loss of MGMT protein expression. (Cancer Epidemiol Biomarkers Prev 2004;13(6):967–75)

Introduction

Head and neck squamous cell carcinoma (HNSCC) represent a highly heterogeneous group of neoplasms with diverse biological behaviors. In USA, they account for 5% of the total cancer burden and there are approximately 50,000 cases each year resulting in about 2000 deaths (1).

It is well recognized that the development of HNSCC is a multistep process with progressive accumulation of adverse chromosomal or genetic aberrations resulting in activation of oncogenes and/or inactivation of tumor suppressor genes and ultimately leading to selective growth advantage and tumor formation (2).

Tobacco smoke contains many well-recognized carcinogens and procarcinogens, such as benzo(*a*)pyrene, polycyclic aromatic hydrocarbon (PAH), arylamines, and tobacco-specific nitrosamines (3). Chronic exposure to these carcinogens can induce DNA damages, leading to irreversible chromosomal and genetic alterations.

Tobacco-specific nitrosamines, a class of alkylating *N*-nitroso compounds, can interact directly with DNA at various sites, forming 13 different types of DNA adducts (4, 5). Of the most biological significance are two mutagenic and carcinogenic DNA adducts, O^6-alkylguanine (O^6-AlkG) and O^4-alkylthymine (O^4-AlkT). Because of structural similarity, DNA polymerase can mistake O^6-AlkG and O^4-AlkT during DNA synthesis as normal bases, adenine and cytosine. If not sufficiently repaired, these two miscoding bases will lead to point mutations, which are, in the case of O^6-AlkG, G:C→A:T transition mutation and in the case of O^4-AlkT, A:T→G:C transition mutation (4, 5).

The O^6-methylguanine-DNA methyltransferase (MGMT) is a specific DNA repair enzyme for O^6-AlkG and involves the direct transfer of an alkyl group from DNA to a cysteine acceptor site in the repair protein, thereby, restoring the DNA structure to its predamaged state in one step and preventing the mutagenic and carcinogenic effects of alkylating *N*-nitroso compounds (6-8).

Received 8/25/03; revised 1/21/04; accepted 1/26/04.

Grant support: Department of Pathology and Otolaryngology, University of Arkansas for Medical Sciences and Biomedical Research Foundation, John L. McClellan Memorial Vetarans' Hospital, Little Rock, Arkansas.

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The biological significance of G:C→A:T transition mutation in alkylating *N*-nitroso compound–induced carcinogenesis is underscored in a *N*-nitroso-*N*-methylurea–induced rat mammary carcinoma model in which the *ras* proto-oncogene has been shown to be activated via this type of mutation (9). In this study, Sukumar et al. (9) identified G:C→A:T transition mutation at codon 12 of the c-Ha-*ras* proto-oncogene in the *N*-nitroso-*N*-methylurea–induced rat mammary carcinoma, presumably due to the persistence of O^6-methylguanine and its mispairing with thymine. This mutation, thus, gives rise to the replacement of glycine by glutamine in the gene product, activating the c-Ha-*ras* proto-oncogene (9). Similar transition mutation at codon 12 of the c-Ha-*ras* proto-oncogene was also identified in a human bladder cancer cell line, resulting in the activation of this oncogene (10).

The *MGMT* promoter hypermethylation has also been shown to be associated with increased frequency of G:C→A:T transition mutations in the *p53* tumor suppressor gene in human brain, colorectal and lung cancer (11-13), and in the K-*ras* proto-oncogene in human gastric and colorectal carcinoma (14, 15).

Gene inactivation can occur via several mechanisms, including homozygous deletion, point mutation in the coding sequence, and CpG hypermethylation in the promoter region (16). While both epigenetic (promoter hypermethylation) and genetic (somatic mutations) factors are responsible for gene silencing in many genes, such as *von-Hippel Lindau* (*VHL*) tumor suppressor gene, *p16* tumor suppressor gene, and *hMLH1* DNA mismatch repair gene, epigenetic alteration in association with promoter hypermethylation is virtually the only identified molecular event in causing loss of function in other genes, including the glutathione-*S*-transferase *Pi* gene and the *MGMT* gene (16).

Promoter hypermethylation of the *MGMT* gene has been reported in a wide variety of human cancer, such as brain tumors (11), testicular germ cell tumors (17), esophageal carcinoma (18), gastric and colorectal cancer (12, 14, 15, 19), hepatocellular (20) and pancreatic (21) carcinomas, prostatic adenocarcinoma (22), and lymphoma (23).

In HNSCC, the *MGMT* promoter methylation status has been analyzed in six separate studies in HNSCC (24-29). The number of the cases involved varied from 21 to 99 with a total number of 315 cases (24-29). In these studies, the incidence of *MGMT* promoter hypermethylation ranged from 20% to 41% with an average frequency of 32% (24-29). However, none of the above studies attempted to correlate *MGMT* promoter hypermethylation with *MGMT* gene expression (24-29).

In the present study, we, for the first time, perform simultaneous analyses of *MGMT* promoter methylation and protein expression patterns by methylation-specific PCR (MSP) and immunohistochemistry and correlate the status of promoter methylation and/or protein expression with clinical follow-up data in a large series of HNSCC.

Materials and Methods

Tissue Collection. Ninety-four consecutive untreated cases of HNSCC were collected from the Anatomic Pathology file (from 1993 to 1998) in the Department of Pathology, John L. McClellan Memorial Veterans' Hospital, Little Rock, Akansas. Both paraffin blocks and H&E-stained slides of tumors from each case were available for study. Case selection was based on the following criteria: (1) primary surgical resection with curative intent or diagnostic biopsy for the purpose of adjuvant therapy and (2) no prior history of HNSCC and adjuvant therapy. The histology of each case was reviewed and representative tissue blocks containing invasive SCC were selected for DNA extraction and promoter methylation analysis. Clinical follow-up was available for all cases up to May 2002. Pertinent patient information was retrieved from the Computerized Patient Record System (CPRS), Department of Veterans Affairs.

H&E Staining. Ninety-four slides of cases were examined to confirm the diagnosis and the representative divisions are selected from the slides for immunohistochemical (IHC) staining. Both paraffin blocks and H&E-stained slides of tumors from each case were available for study.

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. DNA samples from HNSCC cases, negative, and positive control DNA were then subjected to bisulfite modification before MSP using CpGenome DNA modification Kit (Intergen).

Bisulfite Modification of DNA for MSP. DNA samples from HNSCC cases, human placenta (negative control; Sigma Chemical Co., St. Louis, MO), and CpGenome universal methylated human DNA (positive control; Intergen) were modified using the CpGenome DNA Modification Kit (Intergen). Amplification of promoter region of the *MGMT* gene is carried out in a Touchgene Gradient Thermal Cycler (Techne Inc., Princeton, NJ) in 50 μL PCR reaction mixture containing 2 μL of bisulfite-treated genomic DNA, deoxynucleotide triphosphates (each at 200 μmol/L), primers (50 pmol each per reaction), 2.5 mmol/L $MgCl_2$, and 1.25 units Hotstar Taq (Qiagen, Inc., Chatsworth, CA) in 1× PCR buffer. All reagents are supplied with the Qiagen Hotstar Taq Kit (Qiagen) except for the deoxynucleotide triphosphate mix (Roche Mol. Biochem., Indianapolis, IN). Primer sequences of *MGMT* for the unmethylated reaction are 5′-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3′ (sense) and 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′ (antisense), and for the methylated reaction, they are 5′-TTT CGA CGT TCG TAG GTT TTC GC-3′ (sense) and 5′-GCA CTC TTC CGA AAA CGA AAC G-3′ (antisense) as described previously (25). The PCR reactions were carried out as described previously (30) using annealing temperature of 61°C for both unmethylated and methylated reactions.

IHC Staining Procedure. IHC stainings were done on formalin-fixed, paraffin-embedded tissue sections with a monoclonal antibody against MGMT protein (Novus Biologicals, Littleton, CO; 1:1000 dilution). The normal squamous epithelia were used as internal positive controls. The standard avidin-biotin-peroxidase technique was used with microwave antigen retrieval as previously described (30, 31).

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The MGMT IHC staining results were interpreted semiquantitatively as follows: negative, less than 10% positive cells (−); mostly negative, 10% to 20% positive cells (+/−); weakly positive, 20% to 50% positive cells (+); moderately positive, 50% to 70% positive cells (++); and strongly positive, 80% to 100% positive cells (+++). The above percentages were determined by visual estimation of the positively stained area as compared with all areas containing tumor cells. Tumor tissue sections with less than 50% positive cells (negative, mostly negative, or weakly positive) were labeled as ''negative IHC staining result'' or ''apparent loss of protein expression'' while those with more than 50% positive cells (moderate and strongly positive) were labeled as ''positive IHC staining result.'' Two pathologists (C.Y.F., and C.Z.) evaluated IHC staining independently and interpreted the results, unaware of the *MGMT* promoter hypermethylation data.

Statistical Analysis. Survival was measured in months from the date of diagnosis to the date of death or the date of last follow-up. Disease-free survival was the period of time from the initial diagnosis of tumor to the first time of tumor recurrence or the development of second primary cancer of the upper aerodigestive tract. Cause-specific survival was based on death as a direct result of tumor progression as compared with death due to other causes. Overall survival was defined as total death of all causes. The association among various factors, such as *MGMT* promoter methylation, MGMT protein expression, and clinical and pathologic parameters was analyzed with crosstable χ^2 test. Survival functions and survival possibility of various prognostic factors were calculated with the Kaplan-Meier method and logrank test was used to compare difference of survival curve between groups. The prognostic covariates analyzed included sex, age, tumor site, tumor size, nodal status, clinical stage, and presence or absence of chemo/radiation therapies. All P values were two-sided. SPSS software (Version 11.0, SPSS Inc., Chicago, IL) was used for all statistical computation.

Results

All 94 tumor tissues used in the study were derived from primary tumors without prior chemoradiation therapy. These tumor specimens were surgically resected with clean margins by histologic examination. The patient population consisted of all males that ranged in age from 42 to 85 years with a mean of 63.5 years. The mean follow-up for all patients was 53.4 months. The median survival based on all causes of death was 36.2 months. Twenty-nine patients developed recurrences and 34 had regional lymph node metastasis. Among 94 patients studied, 55 died, 37 from their head and neck cancer and 18 from other causes.

Analysis for *MGMT* promoter methylation was done in all 94 cases of HNSCC by MSP. Aberrant *MGMT* promoter hypermethylation was seen in 17 (18.1%) cases with the remaining 77 cases (81.9%) showing no evidence of promoter methylation. Positive and negative controls worked appropriately in each round of PCR reaction. Representative *MGMT* MSP was illustrated in Fig. 1.

The MGMT protein expression was analyzed in 93 cases by IHC staining procedure using a monoclonal antibody

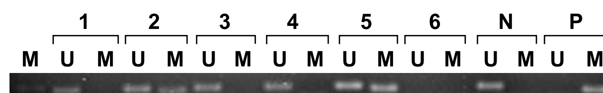

Figure 1. MSP results from five representative HNSCC cases. Both unmethylated- (*U*) and methylated-specific (*M*) MSP primer sets for *MGMT* were used. Samples 1, 3, and 4 were negative for *MGMT* promoter methylation; therefore, only the unmethylated (*U*) amplicons were detected. By contrast, samples 2 and 5 were positive for promoter methylation, as indicated by the presence of both unmethylated (*U*) and methylated (*M*) amplicons. Blank (water, sample 6), negative (*N*; human placental DNA), and positive (*P*; universal methylated human DNA; Intergen) controls were also included in the PCRs. *M* (*far left* on the panel), molecular marker.

against the MGMT protein (Novus Biologicals). In one case, the tissue block was exhausted for DNA extraction, thus, IHC study was not done for this case. Among these 93 cases, 80 contained both surface squamous mucosa (36 morphologically normal, 28 dysplastic, and 16 carcinoma *in situ*) and invasive carcinoma, whereas 13 cases contained only invasive carcinoma. The keratinocytes in surface squamous mucosa were positively stained in all cases, particularly in the actively proliferating basal and parabasal cells, indicative of the presence of endogenous MGMT protein (Fig. 2A and B). Pre-invasive squamous lesions (dysplasia or carcinoma *in situ*) if present, were mostly positive for the MGMT protein. Carcinoma *in situ* was negative only in three cases in which the invasive component was also negative for the protein. One such case is displayed in Fig. 2B and D. The IHC staining patterns for the MGMT protein in HNSCC were in general heterogeneous with both positively and negatively stained tumor cells present in most cases, but at different proportions.

Nineteen (20.4%) cases showed apparent loss of MGMT protein expression and this negative group consisted of four negative cases (−), three mostly negative cases (+/−), and 12 weakly positive cases (+). By contrast, 74 (79.6%) cases showed high levels of MGMT protein expression. This positive group consisted of 17 positive cases (++) and 57 strongly positive cases (+++). The relative percentage of positive and negative tumor cells was measured semiquantitatively and independently by two pathologists (C.Z. and C.Y.F.). There appeared to be a high level of agreement in terms of IHC interpretation with 88 of 93 cases (94.6%) being agreed on by both pathologists. Differences in result interpretation were, however, found in five cases, and all these cases fell into the categories of either weakly (+) or moderately (++) positive. Such discrepancies were resolved by examining the slides in question by both pathologists simultaneously until mutual agreement was reached. Both initial independent interpretation and subsequent re-evaluation on the disputed cases were done, unaware of the *MGMT* promoter hypermethylation data. The final results were then categorized into ''positive'' and ''negative'' groups for statistical analysis (see criteria in Materials and Methods).

Because promoter hypermethylation has been postulated as an important mechanism of gene silencing, we attempted to correlate *MGMT* promoter hypermethylation

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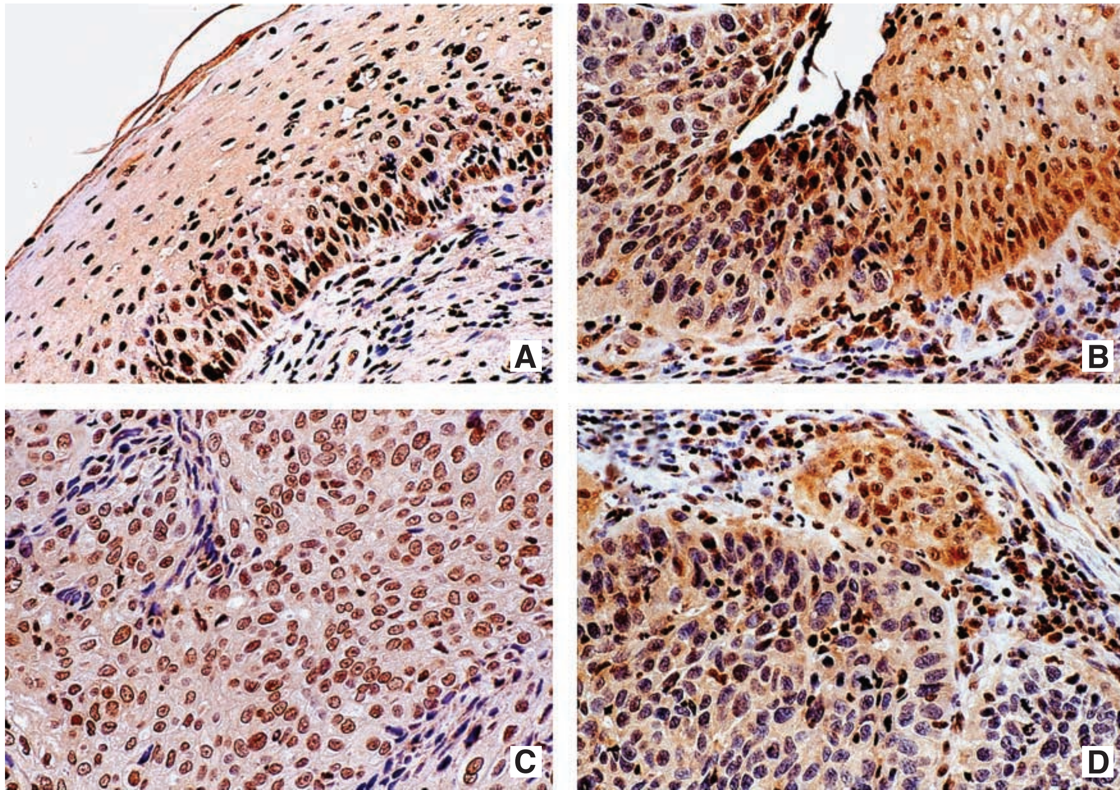

Figure 2. IHC staining for the MGMT protein in HNSCC. **A** and **C** are from a case strongly positive for the MGMT protein, whereas **B** and **D** are from a case that is negative for the protein. In normal squamous epithelia overlying the invasive carcinoma, intense nuclear staining is seen, particularly in the basal and parabasal layers (**A**, **B**). In image **B,** the surface squamous epithelium shows an abrupt transition from mild dysplasia (on the *right*) to carcinoma *in situ* (on the *left*). **B.** MGMT protein is present in the dysplastic epithelium but absent in the carcinoma *in situ*. **C**. Invasive tumor cells in the positive case are mostly positive for the MGMT protein. **D.** By comparison, the carcinoma cells in the negative case were mostly negative.

with its protein expression in 93 cases in which both promoter methylation and protein expression data were available. Among 17 cases that showed *MGMT* promoter methylation, MGMT protein expression was absent in 11 (64.7%) but present in 6 (35.3%) cases. Among 76 cases that displayed no evidence of *MGMT* promoter hypermethylation, MGMT protein expression was absent in 8 (10.5%) but present in 68 (89.5%) cases. In 6 cases, tumors showed *MGMT* promoter hypermethylation yet showed high levels of MGMT protein expression. Among them, 3 were moderately positive (++) and 3 were strongly positive (+++). Eight (8) tumors without *MGMT* promoter methylation displayed apparent loss of protein expression. Among these 8 cases, 3 were mostly negative (+/−) and 5 were weakly positive (+). Overall, the *MGMT* promoter hypermethylation is very significantly correlated with the apparent loss of MGMT protein expression ($P < 0.001$).

We first determined whether *MGMT* promoter hypermethylation or protein expression levels in 94 HNSCC patients correlated with various clinical, pathologic, and treatment parameters by χ^2 test. Neither *MGMT* promoter hypermethylation nor apparent loss of MGMT protein expression in HNSCC was significantly correlated with 65 years of age or older, tumor size, nodal status, clinical stage, history of tobacco or alcohol use, chemotherapy, and radiation therapy (Table 1). The *MGMT* promoter hypermethylation and apparent loss of MGMT protein expression were, however, more frequently seen in the larynx (36.4% and 40.9%, respectively) as compared with the lip (0% and 15.8%, respectively) and oral cavity (17.1% and 10%, respectively; $P = 0.03$, Table 1).

The association of *MGMT* promoter hypermethylation and loss of MGMT protein expression with 2-year disease-free survival (tumor recurrence) was analyzed on the entire patient population (94 patients). The 2-year cutoff was used because majority (>90%) of HNSCC recurrences occur within 2 years following initial curative treatment. Both *MGMT* promoter hypermethylation and apparent loss of MGMT protein expression were significantly correlated with decreased 2-year disease-free survival (increased tumor recurrence; $P < 0.01$ and = 0.02, Table 2). The cumulative probability of surviving 2 years without tumor recurrence is 74% and 72%, respectively, in tumors without *MGMT* promoter hypermethylation and with high levels of MGMT protein. By contrast, the cumulative probability of surviving 2 years without tumor recurrence is only 38% and 47%, respectively, in tumors with *MGMT* promoter hypermethylation or apparent loss of MGMT protein expression.

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Table 1. Association of MGMT hypermethylation and loss of MGMT protein expression with various clinicopathologic characteristics in HNSCC

Prognostic factor	Subgroups	MGMT MSP			χ^2 *P* value*	MGMT IHC[†]			χ^2 *P* value*
		No.	+	%		No.	−	%	
All patients		94	17	18.1		93	19	20.4	
Age	<65 y	44	10	22.7	0.27	43	9	20.9	0.91
	≥65 y	50	7	14.0		50	10	20.0	
Tumor site	Lip	19	0	0.0	0.03	19	3	15.8	0.03
	Oral cavity	41	7	17.1		40	4	10.0	
	Pharynx	12	2	16.7		12	3	25.0	
	Larynx	22	8	36.4		22	9	40.9	
T stage[‡]	1	32	2	6.3	0.16	31	3	9.7	0.33
	2	25	5	20.0		25	7	28.0	
	3	17	5	29.4		17	4	23.5	
	4	20	5	25.0		20	5	25.0	
N stage[‡]	0	60	10	16.7	0.64	60	12	20.0	0.90
	≥1	34	7	20.6		33	7	21.2	
Clinical stage[§]	I	26	1	3.8	0.12	26	2	7.7	0.24
	II	16	3	18.8		16	5	31.3	
	III	16	5	31.3		15	4	26.7	
	IV	36	8	22.2		36	8	22.2	
Tobacco[‖]	No	14	2	14.3	0.72	14	3	21.4	0.80
	Yes	49	9	18.4		48	9	18.8	
Alcohol[‖]	No	20	3	15.0	0.81	20	3	15.0	0.66
	Yes	46	8	17.4		46	9	19.6	
Radiation therapy[‖]	No	40	6	15.0	0.58	40	9	22.5	0.78
	Yes	46	9	19.6		45	9	20.0	
Chemotherapy[‖]	No	56	8	14.3	0.62	56	12	21.4	0.82
	Yes	27	5	18.5		26	5	19.2	

Abbreviations: T, tumor; N, lymphnode.
*P values were obtained after correction for multiple comparisons within each prognostic group.
[†]MGMT IHC results are categorized into ''negative group'' when tumor cells are negative, mostly negative or weakly positive (<30%) for the MGMT protein and as ''positive group'' when tumor cells are moderately or strongly positive (>50%) for the MGMT protein (see Materials and Methods for criteria).
[‡]Stage was determined by pathologic examination.
[§]Clinical stage was determined by combining T, N, and M (distant metastasis) stage.
[‖]The total number for tobacco, alcohol use, radiation, or chemotherapy was less than 94 cases because some of these data were not available in patients' file, thus, were excluded for statistical analysis. All HNSCC tissue samples used in this study were selected retrospectively and before the initiation of adjuvant chemoradiation therapies.

Other potential risk factors that showed significant association with decreased 2-year disease-free survival were 65 years of age or younger ($P < 0.01$), larger tumor ($P < 0.01$), presence of nodal metastasis ($P = 0.03$), and more advanced clinical stage ($P < 0.01$).

Tumors arising in the lip were significantly correlated with increased 2-year disease-free survival as compared with tumors from other sites ($P < 0.01$), probably due to early detection by self-inspection and early treatment.

The association of 5-year cause-specific survival (i.e., patients who did not die of their disease) with *MGMT* promoter hypermethylation or loss of MGMT protein expression was examined on the entire patient population (94 patients). Both *MGMT* promoter hypermethylation and apparent loss of MGMT protein expression were significantly correlated with decreased 5-year cause-specific survival ($P < 0.01$ and $= 0.05$, respectively, Table 2 and Fig. 3). The cumulative probability of surviving 5 years following initial surgery is 64% and 58%, respectively, in tumors without *MGMT* promoter hypermethylation and with high levels of MGMT protein. By contrast, the cumulative

Table 2. Association of *MGMT* promoter hypermethylation and apparent loss of MGMT protein expression with patient survival in HNSCC

Prognostic factor	Results	2-year disease-free*	HR (CI)	Logrank *P*	5-year cause-specific*	HR	Logrank *P*	5-year 2overall*	HR	Logrank *P* value[†]
MGMT MSP	−	0.74	2.38	<0.01	0.64	2.33	<0.01	0.44	1.66	<0.01
	+	0.38	(1.14-7.26)		0.16	(1.08-7.36)		0.07	(1.11-5.18)	
MGMT IHC[‡]	+	0.72	1.89	0.02	0.58	1.52	0.05	0.38	1.13	0.18
	−	0.47	(0.8-6.47)		0.36	(0.76-4.75)		0.30	(0.74-1.13)	

Abbreviations: HR, hazard ratio; CI, confidence interval.
*All survival data are expressed as cumulative probability.
[†]Logrank test is a test of equality of survival function across groups.
[‡]MGMT IHC results are categorized into ''negative group'' when tumor cells are negative, mostly negative or weakly positive for the MGMT protein and as ''positive group'' when tumor cells are moderately or strongly positive for the MGMT protein (see Materials and Methods for criteria).

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probability of surviving 5 years is only 16% and 36%, respectively, in tumors demonstrating *MGMT* promoter hypermethylation or apparent loss of MGMT protein expression.

Other risk factors demonstrating significant association with decreased 5-year cause-specific survival included larger tumor ($P < 0.01$), nodal metastasis ($P < 0.01$), and more advanced clinical stage ($P < 0.01$). Patients who were 65 years of age or older and whose tumor arose from the lip experienced increased 5-year cause-specific survival ($P = 0.05$ and <0.01, respectively).

Correlation of overall survival (i.e., patients who did not die) with *MGMT* promoter hypermethylation and loss of MGMT protein expression in the entire patient population (94 patients) was also done. The *MGMT* promoter hypermethylation but not MGMT protein expression levels was correlated significantly with decreased 5-year overall survival ($P < 0.01$ and = 0.18, respectively). The cumulative probability of surviving 5 years following initial surgery is 44% in tumors absent for *MGMT* promoter methylation but only 7% in those with *MGMT* promoter hypermethylation.

Other prognostic factors showing significant correlation with decreased 5-year overall survival included larger tumor ($P < 0.01$), presence of nodal metastasis ($P < 0.01$), and more advanced clinical stage ($P = 0.01$).

Correlation between *MGMT* promoter hypermethylation with tumor recurrence and patient survival was also determined in patients with various clinical stages. It was found that *MGMT* promoter methylation was significantly correlated with 2-year disease-free ($P = 0.04$), 5-year cause-specific ($P = 0.05$), and 5-year overall survival ($P = 0.04$) in patients with stage II-IV tumors but not with stage I tumors ($P > 0.1$). In addition, MGMT protein expression did not correlate significantly with tumor recurrence or patient survival when it was analyzed in subgroup of patients with different clinical stages ($P > 0.1$).

We next did Cox multivariate regression analyses to elucidate the relationship among different risk factors in predicting tumor recurrences or patient survival. Risk factors analyzed included tumor site, tumor size, nodal status, age, history of chemoradiation therapy, *MGMT* promoter hypermethylation, and protein expression levels. It was found that *MGMT* promoter hypermethylation ($P < 0.01$), apparent loss of MGMT protein expression ($P = 0.04$), and larger tumor size ($P < 0.01$) remained to be significant prognostic factors for decreased 2-year disease-free, independent of other risk factors. It was also found that *MGMT* promoter hypermethylation ($P < 0.01$), apparent loss of MGMT protein expression ($P = 0.05$), tumors arising from the lip ($P = 0.02$), and larger tumor ($P < 0.01$) were independent prognostic factors for decreased 5-year cause-specific survival. In addition, *MGMT* promoter hypermethylation ($P = 0.01$), tumors arising from the lip ($P = 0.03$), and larger tumor ($P = 0.01$) were significant prognostic factors for decreased 5-year overall survival, independent of other risk factors. The above analyses indicate that *MGMT* promoter hypermethylation and apparent loss of MGMT protein expression, as well as tumor size, can independently predict tumor recurrence and patient survival in HNSCC.

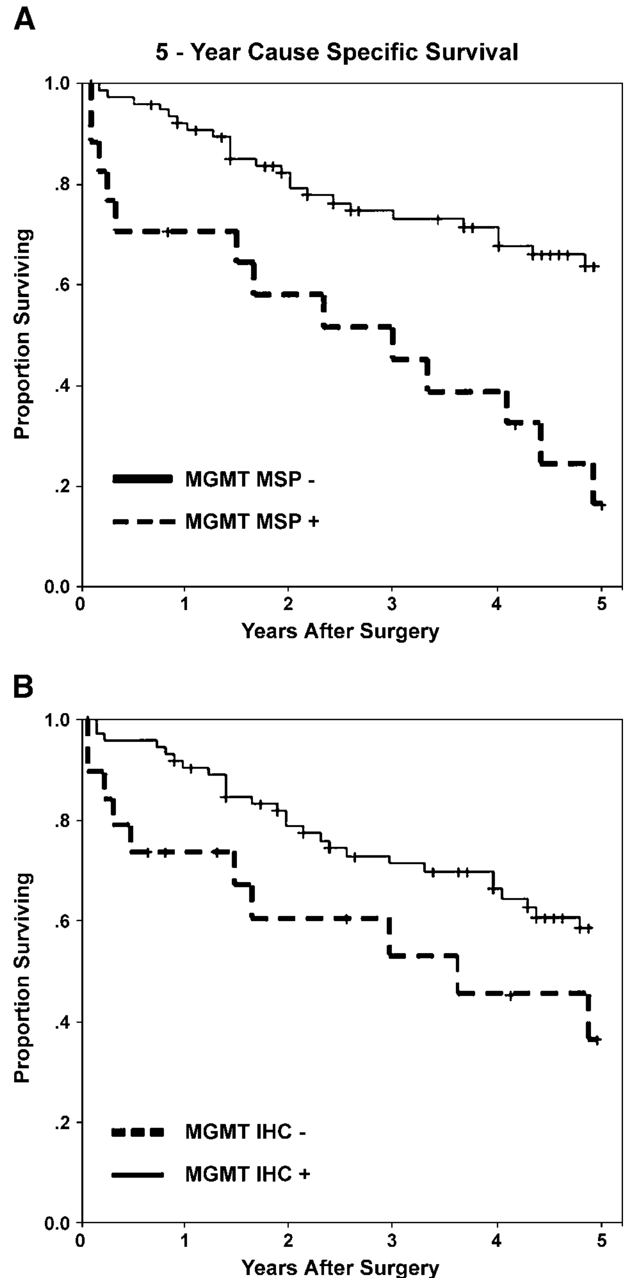

Figure 3. Five-year cause-specific survival among 94 HNSCC patients. Cause-specific survival was calculated with Kaplan-Meier method according to *MGMT* MSP results (**A**: *positive-dotted line*; *negative-solid line*) and IHC staining results (**B**: *positive-solid line*; *negative-dotted line*) in HNSCC patients. *MGMT* promoter hypermethylation or apparent loss of protein expression is significantly correlated with decreased 5-year cause-specific survival, independent of other prognostic factors. The 5-year cause-specific survival rate for HNSCC patients with methylated *MGMT* promoter or apparent loss of protein expression is only 16% and 36%, respectively. By contrast, the cause-specific survival rate for HNSCC patients with unmethylated *MGMT* promoter or high levels of MGMT protein reaches 64% and 58%.

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Discussion

In this study, analyses for *MGMT* promoter hypermethylation and MGMT protein expression were done in 94 consecutive, untreated HNSCC cases and the results obtained were correlated with clinical follow-up data. We found that *MGMT* promoter hypermethylation and apparent loss of MGMT protein expression were present in 17 of 94 (18.1%) and 19 of 93 (20.4%) cases and that both *MGMT* promoter hypermethylation and apparent loss of MGMT protein expression can be used as reliable and independent prognostic predictors for tumor recurrence and patient survival in HNSCC.

The frequency of *MGMT* promoter hypermethylation in this study is 18.1%, similar to those obtained in other two studies (about 20%) on HNSCC (26, 27). One study revealed a particular high frequency of MGMT promoter hypermethylation (41%) involving 99 cases of oral cancer from patients in India (29). This difference may be attributed to different patient populations and/or contributing etiologic factors.

MGMT protein expression pattern has never been analyzed previously in HNSCC. In this study, we characterized MGMT protein expression levels using IHC staining method on 93 cases of HNSCC and found that 19 of 93 cases (20.4%) showed apparent loss of MGMT protein in the nuclei of carcinoma cells. Interestingly, we observed particularly high levels of MGMT protein in the basal and parabasal cells of the normal squamous mucosa, an IHC staining pattern that is identical to that for other two DNA repair enzymes, hOGG1 and hMLH1 (30-32). It is conceivable that the basal and parabasal layers of the squamous mucosa consist of stem cell populations that are in constant proliferative state, and, thus, are more vulnerable to DNA-damaging effects by environmental mutagens or carcinogens. Understandably, this population of cells would be equipped with sufficient amount of functional DNA repair enzymes to allow cells to counteract these mutagenic and carcinogenic effects to cellular DNA.

The presence of *MGMT* promoter hypermethylation is significantly correlated with apparent loss of MGMT protein expression in this study involving 93 cases of primary HNSCC ($P < 0.01$). Similar study also showed that *MGMT* promoter hypermethylation was associated with loss of protein expression in human brain tumors, lymphomas, and colorectal carcinomas (24).

In this study, 11 of 17 (64.7%) cases with *MGMT* promoter hypermethylation showed apparent loss of MGMT protein expression while 68 of 76 (89.5%) cases without *MGMT* promoter hypermethylation showed high levels of MGMT protein expression. Six cases displayed aberrant *MGMT* promoter hypermethylation, yet expressed high levels of MGMT protein. Such lack of correlation between promoter methylation and protein expression may be the result of heterogeneous MGMT protein expression patterns in primary HNSCC. In fact, about 20% of the tumor cells in these six cases did not express the MGMT protein by IHC staining. Thus, this small population of negative cells could very well be the source of methylated DNA. In the future, laser-capture microdissection can be applied to accurately dissect out tumor areas with or without MGMT protein expression for more precise promoter methylation analysis.

In 8 of 76 (10.5%) cases that showed no evidence of *MGMT* promoter hypermethylation, tumor cells showed apparent loss of MGMT protein. The reasons for apparent loss of MGMT protein expression in these 8 cases may result from genetic events, such as inactivating mutations or deletion present in the *MGMT* gene.

The overall significant correlation of the *MGMT* promoter hypermethylation with loss of MGMT protein expression in HNSCC, thus, further supports the conclusion based on studies on other tumor types that epigenetic alterations in association with promoter hypermethylation is primarily the underlying molecular mechanism in causing loss of function of the *MGMT* gene and that genetic factors, such as mutation or gene deletion, are rare, if present at all, in silencing the *MGMT* gene (16).

MGMT plays a significant role in alkylating *N*-nitroso compound–induced carcinogenesis and supportive evidence include the following: (1) MGMT is a specific DNA repair protein that removes mutagenic and carcinogenic adducts, O^6-alkylguanine, in DNA (6-8); (2) Transgenic mice overexpressing MGMT are more efficient in repair for DNA damages and, thus, more resistant to tumor development induced by alkylating carcinogens (33, 34); (3) Mice lacking MGMT (MGMT knockout mice) show deficient DNA repair capacity and, thus, are more susceptible to tumor development by alkylating carcinogens (35, 36).

Because of the apparent clinical relevance of the *MGMT* gene, we attempted to determine whether the *MGMT* promoter hypermethylation and loss of MGMT protein expression would have any significant impact on various clinicopathologic characteristics of HNSCC. Neither *MGMT* promoter hypermethylation nor loss of MGMT protein expression was significantly correlated with tumor size, nodal status, clinical stage, history of tobacco and alcohol use, and chemoradiation therapies (Table 1). However, *MGMT* promoter hypermethylation and loss of MGMT protein expression were significantly correlated with decreased 2-year disease-free and 5-year cause-specific survival. *MGMT* promoter hypermethylation was also significantly correlated with decreased 5-year overall patient survival. These prognostic predictive values were independent of other potential risk factors, such as tumor site, tumor size, nodal status, age, and chemoradiation therapy following Cox multivariate regression analysis.

Several studies have also established that *MGMT* promoter hypermethylation and/or loss of *MGMT* gene expression are predictive of poor survival in patients with hepatocellular (37), gastric (37, 38), breast (37, 39), and lung (40) cancer as well as low-grade diffuse astrocytomas (41). Thus, it seems that *MGMT* promoter hypermethylation or loss of *MGMT* gene expression may represent an important biomarker for biologically aggressive diseases in many human tumor types.

Even though both *MGMT* promoter methylation and apparent loss of MGMT protein expression correlated with increased tumor recurrence (decreased disease-free survival) and worse patient outcome, *MGMT* promoter hypermethylation appeared to be a much stronger predictor as reflected by consistently higher hazard ratios and more statistically significant (see Table 2). The ''weak'' predictive value of MGMT protein expression

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may be because MGMT protein expression was examined on formalin-fixed tissue sections by IHC staining and interpreted semiquantitatively. In the future, studies using fresh HNSCC samples and quantitative real-time reverse transcription-PCR for *MGMT* gene expression should gain more accurate correlation between *MGMT* gene expression and the patient outcome. On the other hand, *MGMT* promoter methylation results are judged objectively and qualitatively by the presence or absence of a PCR band (Fig. 1). Thus, *MGMT* promoter methylation may represent a more reliable and accurate predictive marker for patient survival in HNSCC.

Of particular significance is the clinical relevance of MGMT in the treatment of cancer (42). It has been shown that MGMT enzyme activity correlates inversely with sensitivity of tumor cells to the killing effects of alkylating agents that form O^6-alkylguanine DNA adducts, such as carmustine (BCNU), temozolomide, streptozotocin, and decarbazine (42) and that the full efficacy of these alkylating agents in cell killing depends on a functional DNA mismatch repair system, such as hMLH1 (42, 43). It has been shown that *MGMT* promoter hypermethylation or decreased *MGMT* gene expression improves survival in patients with malignant astrocytomas (44), glioma (45), and diffuse large B-cell lymphoma (23) who were treated with alkylating chemotherapeutic agents, such as carmustine (BCNU) or temozolomide.

Twenty-seven patients had adjuvant chemotherapy (5-fluorouracil and/or cisplatin) and 56 did not receive any forms of chemotherapy (Table 1). Forty-six patients received radiation treatment, whereas 40 did not have this form of treatment (Table 1). Neither *MGMT* promoter hypermethylation nor protein expression correlated with history of chemoradiation therapies (see Results). Because none of the 27 patients with adjuvant chemotherapy in this study received alkylating antitumor agents, it remained to be determined whether *MGMT* gene promoter methylation and/or protein expression would affect the responsiveness of HNSCC to alkylating agents and, thus, overall patient survival.

Our current study may provide guideline or basis for future novel alkylating agent–based chemotherapeutic regimen in the treatment of patients with HNSCC, in particular those with loss of MGMT protein associated with promoter hypermethylation in combination with a normally expressed *hMLH1* gene. In fact, these 94 cases of HNSCC have also been subjected to analyses of *hMLH1* promoter methylation and protein expression patterns.[5] Ten of these 94 cases showed loss of MGMT protein associated with promoter hypermethylation, yet expressed high levels of hMLH1 protein. Among these 10 patients, 8 eventually died with an average survival of 18.4 months. These 8 patients may have benefited from a novel combined chemotherapeutic regimen with the addition of an alkylating agent, such as carmustine (BCNU) or cyclophosphamide.

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[5] Separate manuscript in submission.

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