Association Between Hypermethylated Tumor and Paired Surgical Margins in Head and Neck Squamous Cell Carcinomas

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Abstract **Purpose:** Surgical margin status is reported to be a relevant prognostic factor in head and neck squamous cell carcinoma (HNSCC), associated with a high risk of local recurrence. This study examines whether gene-promoter hypermethylation could be detected in HNSCC surgical margins with no histologic evidence of malignancy, and if so, whether it reflects epigenetic events of primary tumors.

Experimental Design: Promoter methylation status of *MGMT, p16*, and *DAP-K* genes was evaluated by methylation-specific PCR in 20 primary HNSCC tumors. Histopathologically negative surgical margins of hypermethylated tumors were collected, and their methylation status compared with the primary tumor status.

Results: Promoter hypermethylation in at least one of the three tested genes was detected in 65% (13 of 20) of tumors. *MGMT* was hypermethylated in 50% (10 of 20), *DAP-K* in 45% (9 of 20), and *p16* in 20% (4 of 20) of tumors. Methylation status was analyzed in 35 margins from 11 of 13 patients showing promoter hypermethylation in the tumor tissue. Identical methylation events were seen for at least one gene in primary tumor and surgical margins in 9 of 11 cases (82%). Association was found for gene-specific hypermethylation status in tumors and paired surgical margins, and gene-specific concordance was 63% for *MGMT* (κ = 0.24), 90% for *DAP-K* (κ = 0.74), and 90% for *p16* (κ = 0.79).

Conclusions: Our results support the hypothesis that detection of gene promoter hypermethylation in HNSCC tumor cells – free surgical margins may be a helpful biomarker to identify molecularly altered fields in areas adjacent to the tumor.

Despite improvements in the management of head and neck squamous cell carcinoma (HNSCC), a significant percentage of advanced HNSCC patients still have a poor prognosis, with a high percentage (10-30%) of locoregional and distant recurrences (1). The clinicopathologic variables currently used in predicting prognosis of HNSCC, including performance status, tumor-node-metastasis staging, and pathologic grading of differentiation, are often not sufficient to predict the clinical outcome. Resection margins status, usually assessed by histologic analysis, has been reported relevant for the prognosis of HNSCC patients. Specifically, histopathologically positive surgical margins have been suggested as having prognostic value for both locoregional recurrence and distant metastasis (2, 3). However, local recurrences have also been observed in HNSCC patients with histopathologically negative surgical margins, supporting the hypothesis introduced by Slaughter et al. in 1953 (4) that a residual "alterated field" in the area adjacent to the tumor could be the leading cause of treatment failure and local recurrence.

In the last decade, molecular investigation (i.e., p53 mutation, EIF4E expression, and microsatellite analyses) into tumor-specific alterations of apparently tumor-free tissues has been shown to have a potential role in improving prediction of local tumor recurrence or reduced survival in HNSCC patients (5 – 9). Recently, the silencing of cancer-related genes via epigenetic alterations, such as aberrant promoter hypermethylation, has been shown to play an important role in tumor progression and to be an early event in the carcinogenesis process (10). In HNSCC tumors, promoter hypermethylation is thought to be involved in recurrence, second primary tumor occurrence, and patient survival (11, 12). Studies on different cancer sites (13 – 15) have confirmed the relevance of epigenetic alterations in cancer-related genes for prognosis and patient survival. At present, aberrant epigenetic events in HNSCCs have mainly been studied in tumor tissue samples, focusing on genes involved in tumor suppression (*p16*/CDKN2A), apoptosis (*DAP-K*), DNA mismatch repair (hMLH1), DNA repair (*MGMT*), and tumor invasiveness (E-cadherin; refs. 16 – 18).

This study aims at determining whether gene-promoter hypermethylation of three cancer-related genes can be detected in HNSCC surgical margins with no histologic evidence of

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malignancy, and if so, whether it reflects epigenetic events of primary tumors.

Cases were selected for this retrospective analysis that had recurrence and short survival to evaluate whether hypermethylated gene detection in HNSCC surgical margins, diagnosed as tumor-free by conventional histopathologic analyses, might be a helpful biomarker to identify epigenetically altered fields in the area adjacent to the tumor.

Materials and Methods

Study population. Formalin-fixed and paraffin-embedded tumor tissues were obtained from 20 retrospectively identified patients with HNSCC who were diagnosed and surgically treated at the Otorhinolaryngology Clinic of the University of Turin between 1992 and 2001. Patients were selected by availability of surgical paraffin-embedded tumor tissue specimens and histologically tumor-free resection margins recurring between 1993 and 2003, cause-specific death due to HNSCC, and complete follow-up. Information on death was obtained from the Piedmont cancer register and from the hospital and outpatient oncology clinical records. Follow-up of patients was computed from time of surgery to date of death. Disease-free survival was defined as the time between diagnosis and recurrence. Primary tumors from oral cavity, larynx, oropharynx, and pharynx were reviewed, and the histologic diagnosis confirmed. Grading and pathologic staging of the tumors followed the guidelines of the American Joint Committee on Cancer (19). Patient characteristics are shown in Table 1. Primary tumors with distant metastases and patients who had received radiotherapy or chemotherapy before surgery were not included in the study. Surgical margin samples comprised mucosa with a clinically normal appearance, taken at surgery at least 2 cm from the tumor edge after resection. All negative margins were reported "tumor-free" by the pathologist after routine histologic examination.

DNA extraction. Genomic DNA was extracted from three to five (10-μm thick) sequential paraffin sections of primary tumor tissues and adjacent surgical margins and checked for adequacy by PCR amplification of the β-globin gene as previously described (20).

Bisulfite modification. Bisulfite modification was done on DNA samples based on the principle that sodium bisulfite treatment converts unmethylated cytosines to uracil, leaving methylated cytosines unchanged. Methylated and unmethylated DNA sequences thus become distinguishable after bisulfite conversion by sequence-specific primers. Genomic DNA samples underwent bisulfite modification using CpGenome DNA modification kit (Intergen Co.) following the manufacturer's recommendations. Two microliters of a carrier DNA included in the kit (DNA Modification Reagent IV) were added to all samples.

Positive controls for methylated (CpGenome universal methylated DNA, Intergen Co.) and unmethylated status (normal human lymphocyte DNA) were included in each modification set (21). The bisulfite-modified genomic DNA was resuspended in 35 μL of 1× TE buffer and used immediately for methylation-specific PCR or stored at -80°C.

Methylation-specific PCR. Bisulfite-modified DNA was used as a template for PCR amplification using primers specific for either the methylated or the modified unmethylated DNA. The sets of specific primers and their annealing temperatures for methylated and unmethylated forms of *MGMT*, *p16*, and *DAP-K* gene promoters were selected from published sequences (16, 22) and are listed in Table 2. For PCR amplification, 4 μL of bisulfite-modified DNA were added in a final volume of 25 μL PCR mix containing 1× PCR buffer [15 mmol/L Tris (pH 8.0), 50 mmol/L KCl, 6.7 mmol/L $MgCl_2$], deoxynucleotide triphosphates (2 mmol/L each), primers (0.4 μmol/L each per reaction), and 1.25 units of AmpliTaq Gold DNA polymerase (Applera). All PCR amplifications were done in a Gene Amp PCR System 9700 Thermal Cycler (Applera). Bisulfite-modified CpGenome universal methylated DNA (Intergen Co.) was used as positive control for methylated alleles, and bisulfite-modified DNA from normal human lymphocytes was used as a positive control for unmethylated alleles. Negative PCR controls without DNA were included in each PCR run. Ten microliters of each PCR amplification were loaded onto 2% agarose gel stained with ethidium bromide and visualized by UV transillumination. Previous studies have shown this method to have high sensitivity, detecting one methylated nucleotide in 1,000 unmethylated nucleotides (10).

Statistical analyses. The Fisher exact test was used to compare methylation status of genes in tumors and paired surgical margins. Cohen's κ test was used for gene-specific concordance evaluation.

Results

Methylation status in primary HNSCC tumors. The study population included 14 men (70%) and 6 women (30%) with a mean age of 60.95 years (range, 27-89 years). Eight tumors were of the oral cavity, six of the hypopharynx, three of the oropharynx, and three of the larynx. The mean follow-up was 26.9 months (range, 6-83 months). Mean interval from diagnosis to recurrence was 18 months.

Aberrant promoter hypermethylation was found in 65% (13 of 20) of the tumor samples for at least one of the three

Table 1. Baseline characteristics of HNSCC patients

Characteristic	No. cases (%)
Total patients	20 (100)
Age (y)	
Mean	60.9
Range	27-89
Sex	
Male	14 (70)
Female	6 (30)
Primary tumor site	
Oral cavity	8 (40)
Oropharynx	3 (15)
Hypopharynx	6 (30)
Larynx	3 (15)
T classification	
T1	1 (0.5)
T2	10 (50)
T3	5 (25)
T4	4 (20)
TNM stage*	
Stage I	0
Stage II	4 (20)
Stage III	6 (30)
Stage IV	10 (50)
N classification	
N0	6 (30)
N+	12 (60)
Nx	2 (10)
Histologic grading †	
G1	2 (10)
G2	14 (70)
G3	4 (20)

Abbreviation: TNM, tumor-node-metastasis.
*pTNM stage was determined by combining tumor and node stages (tumors with distant metastases were not included).
† Histologic grading following AJCC (19).

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Table 2. Primer sequences for methylation-specific PCR

Gene	Primer sense (5′→3′)	Primer antisense (5′→3′)	Product size (bp)	AT* (°C)
MGMT M †	TTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	80	59
MGMT U †	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	94	59
p16 M †	TTATTAGAGGGTGGGGCGGATCGC	ACCCCGAACCGCGACCGTAA	150	60
p16 U †	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60
DAP-K M †	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	114	59
DAP-K U †	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	116	59
β-*Globin*	TGGGTTTCTGATAGGCACTGACT	AACAGCATCAGGAGTGGACAGAT	152	50

*AT, annealing temperature.
† M, methylated sequence; U, unmethylated sequence.

genes tested. Specifically, *MGMT* showed promoter hypermethylation in 50% (10 of 20), *DAP-K* in 45% (9 of 20), and *p16* in 20% (4 of 20) of the tumors, respectively (Fig. 1). Thirty percent (6 of 20) of the tumors had only one hypermethylated gene, 20% (4 of 20) had two hypermethylated genes, and 15% (3 of 20) had all the three genes hypermethylated.

Methylation status in tumor's paired surgical margins. Each tumor had multiple paraffin-embedded surgical resection margins. A total of 35 paraffin-embedded tumor tissue margins were collected. DNA from paraffin-embedded tumor tissue margins was valuable for 11 of 13 cases, which showed aberrant promoter hypermethylation in the primary tumor; as for the two cases, the DNA quality in paired margins was not adequate for methylation-specific PCR analysis. Considering each tumor sample with the corresponding surgical margins from the same patient, we found that 9 of 11 patients (82%) had at least one surgical margin with the same hypermethylated gene/s previously found in the primary tumor. Among these nine patients, five cases (56%) had at least one identical hypermethylated gene, two cases (22%) had two hypermethylated genes, and two cases (22%) had three hypermethylated genes. The gene-specific frequency of aberrant promoter hypermethylation, both in tumor specimens and in paired margins, was 45% (5 of 11) for *MGMT*, 72% (8 of 11) for *DAP-K*, and 23% (3 of 11) for *p16* (Table 3; Fig. 2). Association was found for gene-specific methylation status in tumors and paired surgical margins (*p16*, $P = 0.02$; *DAP-K*, $P = 0.05$; *MGMT*, $P = 0.54$). Based on the hypothesis of independence of methylation in the three genes, the gene-specific concordance for hypermethylation in the tumor tissue and paired surgical margins (T+M+ plus T-M- versus T+M- plus T-M+) was 7 of 11 (63%) cases for *MGMT* ($\kappa = 0.24$; 95% confidence interval, 0.29 to 0.78), 10 of 11 (90%) cases for *DAP-K* ($\kappa = 0.74$; 95% confidence interval, 0.28-1.00), and 10 of 11 (90%) cases for *p16* ($\kappa = 0.79$; 95% confidence interval, 0.41-1.00; Table 4). In two cases, hypermethylated genes were observed in the surgical margins but not in the corresponding tumor sample.

Correlation between aberrant methylation, patient characteristics, and prognosis. The hypermethylation status of tumors and paired surgical margins did not correlate with any clinicopathologic variables of the patients, such as sex, age, tumor stage, or tumor site (data not shown). Mean disease-free survival was 22 months for patients with one hypermethylated gene, 14 months for those with two hypermethylated genes, and 13 months for those with three hypermethylated genes. However, the correlation between time to relapse and hypermethylation status of any gene or of their combination in tumor specimens and in paired surgical margins was not statistically significant.

Discussion

Head and neck cancers are aggressive tumors with poor prognosis, and the surgical margin status is reported to be a relevant prognostic factor associated with high risk of local recurrence and patient survival (2, 5, 9, 23, 24). Recent studies on lung cancer (25), prostate cancer (26), colorectal cancer (27), and oral squamous cell carcinomas (28, 29) have shown evidence of molecular epigenetic alterations in nonneoplastic cells isolated from regions surrounding the tumor site, supporting the hypothesis that field cancerization occurs in

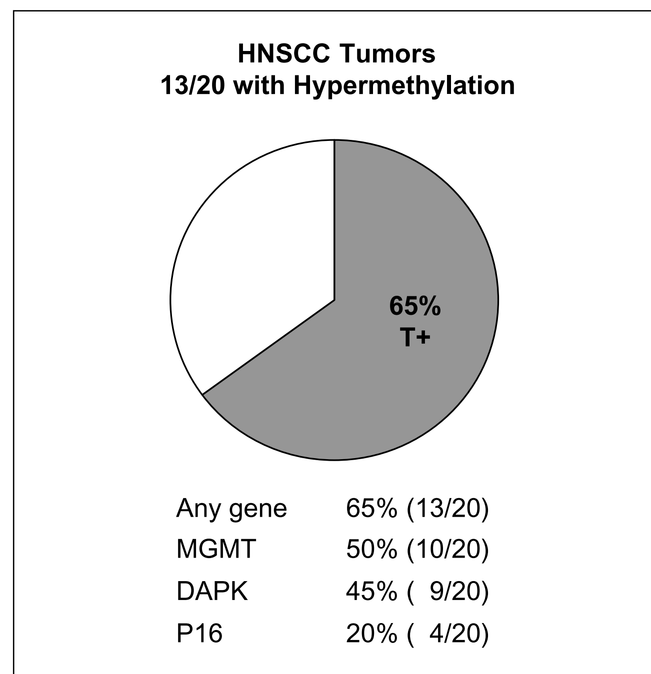

Fig. 1. Gene promoter hypermethylation of 20 primary tumors from HNSCC patients. Percentage of patients with epigenetic alteration in *MGMT, DAP-K*, and *p16* genes and relative frequency of gene hypermethylation events.

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these regions. The present study examined whether histopathologically negative surgical resection margins in HNSCC may hide cells carrying epigenetic alterations in crucial cell regulation genes likely responsible for recurrence and reduced survival. In the 20 HNSCC tumors analyzed, we found a frequency of promoter hypermethylation in the investigated genes that was consistent with data previously reported on head and neck cancers [i.e., 18-37% for *MGMT* (11, 12, 16, 29, 30); 20-65% for *p16* (12, 31–34); and 18-68% for *DAP-K* (12, 29–31)]. The noteworthy finding of our study was the high

Table 3. Promoter hypermethylation status in HNSCC patients

Case no.	Primary tumor site	TNM stage	Histologic grading	Tissue sample	Hypermethylation status	Gene		
						MGMT	*DAP-K*	*p16*
1	Oral Cavity	IV	G3	T	Y	U	M	ne
				SM1	N	U	U	U
				SM2	Y	U	M	ne
2	Oropharynx	IV	G2	T	Y	M	U	U
3	Hypopharynx	II	G2	T	Y	M	M	M
4	Oropharynx	II	G2	T	Y	M	M	M
				SM1	Y	U	U	M
				SM2	Y	M	M	M
5	Oral Cavity	III	G1	T	N	ne	U	ne
6	Hypopharynx	IV	G2	T	Y	M	M	M
				SM1	Y	M	M	U
				SM2	Y	M	M	M
				SM3	Y	M	M	M
7	Larynx	IV	G3	T	N	U	ne	ne
8	Oral Cavity	III	G2	T	N	U	U	ne
9	Larynx	IV	G3	T	Y	M	U	ne
				SM1	N	U	U	U
				SM2	N	U	U	U
				SM3	N	U	U	U
				SM4	N	U	U	U
				SM5	N	U	U	U
10	Hypopharynx	III	G2	T	Y	M	M	ne
				SM1	Y	U	M	ne
				SM2	N	U	U	U
				SM3	N	U	U	ne
				SM4	Y	M	U	ne
11	Larynx	IV	G2	T	N	U	U	U
12	Oral Cavity	III	G2	T	Y	U	M	M
				SM1	Y	U	U	M
				SM2	Y	M	M	U
				SM3	Y	M	U	M
				SM4	N	U	U	U
				SM5	Y	U	M	M
13	Oral Cavity	II	G3	T	Y	M	U	U
				SM1	Y	U	M	M
				SM2	Y	U	U	M
				SM3	Y	M	U	M
				SM4	Y	U	M	M
14	Oral Cavity	IV	G1	T	N	U	ne	ne
15	Oral Cavity	III	G2	T	N	U	U	U
16	Hypopharynx	II	G2	T	Y	M	M	U
				SM1	N	ne	U	U
				SM2	Y	ne	M	ne
17	Hypopharynx	IV	G2	T	Y	M	U	U
				SM1	N	U	U	U
				SM2	N	U	U	U
18	Hypopharynx	IV	G2	T	N	U	ne	U
19	Oral Cavity	IV	G2	T	Y	U	M	U
				SM1	N	U	U	U
				SM2	Y	U	M	U
				SM3	Y	U	M	U
20	Oropharynx	III	G2	T	Y	M	M	U
				SM1	N	U	U	ne
				SM2	Y	U	M	U
				SM3	Y	M	M	U

Abbreviations: T, tumor; SM, surgical margin; U, unmethylated; M, methylated; ne, not evaluable; Y, yes; N, no.

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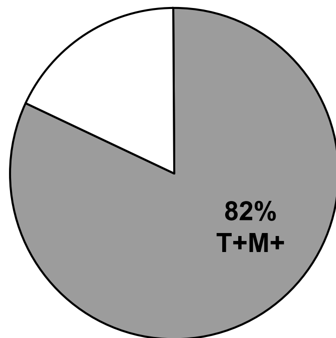

Fig. 2. Gene promoter hypermethylation status of surgical margins from 11 of 13 cases with tumor hypermethylated genes. Percentage of patients with at least one identical hypermethylation event in *MGMT, DAP-K*, and *p16* genes and relative frequency of gene hypermethylation events in tumor tissue and surgical margins.

Table 4. Gene-specific concordance between hypermethylated genes in tumor and paired surgical margins

	T+M+	*T-M+*	*T+M-*	*T-M-*	κ*	*95% CI*
MGMT	5 (45%)	1 (9%)	3 (27%)	2 (18%)	0.24	-0.29 to 0.78
DAP-K	8 (72%)	1 (9%)	0	2 (18%)	0.74	0.28 to 1.00
p16	3 (27%)	1 (9%)	0	7 (63%)	0.79	0.41 to 1.00

Abbreviations: T+, hypermethylated tumors; T-, unmethylated tumors; M+, hypermethylated margins; M-, unmethylated margins; 95% CI, 95% confidence interval.
*Cohen's κ.

proportion of HNSCC cases (82%) showing identical promoter hypermethylation in at least one of the three genes investigated, both in tumor specimens and in paired histopathologically negative surgical margins. Epigenetic changes of *p16, DAP-K*, and *MGMT* genes have been reported to occur at early stages of HNSCC and in the surrounding normal mucosa, exposed to direct damage by etiologic factors, such as smoke, alcohol, denture, dental caries (28–30, 35). However, this is the first time, to our knowledge, that epigenetic altered cells reflecting gene hypermethylation status of the primary tumor have been detected in surgical resection margins diagnosed as tumor-free by conventional histologic analyses. This finding supports the hypothesis of minimal residual disease in the mucosa in close proximity to the tumor not detectable by histopatologic analysis. Alternatively, the epigenetic events may occur early in carcinogenesis as a result of carcinogen exposure (18, 36), leading to a heterogeneous preneoplastic field (polyclonality of tumor cells; refs. 4, 37–39). The latter hypothesis could explain our finding of hypermethylated genes in some surgical margins but not in the corresponding tumor sample. Based on either of these two hypotheses, we may speculate that the detection of early hypermethylation events in HNSCC surgical margins, diagnosed as tumor-free by conventional histopathologic analyses, may become a helpful biomarker to identify subjects at risk of new neoplastic evolution as a consequence of the accumulation over the time of new genetic or epigenetic changes.

Concerning the association with recurrences, unfortunately our study failed to detect significant correlations between the hypermethylation status of any individual gene or of their combination, in tumors and paired surgical margins, and time to relapse. This may be due to the small number of cases, and the absence of controls with longer disease-free survival may have been a limiting factor.

In summary, our results show that histopathologically negative surgical margins of resected HNSCC show a high frequency of gene promoter hypermethylation, reflecting the gene hypermethylation status of the corresponding tumor. Although it is premature to conclude that epigenetic changes in surgical margins may have prognostic significance, we believe that the findings of this preliminary report show a trend justifying a more extensive study to determine whether postoperative gene methylation analysis of surgical resection margins would be an informative tool to identify and monitor subsets of HNSCC patients at increased risk of local recurrence and reduced survival. Furthermore, because epigenetic drugs directed against epigenetic events are likely to become important weapons against cancer (40, 41), information about methylation status of surgical margins may help clinicians to select more specific therapies.

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