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Advances in Brief

Gene Promoter Hypermethylation in Tumors and Serum of Head and Neck Cancer Patients[1]

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Abstract

Promoter hypermethylation is an important pathway for repression of gene transcription in cancer cells. We analyzed aberrant DNA methylation at four genes in primary tumors from 95 head and neck cancer patients and then used the presence of this methylation as a marker for cancer cell detection in serum DNA. These four genes were tested by methylation-specific PCR and included: *p16 (CDKN2A)*, O^6-methylguanine-DNA-methyltransferase, glutathione S-transferase P1, and death-associated protein kinase (DAP-kinase). Fifty-five % (52 of 95) of the primary tumors displayed promoter hypermethylation in at least one of the genes studied: 27% (26/95) at *p16*, 33% (31 of 95) at O^6-methylguanine-DNA-methyltransferase; and 18% (17 of 92) at *DAP-kinase*. No promoter hypermethylation was observed at the *glutathione S-transferase P1* gene promoter. We detected a statistically significant correlation between the presence of *DAP-kinase* gene promoter hypermethylation and lymph node involvement ($P = 0.014$) and advanced disease stage ($P = 0.016$). In 50 patients with paired serum available for epigenetic analysis, the same methylation pattern was detected in the corresponding serum DNA of 21 (42%) cases. Among the patients with methylated serum DNA, 5 developed distant metastasis compared with the occurrence of metastasis in only 1 patient negative for serum promoter hypermethylation ($P = 0.056$). Promoter hypermethylation of key genes in critical pathways is common in head and neck cancer and represents a promising serum marker for monitoring affected patients.

Introduction

HNSCC[4] cancer remains a morbid and often fatal disease. The overall survival has not changed in recent years, despite extensive research on the biological and molecular features of HNSCC. Among the more pressing problems in clinical management are the lack of early detection and the high incidence of local-regional recurrence, even with aggressive surgical therapy (1). Therefore, it is important to develop new molecular targets to be used as diagnostic and prognostic indicators.

An important mechanism for gene transcriptional inactivation is hypermethylation at the CpG islands within the promoter regions (2). Some tumor suppressor genes such as *p16, VHL,* and *MLH1* have been found to harbor promoter hypermethylation associated with loss of protein expression in cancer cells (3–5). Several tumor types have also shown aberrant methylation at CpG islands in other genes, including the detoxifying gene *GSTP1* (6), the DNA repair gene *MGMT* (7), and the potential metastasis inhibitor gene *DAP-kinase* first occurrence. (8). The presence of epigenetic methylation might also be useful as a molecular target for tumor cell detection.

The presence of abnormally high DNA concentrations in the serum of patients with neoplasms of various types was described decades ago (9). Recent publications have demonstrated the tumor origin of this DNA in cancer patients by confirming the presence of tumor-specific molecular abnormalities in the serum. K-*ras* or *p53* mutations have been detected in the serum of colorectal (10, 11), pancreas (12), and breast (13) cancer cases and N-*ras* mutations in some hematological diseases (14). Other DNA abnormalities, such as loss of heterozygosity) and MI, have also been reported in the serum of head and neck (15), lung (16, 17), renal (18), and breast (13) cancer patients. Moreover, recent studies have demonstrated the presence of gene promoter hypermethylation in the serum DNA of lung (8), liver (19), and breast (13) cancer patients. However, the clinical significance of these observations is still not understood.

In the present study, we have analyzed the promoter hypermethylation pattern of the *p16*, *MGMT*, *GSTP1,* and *DAP-kinase* genes in the tumor DNA of 95 head and neck primary tumors. The methylation patterns found in the tumors were used as molecular markers for cancer cell detection in the paired serum DNA. Almost half of the HNSCC patients with methylated tumors were found to display these epigenetic changes in the paired serum.

Materials and Methods

Sample Collection and DNA Extraction. Ninety-five primary tumors where collected from patients diagnosed with HNSCC between the years 1993 and 1999. Patients were treated at the Johns Hopkins University School of Medicine or at the Wayne State University. Fresh tumors were obtained from surgical resection of the HNSCC patients. Serum samples were collected from the same patients at diagnosis and stored at −80°C. Tumor and serum DNA was prepared as described previously (15). A single follow-up serum was collected at 6 to 72 months after treatment in nine patients from Johns Hopkins University School of Medicine.

Bisulfite Treatment. DNA from tumor and serum specimens was subjected to bisulfite treatment, as described previously (20). Briefly, 1 μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using the Wizard purification resin (Promega Corp.), again treated with NaOH, precipitated with ethanol, and resuspended in water.

MSP. The modified DNA was used as a template for MSP. DNA methylation patterns in gene CpG islands were determined by chemical modification of unmethylated cytosines to uracil and subsequent PCR using primers specific for either methylated or the modified unmethylated sequences (20). Appropriate negative and positive controls were included in each PCR reaction. Primer sequences for the *p16* (20), *MGMT* (7), *DAP-kinase* (8), and

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[4] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; MGMT, O^6-methylguanine-DNA-methyltransferase; GST, glutathione *S*-transferase; DAP-kinase, death-associated protein kinase; MI, microsatellite instability; MSP, methylation-specific PCR.

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GSTP1 (21) genes were as described previously: for the *p16* gene, unmethylated reaction: 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ (sense), 5′-CAACCCCAAACCACAACCATAA-3′ (antisense); methylated reaction: 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (sense), 5′-GACCCCGAACCGCGACCGTAA-3′ (antisense); for the *MGMT* gene, unmethylated reaction: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (sense), 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (antisense); methylated reaction: 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ (sense), 5′-GCACTCTTCCGAAAACGAAACG-3′ (antisense); for the *DAP-kinase* gene, unmethylated reaction: 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′ (sense), 5′- CAATCCCTCCCAAACACCAA-3′ (antisense); methylated reaction: 5′-GGATAGTCGGATCGAGTTAACGTC-3′ (sense), 5′-CCCTCCCAAACGCCG-3′ (antisense); for the *GSTP1* gene, unmethylated reaction: 5′-GATGTTTGGGGTGTAGTGGTTGTT-3′ (sense), 5′-CCACCCCAATACTAAATCACAACA-3′ (antisense); methylated reaction: 5′-TTCGGGGTGTAGCGCTCGTC-3′ (sense), 5′-GCCCCAATACTAAATCACGACG-3′ (antisense).

Each PCR product (20 μl) was directly loaded onto 6% nondenaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. Previous studies have demonstrated that this method has a sensitivity of 1:1000 (*i.e.,* can detect one methylated genome in 1000 unmethylated genomes; Ref. 20).

Results

Gene Promoter Hypermethylation Profiles in Primary Head and Neck Tumors. Aberrant methylation at any of the genes studied was detected in 52 of 95 (55%) primary head and neck tumors. Representative results are shown in Fig. 1*A*. The *p16* tumor suppressor gene demonstrated promoter hypermethylation in 26 of 95 (27%), the *MGMT* gene in 31 of 95 (33%), and the *DAP-kinase* gene in 17 of 92 (18%) tumors (Fig. 1*B*). No methylation changes were found at the *GSTP1* gene in 41 tumors analyzed. Nineteen of the 52 (36%) methylation-positive tumors showed epigenetic changes at more than one of the genes tested, and three of them (3%) were positive for all of the three markers simultaneously. However, each of these hypermethylated loci acted as independent events, and the concordance of all of the genes in these three tumors was at the predicted level for a nonassociated event.

We found no association between overall aberrant hypermethylation and the size of the tumor, the presence of lymph node metastasis, or the stage of the disease. Gene promoter hypermethylation at the *MGMT* and *DAP-kinase* gene promoters was more frequent in the tumors located in the oropharynx compared with other sites ($P = 0.018$ and $P = 0.02$; Fisher's exact test, respectively). Interestingly, individual analysis of the different genes revealed that promoter hypermethylation at *DAP-kinase* clustered in the patients with advanced disease (stages III-IV *versus* I-II; $P = 0.016$, Fisher's exact test) and in patients with lymph node metastases. Fourteen of the 15 patients (93%) with *DAP-kinase* aberrant methylation at the tumor DNA had lymph node involvement at the time of diagnosis compared with 37 of 64 (58%) patients with node involvement in the group negative for *DAP-kinase* promoter hypermethylation ($P = 0.014$; Fisher's exact test; Table 1).

Epigenetic Alterations in Serum DNA from HNSCC Patients. We tested for promoter hypermethylation in the serum DNA of 52 of the patients with a known alteration in the primary tumor. We found that 21 of 50 (42%) patients had the same methylation changes in the serum DNA (in 2 cases, MSP was unsuccessful). Representative MSP analysis for *p16, DAP-kinase,* and *MGMT* gene promoter hypermethylation from tumor and paired sera are shown in Fig. 1*A*. The frequency of aberrant serum methylation for each marker was 31% (8 of 26) for *p16,* 48% (14 of 29) for *MGMT,* and 18% (3 of 17) for *DAP-kinase* (Fig. 1*B*). In those patients positive for more than one marker in the primary tumor, the promoter hypermethylation patterns were identical in the serum DNA. However, in two cases with primary tumor hypermethylation in the *MGMT* and *DAP-kinase* genes, methylation was identified only at *MGMT* in the serum DNA. As a control, we screened for abnormal methylation in the serum DNA of 25 patients at those markers for which the corresponding tumor DNA tested negative for the assay. No changes on the methylation patterns were found in the serum DNA of this group control. We then correlated the clinical data of the patients with the molecular results. The presence of aberrant methylation in serum DNA was not associated with stage, tumor size, node involvement, or tumor location (Table 1). Interestingly, aberrant methylation in serum DNA was detected in five patients with distant metastasis, whereas only one patient without detectable methylation changes in serum DNA developed distant metastasis ($P = 0.056$; Fisher's exact test).

Finally, in seven of the patients showing promoter hypermethylation at the tumor DNA, a second serum specimen extracted several months after surgery (between 6 and 72 months) was available for methylation analysis. None of the four patients negative for gene promoter hypermethylation at the serum DNA showed any aberrant methylation in the DNA from the second serum specimen (collected between at 7 and 72 months). All of them were clinically free of disease. One of the patients positive for *DAP-kinase* promoter hypermethylation in the tumor and serum DNA was negative in the serum specimen collected 29 months after surgery, when he did not have any evidence of disease. The remaining two patients were positive for aberrant methylation in the serum collected months after surgery (5–6 months). However, the follow-up of these two patients was too short to make definitive conclusions.

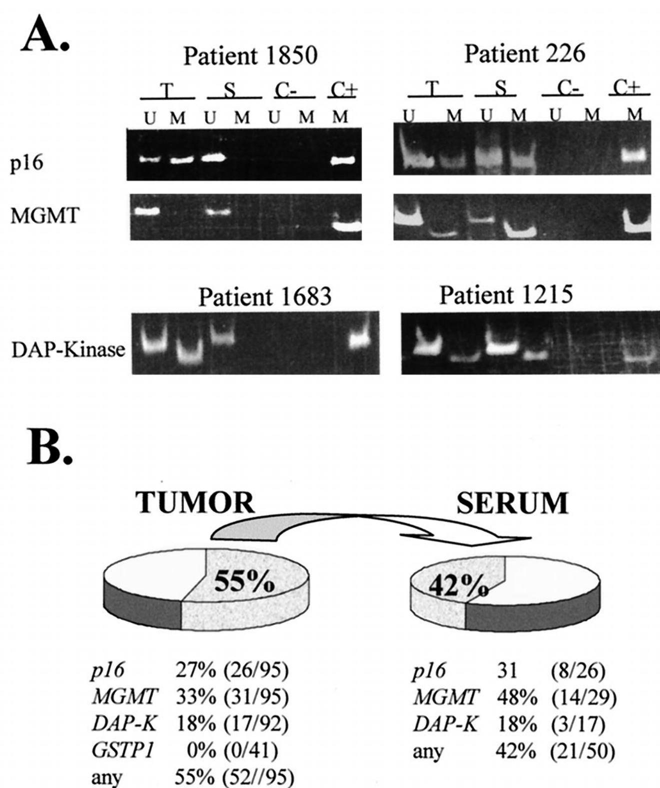

Fig. 1. Gene promoter hypermethylation in primary tumors and serum from HNSCC patients. *A,* representative examples of MSP of *p16, MGMT,* and *DAP-kinase* in tumor (*T*) and serum (*S*) including water as a negative control (*C−*) and a positive methylated control (*C+*). Lanes *U* and *M* correspond to the unmethylated and methylated reactions, respectively. *B,* percentages of patients with epigenetic alterations in the primary tumor and serum. *DAP-K, DAP-kinase*.

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Table 1 *Clinical features and gene promoter hypermethylation in primary tumors and serum in head and neck cancer patients*

	Primary tumors[a]						Serum	
	p16+	*p16−*	*MGMT+*	*MGMT−*	*DAP-K*[b]*+*	*DAP-K−*	+	−
Patients[c]	23	60	26	57	15	64	21	29
Stage								
I–II	4	16 P = NS[d]	7	13 P = NS	0	19 P = 0.016	3	6 P = NS
III–IV	19	44	19	44	15	45	17	20
Node involvement								
N_0	6	22 P = NS	8	20 P = NS	1	27 P = 0.014	5	7 P = NS
N+	17	38	16	37	14	37	15	19
Tumor size								
T_{1-2}	6	26 P = NS	10	20 P =NS	4	26 P = NS	8	8 P = NS
T_{3-4}	17	34	16	37	11	38	12	18
Tumor location[e]								
OC	14	36	12	36	7	39	10	18
L	4	11	5	12	2	11	4	5
HP	1	8	3	6	2	9	3	3
OP	3	3 P = NS	5	1 P =0.018	4	2 P = 0.02	3	2 P = NS
PS	1	2	1	2	0	3	1	1

[a] +/−, presence of absence of gene promoter hypermethylation for any of the three genes.
[b] *DAP-K*, DAP-kinase.
[c] Patients with available clinical data. The patients with recurrent disease at the moment of the first visit are not included in the table.
[d] P = NS represents $P > 0.05$.
[e] OC, Oral cavity; L, Larynx; HP, Hypopharynx; OP, Oropharynx; PS, Perinasal sinus.

Discussion

Hypermethylation of normally unmethylated CpG islands in the promoter regions often occurs in important tumor suppressor genes such as *VHL*, *hMLH1,* and *p16* (3–5). Recently, loss of expression in other interesting genes has been found in cancer cells through promoter methylation. Some of these genes include the detoxifying gene *GSTP1*, commonly altered in prostate, breast, and renal cancer (6, 21), the DNA repair gene *MGMT*, frequently inactivated in brain, colorectal, lung, and lymphomas (7), and the potential metastasis inhibitor *DAP-Kinase* gene altered in lymphomas, leukemias, and lung cancer (8, 22). These epigenetic alterations have been successfully used as indicators of neoplastic serum DNA in lung cancer patients (8).

Approximately one-quarter of the HNSCC analyzed in this study inactivated the *p16* tumor suppressor gene through promoter hypermethylation. These results are in agreement with our previous data (23) and highlight the importance of *p16* methylation as a common pathway of *p16* gene inactivation in HNSCC. Moreover, the *MGMT* gene showed aberrant methylation patterns in one-third of the tumors, similar to recent findings in a limited number of head and neck cancers (7). *GSTP1* was not methylated in any of the HNSCC analyzed, supporting others' results (21) and suggesting a role for this gene in only specific tumor types, such as breast and prostate cancer (6, 21). We found for the first time promoter hypermethylation of the *DAP-kinase* gene in HNSCC. The DAP-kinase protein is a calcium/calmodulin-dependent enzyme that phosphorylates serine/threonine residues. This protein has been classified as a positive mediator of apoptosis induced by IFN-γ. DAP-kinase expression is frequently lost in highly metastatic murine lung tumors compared with their low metastatic counterparts. In addition, restoration of DAP-kinase protein to physiological levels in highly metastatic Lewis carcinoma cells suppressed their ability to form metastasis (24). Interestingly, we found a positive correlation between methylation of *DAP-kinase* and the presence of lymph nodes metastases, supporting a role for this protein in tumor dissemination.

After screening for methylation changes in the primary tumor tissue, we analyzed the paired serum DNA in a subset of the patients. Forty-two % of the tumors with any epigenetic alteration showed the same change in the serum DNA using the MSP assay (sensitivity, ~1:1000). We described previously the detection of loss of heterozygosity and MI in ~20% of paired serum samples from HNSCC patients (15). The lower frequency of detection by microsatellite analysis is probably attributable to its lower sensitivity (approximately 1:200 for MI) compared with MSP (20, 25, 26). Differences in assay sensitivity for distinct markers may also account for the observation of promoter hypermethylation at the *MGMT* but not at the *DAP-kinase* gene in the serum specimen from two patients. Preliminary studies in serum and bronchoalveolar lavage fluid did not detect aberrant methylation in lung cancer patients that lacked hypermethylation patterns in the primary tumors (8, 26). Similarly, we did not detect abnormal promoter hypermethylation in the serum DNA of 25 HNSCC patients in whom the tested markers were not altered in the primary tumor.

The mechanism leading to the presence of free tumor DNA in the serum of cancer patients is not well understood. DNA may simply be released from the tumor tissue from nonviable (apoptotic) neoplastic cells. On the other hand, tumor serum DNA may also originate from cells that have left the primary site and have invaded the circulatory system but are still not capable of metastasis to new organs. After resection of the primary tumor, detection of DNA alterations in the serum may be an indicator of high risk of local or distant recurrence in cancer patients.

The clinical implications of detecting genetic alterations in serum of cancer patients are not clear. It has been reported that DNA concentrations in the serum from cancer patients are higher compared with the normal population, especially in the presence of metastatic disease. Interestingly, it was also observed that DNA levels declined after radiotherapy, especially when the treatment was beneficial. However, there was no correlation between the DNA concentration and several clinical features such as tissue of origin, tumor size, or stage of the disease (9). In addition, recent publications have reported a lack of association between the presence of genetic alterations in serum and the clinicopathological variables such stage, tumor size, lymph node involvement, or histological stage in renal, colorectal, breast, and non-small cell lung cancer (8, 11, 13, 17). However, in pancreatic cancer, plasma K-*ras* mutations have correlated with the development of distant metastasis and shorter survival (27).

Aberrant methylation in the serum DNA was more frequently detected in those patients that developed distant metastasis. Moreover, in our preliminary follow-up evaluation, we did not find aberrant methylation in patients without detectable disease and follow-up longer than 1 year. Our limited results suggest that epigenetic alterations may be an important indicator of metastases or recurrence, but

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larger prospective trials are needed to further establish these observations.

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