Molecular Detection of Early-Stage Laryngopharyngeal Squamous Cell Carcinomas

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Abstract

Purpose: We sought to determine whether early-stage laryngopharyngeal squamous cell carcinomas (SCC) can be detected through molecular analysis of exfoliated cells collected with the use of a pharyngoesophageal brush (PEB).

Experimental Design: Thirty-three patients with a single, untreated, early-stage (T1 or T2) SCC of the supraglottic larynx or pharynx underwent collection of cells with a PEB, followed by endoscopic biopsy of the tumor. PEB specimens were also collected from five healthy subjects. PEB samples and tumor tissue were examined for hypermethylation of $p16^{INK4a}$ (CDKN2) gene promoter CpG islands (assayed by methylation-specific PCR) and UT5085 tetranucleotide microsatellite instability (assayed by GeneScan analysis). PEB samples were also subjected to cytologic analysis.

Results: Eight of 33 (24%) tumors exhibited a bandshift at UT5085, and 14 of 33 (42%) exhibited hypermethylation at the p16 promoter. Overall, 17 of 33 (52%) patients had at least one of the two markers in their tumor. Cytologic analysis of PEB samples revealed tumor in 4 of 33 (12%) patients; cytologic findings were normal in all five control subjects. Molecular analysis of PEB samples revealed tumor DNA in 13 of 17 (76%) patients with at least one of the two molecular markers in their tumor. Eight of 14 (57%) patients with p16 hypermethylation in their tumor and 8 of 8 (100%) patients with UT5085 microsatellite instability in their tumor had similar findings in the PEB samples. None of the PEB samples from the control subjects or patients with neither molecular marker in their tumor displayed abnormality.

Conclusion: Molecular analysis of PEB samples holds promise for the early detection of early-stage laryngopharyngeal SCCs. New molecular markers need to be identified to increase the sensitivity of molecular screening.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide, and an estimated 500,000 people are diagnosed with this malignancy each year (1). In France, HNSCC accounts for 11% of the total cancer burden in men, and an estimated 15,000 new cases are diagnosed each year (2), the majority due to smoking and alcohol consumption (3). Despite intensive efforts to improve diagnosis and treatment, most patients are still diagnosed at advanced stages of disease and the overall 5-year survival rate for patients with HNSCC is only 50% (4). Therefore, a strategy for early identification of individuals with primary HNSCC, metachronous HNSCC, or local recurrence of HNSCC is urgently needed.

Examination of exfoliated cells has never achieved the same success in the diagnosis of HNSCC as it has in the diagnosis of cancer of the uterine cervix (5). However, an encapsulated pharyngoesophageal brush (PEB) technique (Oesotest) for cytologic analysis was recently developed to detect esophageal carcinomas (6). This technique has been reported to have a sensitivity of 88.7% and a specificity of 90.7% in the detection of recurrent and metachronous neoplasms of the upper aerodigestive tract and seems to be safe and minimally invasive (6). Early carcinomas of the supraglottic larynx or of the pharynx are often asymptomatic and are difficult to detect because they are in a hidden part of the upper aerodigestive tract. Therefore, such carcinomas should be ideal targets for cytologic detection using the PEB technique. However, the absence of a marker that is present in all malignant laryngopharyngeal lesions but absent in all benign laryngopharyngeal lesions or in the normal mucosa limits the clinical utility of the PEB cytologic technique in this setting and argues for the use of a combination of markers.

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Many molecular markers, such as nuclear or mitochondrial DNA mutations, loss of heterozygosity, and microsatellite instability (MSI), have been used extensively for early molecular detection of HNSCC (7). They have been shown to detect very low amounts of tumor DNA in samples containing high levels of normal DNA. We previously reported that 11 of 13 HNSCC patients with UT5085 MSI in their tumor (bandshift detected in tumor) had UT5085 MSI detected in DNA from exfoliated cells collected with a PEB, whereas only 6 of the same 13 patients had abnormal cells detected on cytologic analysis (8). Unfortunately, in this previous study, UT5085 MSI was present in only 25% of the primary tumors, and 68% of the primary tumors were advanced-stage and symptomatic tumors.

Recently, the promoter of CDKN2, which codes for p16INK4a, a cyclin-dependant kinase inhibitor, was reported to be inactivated by CpG island hypermethylation in nearly 30% of HNSCCs (9–11). The sodium bisulfite PCR–based technique for promoter CpG island hypermethylation analysis has emerged as an effective tool for HNSCC detection; the technique has high specificity and sensitivity (12, 13).

In the present study, we examined the utility of a combination of two molecular markers, UT5085 MSI and p16 hypermethylation, in the detection of tumor DNA in PEB samples from patients with very-early-stage laryngopharyngeal SCC.

Patients and Methods

Patients and sample collection. Patients with a single, untreated, early-stage (T1 or T2) SCC of the supraglottic larynx or the pharynx (except nasopharynx) were prospectively entered into this study at the Institut Gustave-Roussy (Villejuif, France) between July 1999 and December 2000. We restricted our study to patients with very early tumors (T1 or T2) because no symptom is strongly correlated with early SCC of the larynx or pharynx. We excluded patients with tumors of the vocal cords because these patients always have dysphonia that prompts laryngeal examination, and we excluded patients with oral tumors because these tumors can easily be detected by direct examination. The institutional review board approved the study and informed consent was obtained from all subjects.

PEB sampling was done before panendoscopy as previously described (6, 8). Cytologic findings were considered positive if low-grade or high-grade squamous dysplastic cells or squamous carcinoma cells were detected on at least one slide. This definition of positivity was based on esophageal cytologic criteria proposed by Shu (14) and confirmed by others authors (6). Cytologic findings were considered negative only when there was no significant cytonuclear atypia in squamous cells and the majority of cells were of the intermediate type.

Tumor samples were collected from each patient during panendoscopy and fresh frozen. Specimens of normal mucosa from the larynx or pharynx were also collected from five healthy subjects who were heavy smokers and drinkers but free of SCC. Thin tumor sections from patients were stained with H&E to confirm the diagnosis of invasive SCC. Samples containing more than 70% tumor cells were selected to avoid microdissection and were used for DNA extraction. DNA was extracted from ten to fifteen 10-μm sections with the QIAamp tissue kit (Qiagen, Courtaboeuf, France). DNA quality was checked with GeneQuant II (Amersham Pharmacia Biotech, Piscataway, NJ).

At the time of the initial diagnosis, fresh blood was collected from each patient in EDTA tubes, and lymphocytes were separated for use as a source of normal DNA. DNA was extracted from lymphocytes using the QIAamp blood kit (Qiagen, Courtaboeuf, France).

Methylation-specific PCR analysis of p16 in DNA from tumor tissue and pharyngoesophageal brush samples. At least 2 μg of tumor DNA, mixed with 1 μg of salmon sperm (Invitrogen, Carlsbad, CA), were submitted to chemical modification following the bisulfite protocol described by Herman et al. (15). DNA was denatured with 0.2 mol/L NaOH and then treated with 10 mmol/L hydroquinone and 3 mol/L sodium bisulfite (Sigma Chemical Co., St. Louis, MO). After purification in a Wizard SV Plus kit column (Promega, Madison, WI), the DNA was treated with 3 mol/L NaOH and precipitated with 3 volumes of 100% ethanol, a one-third volume of 10 mol/L NH_4OAc, and 2 μL of glycogen at −20°C. The precipitated DNA was washed with 70% ethanol and dissolved in distilled water. Methylation-specific PCR was conducted with primers specific for either the methylated or the unmethylated version of p16 [methylated-specific primers: 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (sense) and 5′-ACCCCGAACCGCGACCGTAA-3′ (antisense); unmethylated-specific primers: 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ (sense) and 5′-CAACCCCAAACCACAACCATAA-3′ (antisense)]. The 12.5-μL total reaction volume contained 25 ng of modified DNA, 3% DMSO, all four deoxynucleoside triphosphates (each at 200 μmol/L), 1.5 mmol/L $MgCl_2$, 0.4 μmol/L PCR primers, and 0.625 units of HotStar Taq DNA polymerase (Qiagen, Valencia, CA). DNA from lymphocytes was used as a negative control (unmethylated p16) and DNA from H358 cells (ATCC CRL-5807) was used as a positive control (hypermethylated p16). DNA was amplified by an initial cycle at 95°C for 15 minutes as required for enzyme activation, followed by 40 cycles of 95°C for 30 seconds, annealing for 45 seconds, and 72°C for 1 minute, and ending with a 5-minute extension at 72°C in a thermocycler (Applied Biosystems, Foster City, CA). PCR products were separated on 8% polyacrylamide gels and visualized after staining with ethidium bromide or Vistra Green (Amersham Pharmacia Biotech). All results were confirmed twice. PEB DNA samples were then analyzed using the same procedure. We also studied PEB DNA samples from the five control subjects. Two observers assessed all samples independently, and PCR analysis was repeated for cases with borderline results.

Analysis of UT5085 MSI in DNA from tumor tissue and pharyngoesophageal brush samples. The UT5085 tetranucleotide microsatellite was amplified with primers referenced in the GenBank sequence database (accession no. GDB 309286) as previously described (8). To increase the difference between normal DNA and MSI DNA, one primer was labeled in blue with 6-carboxy-fluorescein, and amplified products were run in polyacrylamide sequencing gels, ELLIOSEQ GEL (Ellios Bio Media, Paris, France) mixed with 0.5 μL of GeneScan-500 ROX (Applied Biosystems) and 2.5 μL of formamide blue after a 2-minute denaturing step at 94°C. PCR products were detected on the gel by laser fluorescence on the ABI prism 377 DNA sequencer (Applied Biosystems). Data were analyzed by GeneScan analysis (Applied Biosystems).

All tumors and corresponding lymphocytes were amplified to select unstable UT5085 samples. MSI was defined as the presence of a new pattern in at least one extra band in tumor DNA compared with lymphocyte DNA.

PEB material from each tumor with a shifted band was then amplified and coanalyzed with tumor and lymphocyte DNA to compare the different microsatellite patterns (see figure in ref. 8). We also studied PEB samples from eight uninformative tumors (i.e., those without either of the two molecular markers) and from five control subjects. Two observers assessed all samples independently, and MSI analysis was repeated for cases with borderline results.

Statistical analysis. Statistical analyses were done using Statview 5.0 (SAS Institute, Inc., Cary, NC). Associations between variables were assessed by Fisher's exact test.

Results

Population. Thirty-three patients with a single, untreated, early-stage (T1 or T2) HNSCC were prospectively entered into the study. Tumor sites were as follows: oropharynx, 15 patients; supraglottic larynx, 9 patients; and hypopharynx,

Table 1. Clinical characteristics and findings on cytologic evaluation, analysis of p16 hypermethylation, and analysis of UT5085 MSI for the 17 patients with early-stage laryngopharyngeal SCC who had at least one of the two molecular markers present in their primary tumor

Patient no.	Tumor site	Tumor stage	Cytology findings in PEB sample*	p16 methylation		UT5085 MSI	
				Tumor tissue†	PEB sample*	Tumor tissue†	PEB sample*
1	Oropharynx	T1	Neg	Methylated	Positive	Shift	Positive
2	Larynx	T2	Dysplasia	Methylated	Positive	Shift	Positive
3	Oropharynx	T2	Neg	Methylated	Positive	Shift	Positive
4	Oropharynx	T1	Neg	Methylated	Positive	Neg	Neg‡
5	Oropharynx	T2	Neg	Methylated	Positive	Neg	ND
6	Hypopharynx	T2	Neg	Methylated	Positive	Neg	Neg‡
7	Hypopharynx	T2	Neg	Methylated	Positive	Neg	Neg‡
8	Oropharynx	T2	Dysplasia	Methylated	Positive	Neg	Neg‡
9	Larynx	T2	Neg	Methylated	Neg	Shift	Positive
10	Hypopharynx	T2	Neg	Methylated	Neg	Shift	Positive
11	Hypopharynx	T1	Neg	Methylated	Neg	Neg	Neg‡
12	Oropharynx	T2	Neg	Methylated	Neg	Neg	Neg‡
13	Oropharynx	T2	Neg	Methylated	Neg	Neg	Neg‡
14	Oropharynx	T1	Neg	Methylated	Neg	Neg	Neg‡
15	Larynx	T2	Neg	Unmethylated	Neg‡	Shift	Positive
16	Oropharynx	T2	Neg	Unmethylated	Neg‡	Shift	Positive
17	Larynx	T2	Neg	Unmethylated	Neg‡	Shift	Positive

NOTE: Neg, negative; ND, not done; shift, UT5085 MSI.
*PEB samples consisted of DNA from exfoliated cells collected by pharyngoesophageal brushing with the Oesotest device.
†Tumor tissue was tissue obtained from biopsy of the primary laryngopharyngeal tumor.
‡Negative control.

9 patients. There were 12 T1 and 21 T2 lesions. Twenty-three patients had their tumor discovered because of symptoms (otalgia, dysphagia, or odynodysphagia). Ten patients had asymptomatic tumor and had their tumor discovered during a systematic examination done because of enlarged neck lymph nodes or risk factors. Five healthy control subjects who were heavy smokers and drinkers but free of HNSCC were also studied.

Molecular markers in tumors. Eight of 33 (24%) patients had MSI (i.e., exhibited a bandshift) at UT5085 (Tables 1 and 2). None of the healthy control subjects had MSI at UT5085. Fourteen of 33 (42%) patients had p16 hypermethylation. None of the healthy control subjects had p16 hypermethylation. Overall, 17 of 33 (52%) patients had at least one of the two markers. The tumor sites for these 17 patients were as follows: oropharynx, 9 patients; larynx, 4 patients; and hypopharynx, 4 patients. There were 4 T1 and 13 T2 lesions in this group.

Cytologic findings and molecular markers in pharyngoesophageal brush samples. All PEB samples were available for cytologic interpretation. Four of 33 (12%) PEB samples were positive. One contained squamous carcinoma cells, one contained high-grade dysplastic cells, and two contained low-grade dysplastic cells (Table 1). There was no relationship

Table 2. Cytologic and molecular analysis of Oesotest

Finding	No. (%) patients with finding		
	Tumor sample (n = 33)	PEB samples, all patients (n = 33)	PEB samples, patients with abnormality in tumor sample
Cytologic abnormality	—	4 (12%)	2/17 (12%)*
UT5085 MSI	8 (25%)	8 (24%)	8/8 (100%)
p16 hypermethylation	14 (42%)	8 (24%)	8/14 (57%)
At least one molecular marker	17 (52%)	15 (45%)	13/17 (76%)

*For the calculation of this percentage, patients with at least one molecular marker in their tumor were considered to have an abnormality in the tumor sample.

between the results of the cytologic analysis and the T classification or tumor location. Findings on analysis of PEB samples were normal in all five healthy control subjects.

Eight of 14 (57%) patients with p16 hypermethylation in their tumor also had p16 hypermethylation in the PEB samples (Tables 1 and 2; Fig. 1). Eight of eight (100%) patients with MSI at UT5085 in their tumor had a similar bandshift in the PEB samples (8). When we pooled the results for the two markers, we found that molecular analysis of PEB samples revealed tumor DNA in 13 of 17 (76%) patients with early-stage laryngopharyngeal carcinoma who had at least one of the two markers present in their tumor (Tables 1 and 2). This result was not associated with tumor stage (T1 versus T2) or tumor site (oropharynx versus larynx versus hypopharynx; $P > 0.05$).

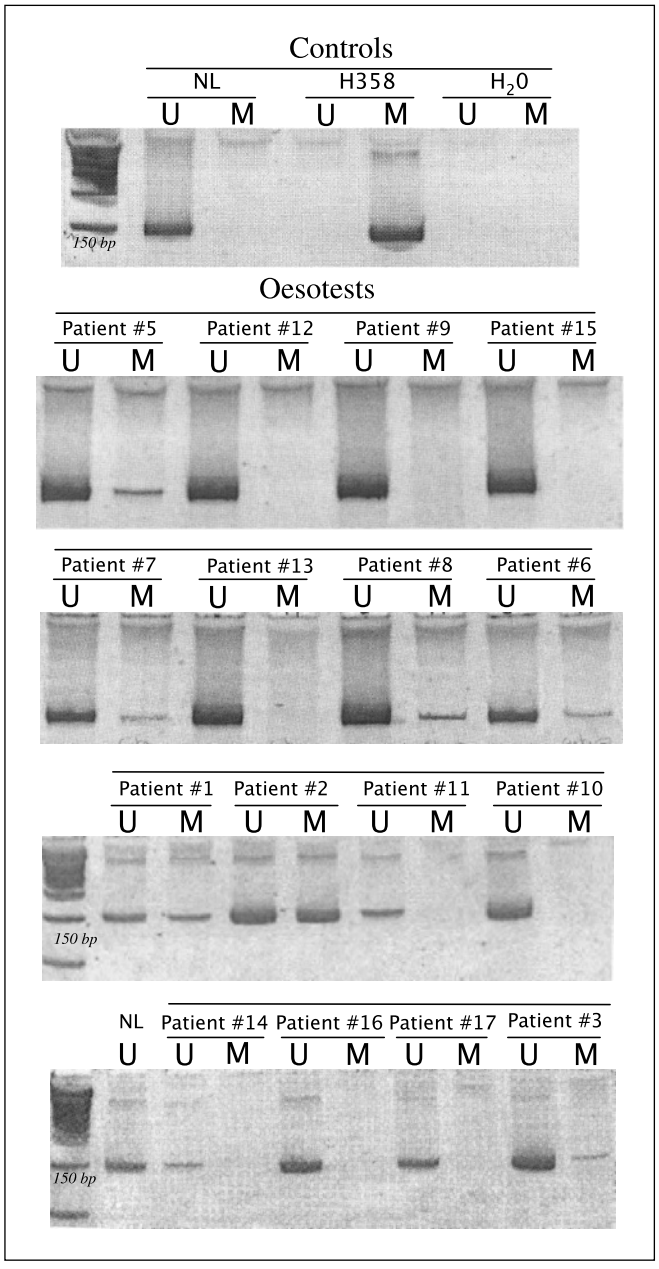

Fig. 1. Methylation-specific PCR of p16INK4a promoter in PEB samples of patients with hypermethylated early-stage laryngopharyngeal SCC. *Lanes U,* presence of a visible PCR product indicates the presence of unmethylated p16; *lanes M,* presence of product indicates the presence of methylated p16. Normal lymphocytes (*NL*) were used as negative controls for methylation; H358 bronchioloalveolar cell line was used as a positive control for methylation; and water was used as a negative PCR control.

All PEB samples that were positive by cytologic evaluation were also positive by analysis of UT5085 MSI and p16 hypermethylation. None of the PEB DNA samples from patients with uninformative tumors (i.e., those without either of the two molecular markers) or the five healthy control subjects showed UT5085 MSI or p16 hypermethylation (i.e., there were no false-positive findings and the specificity was 100%). The sensitivity of testing with the two molecular markers plus cytologic examination was 45% overall [18 of 33 (55%) patients had false-negative findings] and 76% when the molecular analysis of PEB was restricted to the 17 patients with informative tumors [4 of 17 (24%) patients had false-negative findings].

Discussion

We have shown that molecular analysis of PEB samples reveals a large proportion of very-early-stage laryngopharyngeal SCCs. We used two molecular markers to improve informativity and sensitivity. MSI insertion or deletion of repeat DNA microsatellite sequences can easily be detected in tissues containing clonal expanded cells, such as tumor tissues, and is a sensitive (one tumor cell among 200 normal cells) and specific molecular marker for the detection of cancer cells in samples with a substantial background population of normal cells (7, 8, 16, 17). We expect that because we used a laser fluorescence detection assay on an ABI prism 377 DNA sequencer, the sensitivity in our study was slightly higher. Promoter CpG island hypermethylation is also a promising biomarker for molecular detection of HNSCC (10). We found that 42% of these early-stage tumors had p16 hypermethylation, which is consistent with results of previous studies (9, 12). We might have found more informative cases if we had looked for promoter hypermethylation in other genes, such as *MGMT*, *hMLH-1*, *DAPK*, or *E-cadherin* (10).

We confirmed the simplicity and the efficacy of the bisulfite methylation–specific PCR technique, but we had frequent difficulties with repeat false-positive results in normal lymphocytes. We had to repeat the experiment to get appropriate results. We also noticed that in 2 of 14 patients with methylated tumors (patients 9 and 10, Table 1), tumor DNA in PEB samples was detected with MSI analysis but missed by p16 methylation–specific PCR. The sensitivity of methylation-specific PCR needs to be improved before it can be useful as a screening test. On the other hand, we did not find any hypermethylated p16 in the PEB samples from the five healthy control subjects or the 19 patients with unmethylated tumors.

Recently, a real-time quantitative methylation-specific PCR method had been developed with more sensitive results (18, 19). Wong et al. (20) have shown that the concentration of methylated p16 DNA in bodily fluids can be successfully used to discriminate between healthy individuals exposed to carcinogens and patients with HNSCC. We will soon test this new method in our series of patients.

Cytologic evaluation of PEB DNA samples revealed tumor cells in 4 of 33 patients (12%), which is a good result for very-early-stage laryngopharyngeal carcinoma. We are aware

that we did the molecular analysis on only a very small volume of each esopharyngeal brush sample. It is possible that in some of the aliquots tested, no tumor cell DNA was present although tumor cell DNA was present in the corresponding esopharyngeal brush sample. In the next step of this study, we will use much larger sample volumes. Pharyngoesophageal brushing with Oesotest is a safe, easy, and low-cost technique (6, 8) that should be evaluated in a prospective study for detection of laryngopharyngeal SCC in high-risk populations. Cytopathology evaluation is a low-cost and accurate examination that should be done before molecular assay.

Our results complement those of two previous studies in which methylation-specific PCR markers (12) or microsatellite markers (21) were used to detect tumor DNA in oral swab samples of patients with mainly oral SCC. In our study, we confirmed that UT5085 MSI and p16 hypermethylation have a high sensitivity in the detection of early-stage laryngopharyngeal carcinomas, which are more difficult to detect than oral tumors. We excluded oral carcinoma from our study because 90% arise on the visible oral mucosal surfaces of the floor of the mouth, ventrolateral surfaces of the tongue, and retromolar trigone, and sensitive early detection is feasible with direct examination with or without toluidine blue coloration (22, 23). It is noteworthy that one third of the patients in our series had asymptomatic tumors.

In conclusion, we have shown that molecular analysis was able to detect a large proportion of very-early-stage laryngeal and pharyngeal SCCs. This result is important because it was achieved in exactly the population that will be targeted in long-term longitudinal screening studies. However, we need to significantly increase the sensitivity of molecular screening by identifying new molecular markers that are more frequently present in early-stage tumors.

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