Tumor-Specific Methylation in Saliva: A Promising Biomarker for Early Detection of Head and Neck Cancer Recurrence

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Abstract **Purpose:** Our goal was to define tumor and saliva gene methylation profile of head and neck squamous cell carcinoma and to evaluate its prognostic significance and its biomarker potential for early detection of relapse.

Experimental Design: We prospectively analyzed 11 genes by methylation-specific PCR on primary tumors, histologically normal adjacent mucosa, and saliva from 90 French patients at diagnosis and during follow-up as well as on 30 saliva specimens from control-matched patients with nonmalignant head and neck pathology. Five additional genes were analyzed on 50 tumors of the series.

Results: Methylation of *TIMP3, ECAD, p16, MGMT, DAPK*, and *RASSF1* was the most frequently observed in tumors and paired saliva samples were analyzed at diagnosis, with an excellent agreement between both samples. At least one of these six genes was methylated in >75% of the samples without additional positive samples when other genes were analyzed. Methylation profile was similar in newly diagnosed and second primary cancers. Aberrant methylation was not associated with a worse prognosis. Ninety percent of normal adjacent mucosa and all control saliva samples were negative. Twenty-two patients were followed after treatment; abnormal methylation was detectable in the saliva of five patients few months before clinical and 2-deoxy-2[^{18}F]fluoro-D-glucose-positron emission tomography signs of relapse, allowing curable surgery. Saliva samples were negative for the 17 other patients: 16 were in remission and only 1 relapsed.

Conclusions: Gene methylation in saliva is a promising biomarker for the follow-up and early detection of still curable relapses of head and neck squamous cell carcinoma patients.

Head and neck squamous cell carcinoma (HNSCC) affects each year ~780,000 new patients worldwide and >15,000 patients in France (1, 2). Despite advances in therapy, its prognosis has not improved markedly over the past 20 years (3) essentially because of late diagnosis and frequent locoregional recurrences without successful salvage therapy if the tumor is large. Early diagnosis of HNSCC might improve its prognosis but is hindered in patients who often delay seeking medical attention for a variety of reasons linked to tobacco and alcohol intake. Early detection of recurrence is also vital, and effective surveillance is usually easier in patients who have already been managed adequately. HNSCC detection is currently based on expert clinical examination of the upper aerodigestive tract and histologic analysis of suspicious areas, but it may be undetectable in hidden sites, such as crypts of the tongue base or tonsils (4). *De novo* methylation of CpG island within suppressor gene promoters is a well-characterized epigenetic abnormality in cancer that causes gene inactivation (5, 6). Several genes are frequently methylated in HNSCCs (7, 8); they are involved in cell cycle control (*p14*, *p15*, and *p16*), DNA damage repair (*MGMT, hMLH1*, and *ATM*), apoptosis (*DAPK, RASSF1A*, and *RARβ*), and tumor cell invasion (*ECAD* and *DCC*). Some others genes (i.e., *APC, FHIT*, and *TIMP3*) have rarely or never been analyzed in HNSCC but are frequently methylated in non–small cell lung cancer, a tumor also linked to tobacco smoking (7, 9–12). Gene methylation is detectable in a very few malignant cells collected in body cavity fluids at direct contact with the tumor (12, 13); therefore, saliva analysis holds great promise for early detection of primary HNSCC cancer and local recurrence (14). However, gene methylation analysis might be impaired firstly because no single epigenetic signature accounting for all HNSCCs has been identified and secondly because gene methylation might be, as other molecular abnormalities, observed at all stages of the transformation years before clinical manifestations of HNSCC (7, 9), leading to false-positive results in saliva from subjects exposed to risk factors.

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The purpose of this study was firstly to define a gene methylation profile in HNSCC tumors and saliva analyzed at diagnosis and during patient follow-up and secondly to evaluate its prognosis significance as well as its value as biomarker for early detection of recurrence.

Materials and Methods

Study design. Between 2003 and 2005, 120 patients ages <75 years with a primary nonmetastatic HNSCC of the oral cavity, pharynx, and larynx were included after they gave informed consent in a monocentric prospective study; this study was designed as recommended by Mc Shane (15) and approved by Grenoble University Hospital Ethical Committee (Grenoble, France). Thirty patients were excluded because the tumors contained <70% malignant cells (Fig. 1). Diagnostic staging included tobacco/alcohol addiction and demographic characteristics recording, physical examination by a head and neck surgeon, endoscopy of the upper aerodigestive tract, cervicothoracic computed tomography scan, and liver ultrasound. Tumor staging and histology were defined according to the International Union Against Cancer and WHO 1991 classifications (16, 17). Patients were treated by surgery and/or radiotherapy and followed with regular physical examinations, saliva samples, and 2-deoxy-2[^{18}F]fluoro-D-glucose-positron emission tomography (FDG-PET).

Sample collection. Tumor samples and histologically normal adjacent mucosa were obtained during surgery (*n* = 53) or by biopsy during initial endoscopy (*n* = 37), frozen in liquid nitrogen, and stored at −80°C. DNA was extracted from 10-μm sections that contained >70% infiltration by SCC as quantified by two pathologists on the first Giemsa-stained section. Paired saliva samples were collected just before surgery or biopsy and during posttreatment staging after 3 min of swishing and gargling with a 25 mL sterile NaCl solution at 0.9%. Samples were treated in Hank's solution with 1% DTT and centrifuged, and cell pellets were frozen at −80°C before analysis. Saliva was also collected from 30 control patients (20 males) and received in the Head and Neck Department after a head and neck surgeon had confirmed the absence of clinically detectable mucosal or malignant lesion.

DNA extraction. Genomic DNA from tumor and exfoliated cells in saliva was digested with proteinase K and extracted with QIAmp tissue kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations.

Methylation-specific PCR. Methylation analysis was done on tumors, normal adjacent mucosa, and saliva after bisulfite DNA conversion with CpGenome DNA modification kit (MP Biomedicals, Illkirch, France); DNA was amplified with FastStart DNA polymerase (Roche, Meylan, France) using specific primers for the methylated or unmethylated sequences of the genes (Table 1; ref. 18). *MyoD* amplification, with primers selected in an unmethylated region, served as DNA quantity control after bisulfite treatment. DNA from peripheral blood lymphocytes, treated or not with *Sss*1 methyltransferase (New

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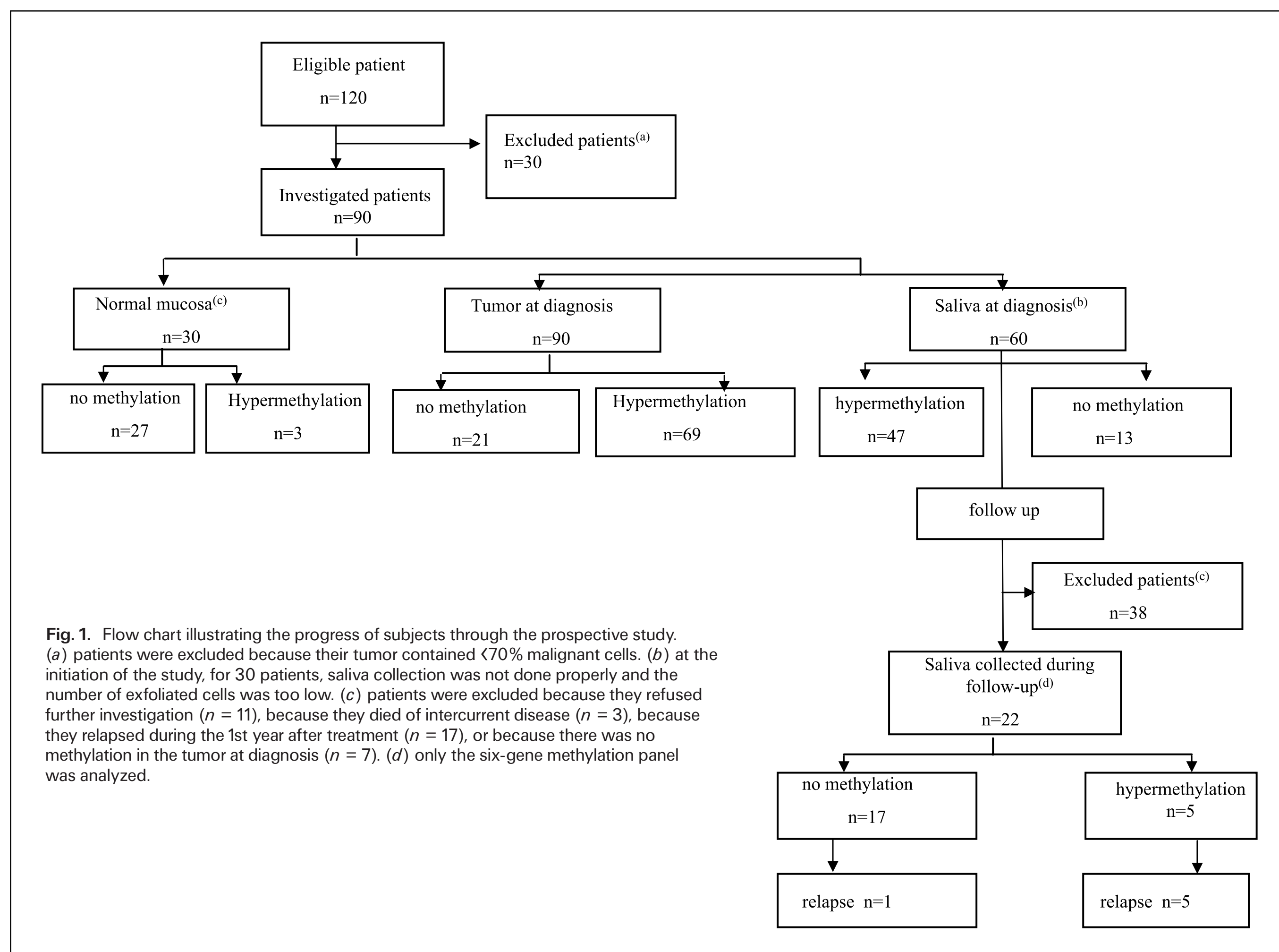

Fig. 1. Flow chart illustrating the progress of subjects through the prospective study. (*a*) patients were excluded because their tumor contained <70% malignant cells. (*b*) at the initiation of the study, for 30 patients, saliva collection was not done properly and the number of exfoliated cells was too low. (*c*) patients were excluded because they refused further investigation (*n* = 11), because they died of intercurrent disease (*n* = 3), because they relapsed during the 1st year after treatment (*n* = 17), or because there was no methylation in the tumor at diagnosis (*n* = 7). (*d*) only the six-gene methylation panel was analyzed.

Table 1. Primers for methylation-specific PCR

Gene	Primer sense (5′-3′)	Primer antisense (5′-3′)	Hybridization temperature (°C)	Product size (bp)	References
*MyoD**	TGATTAATTTAGATTGGGTTTAGAGAAGGA	CCAACTCCAAATCCCCTCTCTAT	60	152	(36)
p14 ARF (M)	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	60	122	(37)
p14 (U)	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	60	132	
p15 INK4b (M)	GCGTTCGTATTTTGCGGTT	CGTACAATAACCGAACGACCGA	60	148	(38)
p15 (U)	TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	60	154	
p16 INK4a (M)†	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	65	151	(18)
p16 (U)†	GGTTATTAGAGGGTGGGGTGGATTGT	CCCAACCCCAAACCACAACCATAA	60	151	
DAPK (M)†	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	60	98	(39)
DAPK (U)†	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	60	106	
RASSF1A (M)	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	60	93	(40)
RASSF1A (U)	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA	60	105	
FHIT (M)	TTGGGGCGCGGGTTTGGGTTTTTACGC	CGTAAACGACGCCGACCCCACTA	64	74	(41)
FHIT (U)	TTGGGGTGTGGGTTTGGGTTTTTATG	CATAAACAACACCAACCCCACTA	64	74	
MGMT (M)	TTTCGAACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	55	81	(39)
MGMT (U)	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	55	93	
hMLH1 (M)	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	63	114	(42)
hMLH1 (U)	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	60	120	
ECAD (M)	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	60	173	(38)
ECAD (U)†	TAATTTTAGGTTAGAGGGTTATTG	CACAACCAATCAACAACACA	56	173	
APC (M)	GAACCAAAACGCTCCCCAT	TTATATGTCGGTTACGTGCGTTTATAT	65	74	(43)
APC (U)	AAACCAAAACACTCCCCATTC	AGTTATATGTTGGTTATGTGTGTTTAT	60	76	
TIMP3 (M)	GCGTCGGAGGTTAAGGTTGTT	CTCTCCAAAATTACCGTACGCG	59	116	(44)
TIMP3 (U)†	TGTGTTGGAGGTTAAGGTTGTTTT	ACTCTCCAAAATTACCATACACACC	59	122	
RARβ (M)	TCGAGACGCGAGCGATTCG	GACCAATCCAACCGAAACGA	59	146	(45)
RARβ (U)	TTGAGAATGTGAGTGATTTGA	AACCAATCCAACCAAAACAA	59	146	
DCC (M)	CGTTGTTCGCGATTTTTGGTTTC	ACCGATTACTTAAAAATACGCG	60	145	(20)
DCC (U)	GTTGTTGTTGTTTGTGATTTTTGGTTTT	CCACTTACCAATTACTTAAAAATACACA	60	134	
ATM (M)	GGAGTTCGAGTCGAAGGGC	CTACCTACTCCCGCTTCCGA	59	239	(29)
ATM (U)	GTTTTGGAGTTTGAGTTGAAGGGT	AACTACCTACTCCCACTTCCAA	56	246	
THSB1 (M)	TGCGAGCGTTTTTTTAAATGC	TAAACTCGCAAACCAACTCG	58	74	(46)
THSB1 (U)	GTTTGGTTGTTGTTTATTGGTTG	CCTAAACTCACAAACCAACTCA	60	145	
CASP8 (M)	TAGGGGATTCGGAGATTGCGA	CGTATATCTACATTCGAAACGA	58	322	(47)
CASP8 (U)	TAGGGGATTTGGAGATTGTGA	CCATATATATCTACATTCAAAACAA	50	321	

Abbreviations: U, unmethylated; M, methylated; *DAPK,* death-associated protein kinase 1; *RASSF1A,* Rass association domain family 1A; *FHIT,* fragile histidine triad; *MGMT,* O^6-methylguanine DNA methyltransferase; *hMLH1,* human homologue of bacterial MutL; *ECAD,* E-cadherin; *APC,* adenomatosis polyposis coli; *TIMP3,* tissue inhibitor metalloproteinase 3.

*Myogenic differentiation; *MyoD* amplification, with primers selected in an unmethylated region, served as DNA quantity control after bisulfite treatment.

†DMSO requirement.

England Biolabs, Ozyme, St. Quentin Yuelines, France), was used as a positive and negative control. PCR products were analyzed by 3% agarose gel electrophoresis. The methylation-specific PCR allows detecting reproducibly 0.01% to 1% of methylated DNA in samples (12).

Statistical analyses. Associations between gene promoter hypermethylation and tumor and patients' characteristics were analyzed through the use of the Mann-Whitney or Fisher's exact test as appropriate. κ coefficients between saliva and tumor samples were calculated. Variables associated with relapse or death were tested using Kaplan-Meier estimates and compared using a log-rank test. A multivariate Cox's model was used to determine independent predictors of relapse or death and, in particular, to test the association between gene promoter hypermethylation and prognosis. We used a two-sided test and a P value of <0.05 denoting statistical significance. Statistical Analysis System version 9.1 (SAS Institute, Inc., Cary, NC) and MedCalc 8.8.1.0 (MedCalc, Ghent, Belgium) softwares were used.

Results

Prevalence of aberrant methylation in tumors and in histologic normal adjacent mucosa. Ninety HNSCC patients were included (median age, 57 years; range, 33-74). With the exception of five patients who had no tobacco and/or alcohol addiction, in the other ones, average tobacco and alcohol consumption was of 40 pack-years (range, 20-80) and 120 g/24 h (range, 50-160). Methylation of 11 genes was analyzed, and percentage of methylation for each specific gene is shown on Table 2. Sixty-nine patients had newly diagnosed primary tumors and 22 ones had a second primary HNSCC, the first tumor being treated with surgery and radiotherapy; the profiles of methylation were similar in the two cohorts, and in the remainder of the study, analysis was done on the whole series of patients. Overall, 69 (77%) of the tumors were positive (i.e., they had at least one methylated gene). The six most frequently methylated genes were *TIMP3* (46%), *ECAD* (36%), *MGMT* (29%), *p16* (29%), *DAPK* (27%), and *RASSF1A* (20%); furthermore, in positive tumors, one of these six genes was always positive and analysis of the five other genes (i.e., *p14*, *p15*, *FHIT*, *hMLH1*, and *APC*) did not increase the number of positive tumors. Specificity of those six genes methylated for the tumoral process was evaluated by the analysis of 30 histologically normal mucosa samples harvested over 3 cm away from the tumor. Methylation of the six genes was significantly different between tumors and normal mucosa (P < 0.001); indeed, only three mucosa

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Table 2. Methylation profile in the general population and the two subgroups of newly diagnosed and second primary HNSCC

	Positive samples, *n* (%)*	% of methylated samples										
		TIMP3	*ECAD*	*MGMT*	*p16*	*DAPK*	*RASSF1A*	*p15*	*p14*	*APC*	*FHIT*	*hMLH1*
General population (N = 90)	69 (77)[†]	46	36	29	29	27	20	18	11	11	10	2
Newly diagnosed primary cancers (n = 68)	54 (79)[†]	47	38	31	29	29	20	19	12	12	10	1.5
Second primary cancers (n = 22)	15 (64)[†]	41	27	23	27	18	18	14	9	9	9	4

*Positive samples contained at least one methylated gene.
[†]The median number of methylated genes per sample was 2 ± 1.6 in the global population, 2.7 ± 1.9 in newly diagnosed cancers, and 1.5 ± 1.8 in second primary cancers.

samples taken in patients with the most advanced tumors were methylated, with *TIMP3* positivity in two mucosa and *DAPK* in one.

In an attempt to increase the percentage of positive tumor samples, five other genes were analyzed but the analysis was stopped. Indeed, *ATM, CASP8*, and *THSP1* genes were not methylated on the 50 first tumors analyzed; methylation of *DCC* was observed in 24% of the tumors but was always associated with methylation of one of the six genes described above. Finally, methylation of *RARβ* was positive on 56% of the tumor samples but the abnormality was also observed on 20% normal mucosa and on control saliva samples as shown below.

Correlation between aberrant methylation, patients' characteristics, and prognosis. Patients' characteristics (age, sex, tumor location, stage of the disease, histologic grade, and classification) according to the presence or absence of a gene methylation in the tumor are shown on Table 3; the presence of gene methylation was more frequent in high clinical T and N stages. The median follow-up was 564 days, and the risk of relapse or death (Kaplan-Meier estimate) was 73 ± 5% at 1 year and 52 ± 6% after 2 years. As expected, known risk factors of relapse or death were present on the multivariate Cox's model [i.e., clinical stage (I or II versus III or IV), histologic grade (1 versus 2 and 3), and previous history of HNSCC; data not shown], but the presence or absence of gene methylation in the tumor was not associated with a worse prognosis (P = 0.6, log-rank test); the absence of correlation between methylation status and prognosis was confirmed when the final model was forced (hazard ratio, 0.5922; 95% confidence interval, 1.26-0.54; P = 0.28). The comparison between each of the six most frequently methylated genes defined above and patients' characteristics only revealed that *RASSF1A* methylation was more frequent in poorly differentiated tumors (P = 0.019) and in those with lymph node metastases (P = 0.048).

Prevalence of aberrant methylation in saliva collected at diagnosis in HNSCC patients and in control patients. Saliva samples, obtained in 60 patients, were analyzed and the concordance with paired tumors was measured by the κ coefficient (Table 4). Representative examples of methylation-specific PCR in tumors and paired saliva are available online (Supplementary Fig. S1). As in tumors, *TIMP3, ECAD, p16, MGMT, DAPK*, and *RASSF1A* were the most frequently methylated genes in saliva and their analysis was sufficient to define the 47 positive samples. There was no false-positive result in saliva screening (i.e., aberrant methylation in saliva was always observed with aberrant methylation in the tumor); only two saliva samples were negative despite positivity of the paired tumors. The κ coefficient between tumors and saliva for at least one aberrant methylation was 0.833 (95% confidence interval, 0.66-1.01). The percentage of positive saliva samples in saliva and tumor for each specific gene are given in Table 4. In 24 of the 47 positive saliva, the methylation profile (i.e., positive genes) was fully identical in tumor and paired saliva. For the 23 others, when a specific gene was methylated in saliva, it was always methylated in paired tumor but some specific genes methylated in tumor were not methylated in the corresponding saliva. This is reflected by the κ coefficient between saliva and tumors for each gene, which was excellent except for *ECAD* (11 of 23 false-negative saliva) and *TIMP3* (13 of 30 false-negative saliva). The κ coefficient between tumors and saliva was excellent (κ = 1) when the tumors were located in the oral cavity, and it was not different in newly diagnosed HNSCC (κ = 0.762; 95% confidence interval, 0.51-1.01) and in second primary cancer (κ = 1, no discordant pairs). Methylation of the 11 genes was undetectable in saliva samples collected in 30 control patients (median age, 45 years; range, 31-50), although 15 had tobacco/alcohol addiction (average number of pack-years, 35; range, 20-60 and absolute alcohol absorption, 100 g/24 h; range: 60-130). Thus, the methylation process was specific to the head and neck malignant process. In contrast, *RARβ* gene was methylated in 7 of the 30 control salivas.

Detection of gene methylation in saliva collected during patient follow-up and relapse diagnosis. Among the 60 patients in whom saliva was analyzed at diagnosis, 11 had no detectable methylation in the tumor or in saliva and 10 others refused further investigations; 3 patients died of intercurrent disease and 14 relapsed before the first complete staging (Fig. 1). Therefore, 22 patients were followed after diagnosis and treatment (median, 25 months; range, 19-31), with physical examination every 8 weeks and a first complete staging at 14 months after diagnosis (range, 8-20 months), including physical examination, saliva sampling, and FDG-PET; then, subsequent staging included physical examination and saliva sampling every 2 to 6 months. Patient median follow-up was not significantly different whether they relapsed or were disease-free (data not shown).

Methylation of *TIMP3, ECAD, p16, MGMT, DAPK*, and *RASSF1A* genes was analyzed in saliva samples collected during

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follow-ups (Table 5). Four patients (nos. 1-4) had a positive saliva at first postdiagnosis staging but the FDG-PET was negative; all four patients relapsed within 2 to 14 months. Eighteen patients had negative saliva at first postdiagnosis staging; 16 (nos. 7-22) were still disease-free with a negative FDG-PET at last examination (median, 22 months; range, 18-29). In the 17 patients (no. 5), saliva became positive at 21-month staging but FDG-PET was negative; he relapsed 1 month later. Patient no. 6 was the only false negative of this cohort: saliva was negative at first staging but the FDG-PET was positive; he relapsed 1 month later and died at 20 months of a very rapidly growing tumor. In patient nos. 1 to 5, methylation detection and subsequent careful clinical follow-up allowed to diagnose relapse when the tumor was still small (diameter, within 5 to 10 mm) and surgically curable; furthermore, saliva was normalized after the second surgery. In those five patients, methylated genes, detected in saliva before relapse, were already abnormal in samples analyzed at diagnosis.

Discussion

Based on the analysis of a first set of 11 genes and then of 5 others, we have defined a six-gene methylation panel (i.e., *TIMP3*, *ECAD*, *p16*, *MGMT*, *DAPK*, and *RASSF1A*) that allows to detect aberrant methylation in >75% of primary HNSCC. Aberrant methylation of those six genes in exfoliated malignant cells of the saliva reflects tumor status as previously suggested for other molecular abnormalities (4, 19) and is highly specific of the malignant process. Moreover, in our series, HNSCC recurrence was always associated with the persistence or reappearance of an aberrant methylation of one of these six genes in saliva analyzed during posttreatment follow-up. Methylations of *ECAD*, *p16*, *MGMT*, *DAPK*, and *RASSF1A* are among the most frequently reported epigenetic alterations in HNSCC (7, 14, 20–22) but *TIMP3* methylation had never been described in this pathology, although it is a frequent alteration in many human tumors, including non–small cell lung cancers

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Table 3. Correlation between clinicopathologic and histologic characteristics and methylation status

Variables	No. patients (%)	No methylation (%)	Hypermethylation (%)	P
Patients				
Total	90 (100)	21 (23)	69 (77)	—
Age	57 ± 12	57.5 ± 13	57 ± 10	0.8
Males	77	18 (88)	59 (75)	0.7
Females	13	3 (12)	10 (25)	
Addictions				
Tobacco use* (current or former)	84	20 (95)	64 (90)	1
Alcohol use† (current or former)	64	13 (75)	51 (68)	0.28
Second primary cancer‡	22 (31)	8 (38)	14 (20.6)	0.14
Tumor location				
Oral cavity	25	4 (19)	21 (30)	0.3
Oropharynx	32	9 (43)	23 (34)	
Hypopharynx	17	6 (27.5)	11 (16)	
Larynx	16	2 (8.5)	14 (20)	
Tumor stage§				
I	8	3 (14.3)	5 (7.2)	0.06∥
II	32	8 (38.1)	24 (34.8)	
III	22	8 (38.1)	14 (20.3)	
IV	28	2 (9.5)	26 (37.7)	
Node stage§				
N_0	58	18 (86)	40 (58)	0.02
$>N_0$	32	3 (14)	29 (42)	
Clinical stage¶				
I	7	3 (14.3)	4 (5.8)	0.03∥
II	22	6 (28.6)	16 (23.2)	
III	21	8 (38.1)	13 (18.8)	
IV	40	4 (19)	36 (52.2)	
Histologic grade**				
1	61	14 (66.7)	47 (68.1)	0.94∥
2	16	4 (19)	12 (17.4)	
3	13	3 (14.3)	10 (14.5)	
Histologic classification††				
Verrucous carcinoma	85	19 (90)	66 (86)	0.7
Basaloid SCC	5	2 (10)	3 (14)	

NOTE: Results for methylation are presented as mean ± SD for quantitative variables and as *n* (%) for qualitative ones.
*Smokers: current (*n* = 71) and former (*n* = 13).
†Alcohol use: current (*n* = 59) and former (*n* = 4).
‡Patients received radiotherapy before samples.
§Tumor and lymph node stages were determined by clinical and radiological examination.
∥Cochrane-Armitage trend test.
¶Clinical stage was determined by combining tumor and node stages.
**International Union Against Cancer classification: 1, well differentiated; 2, moderately differentiated; 3, poorly differentiated.
††According to WHO classification (17).

Table 4. Gene promoter hypermethylation in 60 tumors and paired saliva samples at diagnosis

	No. positive samples (%)		κ coefficient*
	Tumor	Saliva	
At least one methylated gene	49 (82)	47 (78)	0.833 (0.66-1.01)
TIMP3	30 (50)	17 (28)	0.56 (0.38-0.75)
ECAD	23 (38)	12 (20)	0.57 (0.37-0.78)
p16	20 (33)	16 (27)	0.8 (0.64-0.96)
MGMT	16 (27)	13 (22)	0.86 (0.73-1.01)
DAPK	14 (23)	9 (15)	0.7 (0.49-0.92)
RASSF1A	14 (23)	10 (17)	0.8 (0.62-0.98)
p15	10 (17)	7 (12)	0.79 (0.57-1.01)
p14	6 (10)	2 (3)	0.47 (0.05-0.89)
APC	6 (10)	4 (7)	0.78 (0.49-1.07)
FHIT	5 (8)	2 (3)	0.47 (0.05-0.84)
hMLH1	1 (1.5)	0 (0)	—

*κ coefficient indicates the overall agreement between aberrant methylation from tumors and saliva.

(9, 23). *TIMP3* encodes for a metalloproteinase inhibitor that suppresses tumor growth, angiogenesis, invasion, and metastasis (23, 24); it is located on chromosome arm 22q, a region where losses of heterozygosity have never been described in HNSCC, although they are frequent in other chromosomal regions (25–27). Therefore, methylation might be an important mechanism to silent *TIMP3* in HNSCC; additional mechanisms, such as microRNA, might be involved and are currently investigated. Recently, methylation of several other genes has been described in HNSCC, and a panel of four genes, including only three of our series (i.e., *ECAD*, *p16*, and *MGMT*), has been shown to allow methylation detection in 86.7% of HNSCC from Indian patients (28). Variability in epigenetic abnormalities between tumor series might be linked to race and/or risk factors. In our series, patients were French and Caucasians, with a great proportion of oropharynx and hypopharynx localizations in relation to tobacco/alcohol consumption (2), and were representative of the cohorts of HNSCC patients treated in France. Methylation of these six genes was never observed in saliva from patients with nonmalignant head and neck disease, including those with alcohol and/or tobacco consumption, and in only 10% of histologically normal mucosa samples taken in the vicinity of the largest tumors, a result suggesting that methylation of these six genes is a late event during transformation of the oral mucosa in HNSCC. In our series, we have observed only one correlation between HNSCC characteristics and methylation status of a specific gene (i.e., *RASSF1* methylation was more frequent in poorly differentiated tumors or in those with lymph node metastasis). Although several publications (14, 29, 30) suggested that methylation of a specific gene or a combination of genes might correlate to tumor characteristics and/or prognosis, patient cohorts were usually small; therefore, there is not yet consensus on an emerging methylation gene panel that may facilitate prognostic profiling of tumors particularly for HNSCC. Moreover, we did not observe any correlation between the presence of gene methylation in the tumors (or saliva; data not shown) and prognosis. It was very convincingly shown that the number of methylated genes in individual samples correlated to survival in T-cell acute leukemia (31) but it was only suggested in a small retrospective study of 51 HNSCCs (32). Several studies have suggested the potential diagnosis value of new salivary biomarkers that distinguished between saliva from normal and HNSCC patients, such as salivary transcriptome, mutation of p53, and microsatellite analysis (4, 19, 33). None of those markers have yet been evaluated for early detection of primary or relapsing HNSCC. In this prospective study, we have shown that methylation analysis of a panel of six genes in saliva of HNSCC patients followed after initial treatment allowed to detect abnormality few months before clinical relapse; then, careful follow-up of patients with positive saliva allowed diagnosing of tumors when they were still very small and thereby surgically curable. Based on our results, we cannot conclude whether this aberrant methylation had persisted since diagnosis or had reappeared after treatment; normalization of the saliva after the second surgery in patients who had relapsed suggested that the second hypothesis is frequent. This point will be investigated because if some patients have persistent abnormalities in the saliva just after treatment, they could be followed more carefully or treated more aggressively. The only false-negative analysis of our series was observed in a very rapidly growing tumor that has led to death in 3 months, and there was no false positive. Methylation detection in saliva better predicted relapse than FDG-PET, a technique used to assess HNSCC response to therapy and relapse (34). Furthermore, methylation profiles of second primary and newly diagnosed tumors were similar, a result that reinforces its potential usefulness in posttreatment surveillance. The 14-month time point for first saliva collection

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Table 5. Detection of gene methylation in saliva collected during follow-up of patients who have relapsed

Patient no.	Methylation status at diagnosis		Methylation status during follow-up (mo after diagnosis)	Relapse (mo after diagnosis)
	Tumor	Saliva		
1	*TIMP3, ECAD, MGMT, p16*	*TIMP3, ECAD, MGMT, p16*	*MGMT, p16* (14)	Yes (16)
2	*RASSF1A, TIMP3, DAPK*	*RASSF1A*	*RASSF1A* (16)	Yes (30)
3	*RASSF1A, p16*	*RASSF1A, p16*	*p16* (10)	Yes (17)
4	*p16, MGMT, RASSF1A*	*p16, MGMT*	*MGMT* (20)	Yes (28)
5	*p16*	*p16*	No methylation (14), no methylation (17), p16 (21)	Yes (22)
6	*RASSF1A, MGMT*	*RASSF1A*	No methylation (16)	Yes (17), death (20)
7-22	Positive	Positive	No methylation	No

after treatment was choose firstly because, in our clinical practice, all patients have a first staging between 12 and 15 months and secondly because the full treatment requires 5 months and a wait and watch period allows to observe patients who progress within the few months following treatment. Methylation-specific PCR had sufficient sensitivity and specificity to detect HNSCC exfoliated cells in saliva few months before clinical relapse. Quantitative real-time PCR, as used in some studies (35), might improve the level of detection, but it remains to be proved that it will not increase false positives. Therefore, we will continue the study to confirm the results with the same design. In conclusion, we have defined, in a French cohort of HNSCC patients, a minimal gene methylation panel that is specific of the tumoral process and sensitive enough to detect malignant exfoliated cells in saliva few months before clinical diagnosis of relapse; those results open new perspectives in HNSCC management. Another application would certainly be early detection of HNSCC in high-risk subjects with tobacco and alcohol consumption, with some limitations due to the poor compliance of this population.

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