Molecular and Cytogenetic Subgroups of Oropharyngeal Squamous Cell Carcinoma

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Abstract **Purpose:** The aim of this study was to acquire further insights into the pathogenetic pathways of head and neck squamous cell carcinomas (HNSCC) that may be useful for identifying new biomarkers instrumental in developing more specific treatment approaches.

Experimental Design: Cell cycle regulators and epidermal growth factor receptor (*EGFR*) and *BRAF* genes were analyzed in a series of 90 oropharyngeal SCCs of a cohort of surgically treated patients from a single institution, and the results were matched with the presence of high-risk human papillomavirus (HR-HPV) DNA and the *TP53* status.

Results: At least four distinct groups of tumors were identified sharing a common histology but displaying different molecular/cytogenetic patterns: (*a*) 19% were HPV-positive SCCs whose lack of alterations of the investigated genes could explain their particular natural history, which requires less aggressive treatment; (*b*) 37% were HPV-negative SCCs carrying *TP53* mutations, which may be more effectively treated by drugs acting through p53-independent apoptosis; (*c*) 34% were HPV-negative SCCs carrying wild-type *TP53* and loss of 9p21 ($p16^{INK4a}$ and $p15^{INK4b}$) and/or cyclin D1 overexpression that justify treatment with DNA-damaging drugs followed by cell cycle inhibitors; and (*d*) 10% were HPV-negative lacking tumor suppressor genes and cell cycle alterations. The second, third, and fourth groups also showed an increased copy number of *EGFR* and chromosome 7 (43%) that might justify the additional or alternative use of EGFR inhibitors.

Conclusions: Our findings suggest that assessing HPV, *TP53*, 9p21, and *EGFR* status may be crucial to finding more tailored and beneficial treatments for oropharyngeal SCCs.

Head and neck squamous cell carcinomas (HNSCC) are usually treated with surgery and/or radiotherapy, and advanced cases may be also treated with concomitant chemotherapy and radiotherapy. However, a greater understanding of the complex process leading to the transformation and maintenance of the malignant phenotype of HNSCC might help in the identification of diagnostic, prognostic, or predictive markers as well as of new therapeutic targets.

Despite their shared histology, the presence or absence of high-risk human papillomavirus (HR-HPV) identifies two distinct HNSCC entities with different clinical properties and genetic-molecular patterns. Clinically, the tumors with integrated viral DNA show a more favorable outcome than those that are HR-HPV negative (1). At molecular level, the HR-HPV–positive HNSCCs are characterized by the lack of $p16^{INK4a}$ gene deletion coupled with p16 protein expression (2) and a unique gene expression profile (3), with a decreased occurrence of *TP53* mutations, cyclin D up-regulation/amplification, 14-3-3σ and RASSF1A promoter methylation (4), and loss of heterozygosity at 17p, 9p, and 3p (5, 6). These latter are common alterations in patients with HPV-negative HNSCCs.

Clinical behavior of HPV-negative HNSCCs is heterogeneous, and it is warranted to better characterized this gray zone. The most frequent molecular alteration carried by patients with HPV-negative HNSCCs is the *TP53* mutation, which seems to correlate with a poor response to both radiotherapy (7) and chemotherapy (8, 9) as well as with a poor prognosis (10). Few information is available regard the role of cell cycle regulators and epidermal growth factor receptor (*EGFR*) gene status in HPV-negative HNSCCs (11).

The aim of this study was to acquire further insights into the pathogenetic pathways of the subsets of HR-HPV–positive and

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HR-HPV–negative oropharyngeal SCCs that may be useful for identifying major new biomarkers and help in the development of more tailored treatment approaches. To this end, we undertook molecular/cytogenetic analyses of tumor suppressor genes/cell cycle regulators, as well as receptor tyrosine kinase and serine/threonine kinase in a series of 90 patients with surgically resected oropharyngeal SCCs who had been previously characterized in terms of HR-HPV DNA and *TP53* status.

Our findings highlight the heterogeneous biology underlying oropharyngeal SCCs, and the fact that the frequently overlapping cell cycle protein and receptor tyrosine kinase alterations are mainly restricted to HPV-negative tumors. These findings may have therapeutic implications that warrant further investigation in prospective studies.

Materials and Methods

Patients and tumor specimens

Ninety consecutive cases of oropharyngeal SCCs (eight stage I-II and 82 stage III-IV) surgically treated at the National Cancer Institute of Milan between 1990 and 1999 were fully evaluable in terms of the availability of pathologic material and adequate long-term follow-up information. Fifty-eight patients (64%) had received postoperative radiotherapy because of the presence of commonly accepted postoperative pathologic risk features. None of them had undergone chemotherapy. A written informed consent was obtained from all the patients.

All of the formalin-fixed, paraffin-embedded tumors had been previously investigated for HR-HPV16 DNA and physical status by means of real-time PCR, for the *TP53* mutation, p16 immunophenotyping, and homozygous deletion (2).

Detection and relative quantification of HPV16 E6 and E7 expression

Total RNA from microdissected samples was isolated by means of the TRIzol method (Life Technologies, Carlsbad, CA) and reverse transcribed with Superscript reverse transcriptase (Life Technologies) using both oligo dT and random hexamers. The HPV16 DNA–positive samples were investigated for E6 and E7 mRNA expression by means of real-time quantitative PCR (ABIPRISM 5700 PCR Sequence Detection Systems, Applied Biosystems, Foster City, CA) using a Taqman-based analysis (12). Formalin-fixed, paraffin-embedded CaSki cells were used as a positive control.

Immunohistochemistry

p53, cyclin D1, and cyclin-dependent kinase 4. Immunohistochemistry was done on 2-μm cut formalin-fixed and paraffin-embedded tumoral sections using the peroxidase-streptavidin method (1:300 in PBS, DAKO, Carpinteria, CA). All of the stains were developed using 3,3-diaminobenzidine (Sigma, St. Louis, MO). The primary antibodies were a p53 mouse monoclonal antibody diluted 1:400 (YLEM, Rome, Italy) and cyclin D1 rabbit monoclonal antibody diluted 1:100 (Clone SP4, Lab Vision, Fremont, CA), with antigen retrieval using 5 mmol/L citrate buffer (pH 6) in an autoclave at 95°C for 6 to 15 minutes; and cyclin-dependent kinase 4 (Cdk4) rabbit polyclonal antibody diluted 1:400 (C-22, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

The positive controls were a serous ovarian carcinoma with a known *TP53* mutation for p53, a mantle cell lymphoma for cyclin D1, and a WD/DD liposarcoma for Cdk4.

For p53, a cutoff value of ≥50% positive nuclei was used as a tentative marker of predicted mutation (13).

EGFR. EGFR was immunostained using the EGFR-DAKO kit. A ready-to-use monoclonal mouse anti-human EGFR antibody and CAMA-1 and HT-29 cell lines (negative and positive controls) were provided. EGFR-immunostained cells were quantitatively evaluated, and the staining intensity was scored as detailed in Table 1. A final score obtained by combining the two score values was calculated.

Fluorescence *in situ* hybridization (FISH) on locus *9p21* and the *EGFR* gene

Tumoral sections of 2 μm were placed on silanized slides, air-dried, and baked at 56°C overnight in Hybraite Thermoblock (Vysis, Downers Groove, IL), before being deparaffined with three 10-minute xylene washings, rehydrated in a graded alcohol series, washed in Tris-EDTA (15 minutes at 96°C), and placed in double-distilled water for 2 minutes. After digestion with 10 mg/mL Pepsin in 0.01 N HCl for 15 to 20 minutes at 37°C, the specimens were dehydrated in alcohol for 3 minutes. The probes were placed on the target area, covered with a 18 × 18 mm coverslip, and sealed with rubber cement. Denaturation (85°C for 1 minute) and hybridization (37°C overnight) were done in Hybrite Thermoblock. After being washed in 2×SSC at 73°C for 2 minutes, the slides were treated with mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole.

For the *9p21* locus, the cosmid C5, spanning the chromosomal region from the $p16^{INK4a}$ to the $p15^{INK4b}$ gene (about 50 kb), and the specific probe BAC clone RP11-149I2 (kindly provided by Prof. M. Rocchi, University of Bari, Bari, Italy), spanning 100 bp and directly labeled with Spectrum Orange dUTP by the Vysis Nick Translation kit were used associated with the Spectrum Green-labeled control probe CEP9 (Vysis). The 9p21 hybridization pattern was evaluated on the basis of previously described criteria (14).

The LSI EGFR dual-colour probe (Vysis) was used for *EGFR*. The *EGFR* copy number in each nucleus was assessed in relation to the chromosome 7 copy number, with the presence of an ''amplification cluster'' of *EGFR* indicating gene amplification. The numerical status of chromosome 7 was defined as being balanced polysomic when the cancer cell population showed multiple (>3) 7 centromere signals associated with the same number of *EGFR* signals. A distinction was made between low (trisomic and tetrasomic hybridization patterns) and high polysomy, when the chromosome 7 copy number was ≥5.

Homozygous deletions of $p14^{ARF}$ and $p15^{INK4b}$ genes

DNA was extracted from 7-μm serial sections of the most representative tumoral block as previously described (15), and its quality was tested in each sample by amplifying the human *β-globin* gene fragment (200 bp).

Table 1. EGFR scoring system

Quantitative evaluation		Staining intensity		Final evaluation	
% Immunopositive cells	Score	Intensity	Score	Combined score level	Expression
<1	0	Weak	1	0-2	Low
1-20	1	Moderate	2	3-5	Intermediate
20-50	2	Strong	3	6-7	High
50-80	3				
>80	4				

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Table 2. Primers used for comparative duplex PCR

TSG exon	Primer sequence (5′→3′)		Annealing temperature (°C)
	Sense	Antisense	
$p15^{INK4b}$ exon 1	AATGCGCGAGGAGAACAAG	CGGCTTCCAGGAGCTGTC	60
$p15^{INK4b}$ exon 2	CTTCCTGGACACGCTGGT	GCAGGTACCCTGCAACGTC	60
$p14^{ARF}$ exon 1β	AGTTAAGGGGGCAGGAGTG	ATCCGGAGGGTCACCAAG	61
β-globin	ACACAACTGTGTTCACTAGC	GCAGACTTCTCCTCAGGAG	

The homozygous deletion assays were done using 30 cycles of comparative duplex PCR to investigate *$p14^{ARF}$* exon 1β and *$p15^{INK4b}$* exons 1 and 2. Each exon was coamplified with a fragment of human *β-globin* as previously described (2). The primers and annealing temperature used for each PCR are shown in Table 2. The negative control was the K562 cell line, which has a homozygous deletion at the *INK4A* locus.

Mutational analysis

EGFR. Mutational analysis, restricted to exon 19 of the *EGFR* gene in which mutations have been found in HNSCC (16, 17), was done as previously described (18). After purification, the PCR products underwent automated DNA sequencing (3100 Genetic Analyzer, Applied Biosystems).

BRAF. Mutational analysis was restricted to exon 15 of the *BRAF* gene. The DNA was amplified using previously described specific primers (19).

Results

HR-HPV

Assessment of HPV status. As previously reported (2), we successfully assessed HPV physical status in 16 of 17 oropharyngeal SCCs that were positive for HPV-DNA16 (HPV$^+$), and we found viral integration in all 16 HPV$^+$ cases. In detail, two cases showed full integration, whereas the others had fewer E2 than E6 transcripts, a finding that is consistent with the presence of episomal and integrated viral forms. The E2/E6 ratios are shown in Table 3.

HPV16 E6 and E7 mRNA expression detection. The samples that were positive for HPV16 DNA were evaluated for E6 and E7 mRNA expression by means of relative real-time PCR analysis, and the detection of both in all 17 HPV$^+$ cases (Table 3) suggested an active role of the virus. The *hRnaseP* control was positive and amplified in all samples, thus confirming the adequate quantity and quality of the cDNA.

Tumor suppressor genes and cell cycle regulators

P53

TP53 *mutation analysis.* Overall, as previously reported (2), 35 of 90 (39%) cases harbored *TP53* mutations (*TP53* mut), and 55 cases (61%) carried *TP53* wild type (*TP53* wt). The frequency of *TP53* mutations was significantly lower ($P < 0.025$) in the HPV$^+$ SCCs (2 of 17, 12%; Table 3) than in the HPV$^-$ tumors (33 of 73, 45%; Tables 3 and 4).

Table 3. HR-HPV–positive oropharygeal SCC

Case	Physical status	E2/E6	mRNA		TP53/p53		Homozygous deletion					9p21 FISH	Cyclin D1 IHC	EGFR		
							$p15^{INK4b}$		*$p14^{ARF}/p16^{INK4a}$*							
			E6	E7	*TP53* mut	IHC	Exon 1	Exon 2	Exon 1β	Exon 1α	Exon 2			IHC	FISH	Mutation
1	Mixed	0.31	+	+	wt	–	N	N	N	N	N	Disomy	–	High	Disomy	wt
2	Mixed	0.04	+	+	wt	–	N	HD	N	N	N	Disomy	–	High	Disomy	wt
3	Mixed	0.06	+	+	$Gly^{199}Arg$/ GGA>AGA	–	NE	NE	NE	N	NE	Disomy	–	High	Disomy	wt
4	Mixed	0.03	+	+	wt	–	N	N	N	N	N	Disomy	–	Intermediate	HED	wt
5	Mixed	0.2	+	+	wt	–	N	N	N	N	N	Disomy	–	High	Disomy	wt
6	Mixed	0.2	+	+	wt	<20%	N	N	N	N	N	Disomy	–	High	Disomy	NE
7	Mixed	0.09	+	+	wt	–	N	N	N	N	N	Disomy	–	High	Disomy	wt
8	Mixed	0.04	+	+	wt	–	N	N	N	N	N	Disomy	–	Intermediate	Disomy	wt
9	Mixed	0.05	+	+	wt	–	N	N	N	N	N	Disomy	–	High	Disomy	wt
10	Mixed	0.02	+	+	wt	–	N	N	N	N	N	Disomy	–	High	Disomy	wt
11	Mixed	0.04	+	+	$Asp^{281}Asn$/ GAC>AAC	80%	N	N	N	N	N	Disomy	NE	High	Disomy	wt
12	Mixed	0.01	+	+	wt	–	NE	NE	NE	NE	NE	Disomy	60%	Intermediate	Disomy	wt
13	Mixed	0.03	+	+	wt	<20%	N	HD	HD	N	N	Disomy	–	Intermediate	Monosomy	NE
14	Mixed	0.04	+	+	wt	–	NE	NE	NE	NE	NE	Disomy	–	High	Disomy	wt
15	Integrated/ ND		+	+	wt	–	N	N	HD	N	N	Disomy	–	High	Disomy	wt
16	ND		+	+	wt	<20%	N	N	N	N	NE	Disomy	–	Intermediate	Monosomia	wt
17	Integrated		+	+	wt	–	N	N	NE	N	N	Disomy	–	Intermediate	High polysomy	wt
			100%		12%	6%			20%				0%	100%	6%	0%

Abbreviations: ND, not done; +, positive; –, negative; IHC, immunohistochemistry; N, normal; HD, homozygous deletion; NE, not evaluable; HED: hemizygous deletion.

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Table 4. HR-HPV–negative *TP53* mutated oropharyngeal SCC

Case	TP53/p53		Homozygous deletion					9p21 FISH	cyclin D1 IHC	EGFR		
			$p15^{INK4b}$		$p14^{ARF}/p16^{INK4a}$							
	TF53 mutation	IHC	ex1	ex2	ex1β	ex1α	ex2			IHC	FISH	Mutation
18	Exon 4, A1a^{39}Pro/ GCA>CCA	80%	N	HD	HD	HD	HD	HD	30%	Intermediate	Disomy	wt
19	Exon 5, Lys^{132}Arg/ AAG>AGG	50%	N	HD	HD	N	N	Disomy	–	High	Disomy	wt
20	Exon 5, Ala^{161}Asp/ GCC>GAC	70%	N	N	N	N	N	HD	>70%	High	High polysomy	wt
21	Exon 7, Arg^{248}Trp/CGG>TGG	90%	N	N	N	N	N	Disomy	–	High	Low polysomy	ND
22	Exon 6, Tyr^{220}Cys/TAT>TGT	>80%	N	HD	HD	HD	HD	HD	–	High	Amplification	ND
23	Exon 6, His^{193}Arg/CAT>CGT	>80%	N	N	N	N	N	Disomy	–	High	Disomy	wt
24	Exon 6, Tyr^{205}Cys/TAT>TGT	>80%	N	N	N	N	N	Disomy	60%	–	Monosomy	wt
25	Exon 6, Tyr^{220}Cys/TAT>TGT	50%	N	HD	NE	NE	NE	Disomy	40%	High	Disomy	ND
26	Exon 6, Arg^{213}Trp/CGA>TGG	50%	N	N	N	N	N	HD	20%	High	Low polysomy	wt
27	Exon 6, Tyr^{205}Cys/TAT>TGT	90%	N	HD	HD	HD	N	HD	–	NE	HD	ND
28	Exon 7, Arg^{248}Trp/CGG>TGG	90%	N	N	N	N	N	HED	–	Intermediate	Disomy	ND
29	Exon 7, Arg^{248}Gln/CGG>CAG	90%	N	HD	HD	HD	HD	Nv	40%	NE	Disomy	wt
30	Exon 7, Arg^{306}Thr/GCA>ACA	>80%	N	HD	HD	N	NE	Disomy	>70%	High	Disomy	ND
31	Exon 7, Arg^{248}Trp/CGG>TGG	>80%	N	HD	HD	HD	HD	HD	>70%	High	Amplification	ND
32	Exon 7, Cys^{242}Ser-TGC>TCC	50%	N	HD	HD	HD	low	HD	–	Intermediate	Low polysomy	ND
33	Exon 7, Tyr^{234}Cys/TAC>TGC	80%	N	N	N	N	N	Disomy	>70%	High disomy		Gly^{719}Glu GGA>GAA
34	Exon 8, Gly^{266}Val/GGA>GTA	90%	N	HD	HD	N	N	Disomy	>70%	Intermediate	Low polysomy	ND
35	Exon 8, Pro^{278}Leu/CCT>CTT	60%	N	N	N	N	N	Disomy	–	Intermediate	Low polysomy	ND
36	Exon 8, Arg^{282}Trp/CGG>TGG	>80%	N	HD	HD	NE	N	HD	>60%	High	Monosomy	wt
37	Exon 8, Arg^{282}Gly/CGG>GGG	60%	N	N	N	N	N	HD	50%	Intermediate	Low polysomy	ND
38	Exon 9, Gly^{325}Glu/GGA>GAA	70%	N	HD	HD	HD	HD	HD	–	Intermediate	High polysomy	ND
39	Exon 10, Leu^{348}Ser/TGG>TCG	60%	NE	NE	HD	HD	HD	HD	–	Intermediate	Disomy	wt
40	Exon 6, Glu^{204}Stop/GAG>TAG	>80%	N	N	HD	HD	HD	HD	20%	–	Disomy	wt
41	Exon 7, Glu^{258}stop/GAA>TAA	–	N	N	N	N	N	HD	–	High	Low polysomy	ND
42	Exon 6, Gly^{244}stop/GAG>TAG	–	N	N	N	HD	N	Disomy	30%	Intermediate	High polysomy	ND
43	Exon 6, Arg^{213}Stop/CGA>TGA	–	NE	HD	HD	HD	HD	HD	20%	High	HED	wt
44	Exon 6, Arg^{213}Stop/CGA>TGA	–	N	HD	N	N	N	Disomy	60%	High	Amplification+ low polysomy	ND
45	Exon 8, Glu^{298}Stop-GAG>TAG	–	N	HD	HD	HD	HD	HD	30%	High	Disomy	ND
46	13317-13329 delTAGGTCTGGCCCC	–	N	HD	HD	HD	HD	HD	20%	NE	Amplification+ low polysomy	ND
47	14510-14524 delGACCGGCGCACAGAG	50%	NE	N	N	low	HD	Disomy	70%	Intermediate	Amplification+ low polysomy	ND
48	Exon 10, del17661	50%	N	low	low	low	low	HED	30%	High	Disomy	wt
49	INTRONE 9, A17708T	90%	N	HD	HD	NE	HD	HD	>70%	Low	Disomy	wt
50	Exon 7, Ser^{261}Ser/AGT>AGC	–	NE	NE	NE	NE	NE	HED	20%	Intermediate	Low polysomy	ND
	45%				66%			62%	21%	90%	48%	

Abbreviations: ND, not done; +, positive; –, negative; IHC, immunohistochemistry; N, normal; HD, homozygous deletion; NE, not evaluable; HED, hemizygous deletion.

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p53 immunohistochemistry. A cutoff of ≥50% positive nuclei for p53 immunoreactivity was used as a tentative marker of predicted mutation (13). The fact that p53 immunoreactivity was found in 27 of 35 (77%) *TP53* mut cases (Tables 3 and 4), whereas none of 55 *TP53* wt tumors was p53 immunopositive ($P < 0.001$; Tables 3 and 5), indicates that ≥50% nuclear immunoreactivity may make a good surrogate of *TP53* mutations.

The frequency of p53 immunoreactivity was significantly lower ($P \leq 0.025$) in the HPV$^+$ SCCs (1 of 17, 6%; Table 3) than in the HPV$^-$ tumors (27 of 73, 37%).

9p21 locus

Homozygous deletion of p16^{INK4a}, p14^{ARF}, *and* p15^{INK4b} *tumor suppressor genes.* We have previously shown that 100% of the HPV$^+$ cases carried a normal *p16^{INK4a}* gene in keeping with p16 overexpression, whereas a *p16^{INK4a}* homozygous deletion was found in 47% of the HPV$^-$ cases, similarly distributed among the *TP53* mut (48%) and *TP53* wt tumors (46%; ref. 2).

To characterize the DNA status of the two other tumor suppressor genes (TSG) mapping in tandem to the *9p21* locus (*p14^{ARF}* and *p15^{INK4b}*), we assayed homozygous deletions by means of comparative duplex PCR. A homozygous deletion of one or more of the three TSGs was found in 43 of 76 SSCs (56%), in 23 of which (53%) it encompassed all three TSGs.

The frequency of an homozygous deletion of one or more TSGs at the *9p21* locus was significantly lower ($P < 0.001$) in the HPV$^+$ (3 of 15, 20%) than the HPV$^-$ tumors (40 of 61, 65%; Fig. 1), both *TP53* mut (21 of 32) and *TP53* wt (19 of 29; Tables 4 and 5, respectively). In the HPV$^+$ tumors, the homozygous deletion involved both *p14^{ARF}* and *p15^{INK4b}* in

Table 5. HR-HPV–negative *TP53* wild-type oropharyngeal SCC

Case	TP53/p53		Homozygous deletion					9p21 FISH	cyclin D1 IHC	EGFR		
			p15^{INK4b}		*p14^{ARF}/p16^{INK4a}*							
	TF53 mut	IHC	Exon 1	Exon 2	Exon 1β	Exon 1α	Exon 2			IHC	FISH	Mutation
51	wt	20%	NE	NE	NE	NE	NE	Disomy	–	–	Low polysomy	ND
52	wt	30%	NE	NE	NE	NE	NE	NE	20%	Low	NE	ND
53	wt	20%	N	HD	HD	NE	HD	Monosomy	40%	Intermediate	High polysomy	ND
54	wt	30%	NE	NE	NE	NE	NE	Disomy	80%	Intermediate	Low polysomy	wt
55	wt	30%	N	HD	HD	HD	HD	HD	30%	Low	High polysomy	ND
56	wt	20%	N	HD	N	N	N	Disomy	–	Intermediate	Disomy	wt
57	wt	30%	NE	NE	NE	NE	NE	Disomy	30%	Intermediate	Disomy	ND
58	wt	30%	HD	HD	HD	HD	HD	HED	–	Intermediate	Disomy	ND
59	wt	–	N	N	N	N	N	HD	–	High	High polysomy	wt
60	wt	–	N	N	N	N	N	Disomy	40%	High	High polysomy	ND
61	wt	–	N	HD	HD	HD	N	HD	–	Intermediate	Low polysomy	ND
62	wt	–	NE	NE	NE	NE	NE	HED	40%	High	Disomy	wt
63	wt	–	N	N	nv	N	N	HD	70%	Intermediate	High polysomy	ND
64	wt	–	N	low	low	low	NE	HED	–	High	Disomy	ND
65	wt	–	NE	NE	NE	NE	NE	Disomy	–	Intermediate	Disomy	ND
66	wt	–	NE	NE	NE	NE	NE	NE	–	Intermediate	NE	ND
67	wt	–	N	N	N	N	N	HD	30%	High	Disomy	wt
68	wt	–	N	HD	HD	HD	HD	HD	–	Intermediate	Disomy	ND
69	wt	–	N	N	HD	HD	HD	HD	–	High	Disomy	ND
70	wt	–	N	HD	HD	na	HD	Disomy	30%	NE	NE	ND
71	wt	–	HD	HD	HD	HD	HD	HD	>70%	High	Disomy	ND
72	wt	–	N	N	N	N	N	Disomy	–	Intermediate	Low polysomy	ND
73	wt	–	N	N	N	N	N	Disomy	–	High	Disomy	ND
74	wt	–	N	HD	HD	HD	HD	Monosomy	20%	High	High polysomy	ND
75	wt	–	NE	NE	NE	NE	NE	Monosomy	50%	High	Disomy	ND
76	wt	–	NE	NE	NE	NE	NE	HD	40%	Intermediate	Disomy	ND
77	wt	–	NE	NE	NE	NE	NE	HD	nv	Low	NE	ND
78	wt	–	N	HD	HD	HD	HD	HED	–	Intermediate	Disomy	ND
79	wt	–	HD	HD	low	low	NE	Disomy	–	Intermediate	Disomy	ND
80	wt	–	HD	HD	HD	HD	HD	Monosomy	60%	–	HED	ND
81	wt	–	N	N	N	N	N	HD	30%	High	Disomy	wt
82	wt	–	N	HD	HD	HD	HD	HD	–	High	Low polysomy	ND
83	wt	–	N	N	N	N	N	Disomy	–	High	Disomy	wt
84	wt	–	HD	HD	HD	NE	NE	NE	–	Intermediate	Disomy	ND
85	wt	–	N	N	N	N	N	HD	–	High	Low polysomy	wt
86	wt	–	N	HD	N	N	N	Disomy	–	Intermediate	Disomy	wt
87	wt	–	N	HD	HD	N	N	Disomy	–	Low	Low polysomy	wt
88	wt	–	N	HD	HD	N	N	Disomy	70%	High	Disomy	ND
89	wt	–	N	HD	HD	HD	HD	HD	20%	–	Disomy	ND
90	wt	–	NE	NE	NE	NE	NE	HD	–	High	Low polysomy	wt
					65%			62%	10%	82%	39%	

Abbreviations: ND, not done; +, positive; –, negative; IHC, immunohistochemistry; N, normal; HD: homozygous deletion; NE: not evaluable; HED, hemizygous deletion.

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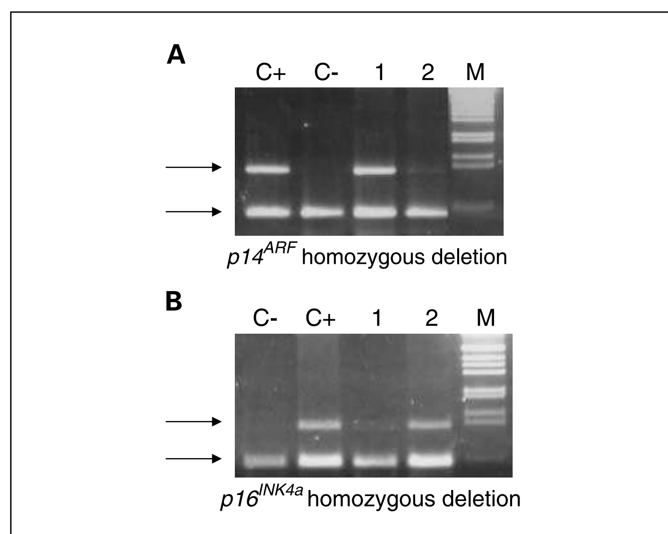

Fig. 1. Homozygous deletion analysis by using comparative duplex PCR of $p14^{ARF}$ and $p16^{INK4a}$. The lower band corresponds to β-globin fragment gene coamplified with the gene of interest (upper band). *A,* retention of $p14^{ARF}$ exon 1β in HPV^+ case 5 (*lane 1*) and absence in HPV^- case 18 (*lane 2*). *B,* absence of $p16^{INK4a}$ exon 1α in HPV^- case 68 (*lane 1*) and retention in HPV^+ case 5 (*lane 2*). *C+,* positive control, normal genomic DNA; *C−,* negative control, DNA from K562 cell lines carrying the *9p21* locus homozygous deletion; *M,* 1-kb molecular marker.

one case and only $p14^{ARF}$ or $p15^{INK4b}$ in the remaining two (Table 3).

FISH. Forty-three of the 86 analyzed cases (50%) showed a 9p21 loss: 32 (74%) homozygous deletions and 11 (26%) a hemizygous deletion or monosomic hybridization pattern.

All 17 HPV^+ cases showed a normal hybridization pattern (Table 3), whereas 62% of the HPV^- cases carried a 9p21 loss ($P \le 0.01$; Fig. 2): 20 of 32 *TP53* mut (Table 4) and 23 of 37 *TP53* wt (Table 5).

Combined homozygous deletion and 9p21 FISH analyses. Based on combined homozygous deletion and FISH analyses, a cumulative 9p21 loss occurred in 61 of 88 SCCs (69%): 3 HPV^+ (with homozygous deletion of p14 and/or p15), 28 HPV^-/*TP53* mut, and 30 HPV^-/*TP53* wt.

The frequency of 9p21 loss was significantly lower ($P < 0.001$) in HPV^+ tumors (3 of 17, 18%) than HPV^- tumors (58 of 71, 82%).

Cyclin D1 and Cdk4 immunohistochemical analysis

Cyclin D1. Eighty-eight cases were suitable for cyclin D1 analysis. Taking a cutoff of ≥70% positive cells, no immunoreactivity was found in 16 HPV^+ cases (one was not evaluable; Table 3), whereas 11 of 72 (15%) HPV^- cases were positive. Cyclin D1 overexpression was coupled with 9p21 loss in 9 of 11 cases (82%). In terms of *TP53* status, cyclin D1 immunoreactivity involved 7 of 33 *TP53* mut cases (21%) and 4 of 39 *TP53* wt cases (10%; Tables 4 and 5).

Cdk4. None of the 89 analyzed cases showed any Cdk4 immunoreactivity (data not shown).

Cumulative evidence of TSG and cell cycle alterations. Despite their similar phenotype, we identified four different molecular groups by means of HR-HPV, *TP53*, 9p21, and cyclin D1 analyses:

17 HPV^+ cases, showing occasionally (*TP53* mut, 9p21 loss) or null (cyclin D1 expression) nonoverlapping alterations (19%);
33 HPV^-/*TP53* mut cases, including 29 with evidence of additional cell cycle alterations consisting of 9p21 loss and/or cyclin D1 overexpression (37%);
31 HPV^-/*TP53* wt cases showing evidence of the same cell cycle alterations of the group II (34%);
9 HPV^-/*TP53* wt cases lacking cell cycle alterations (10%).

Groups II and III showed additional homozygous deletion of the $p14^{ARF}$ gene in 22 and 23 cases, respectively.

The concurrent abnormalities of *TP53* and $p14^{ARF}$ and $p16^{INK4b}$, $p15^{INK4b}$, and cyclin D1 provide further evidence of the possible collaborative role of multiple components of the same pathway (6, 20).

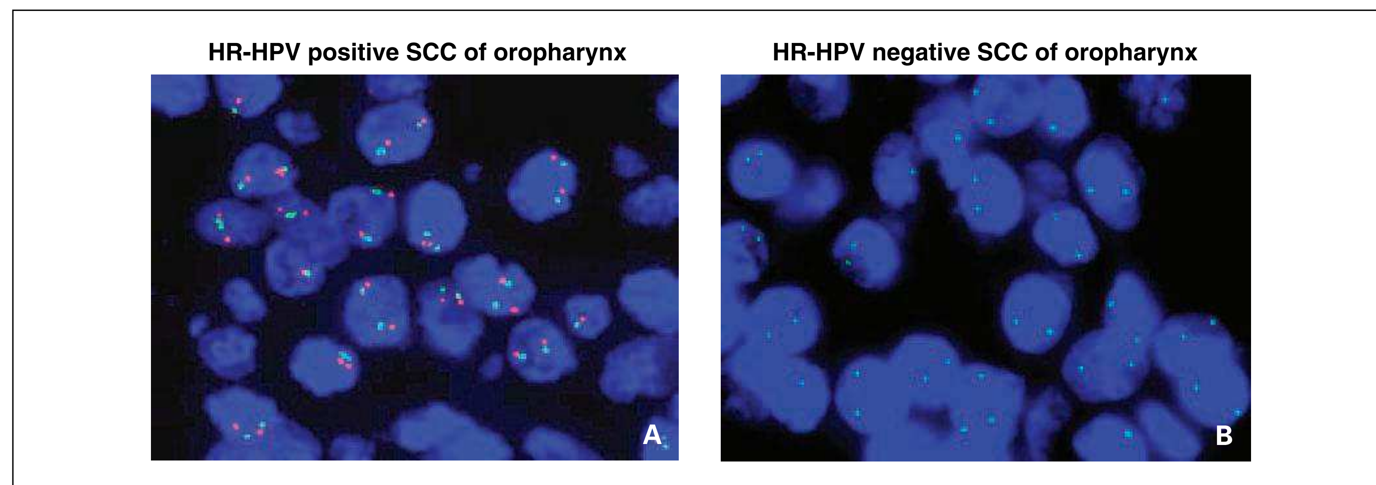

Fig. 2. FISH analysis using a CEP9/9p21 probe to interphasic nuclei. *A,* in HPV-positive case 5, the nuclei showed a normal disomic pattern of 9p21 represented by two centromere 9 signals (*green spots*) coupled with two 9p21 signals (*red spots*). *B,* in HPV-negative case 46, the nuclei showed a homozygous 9p21 deletion represented by two centromere 9 signals and no 9p21 signals.

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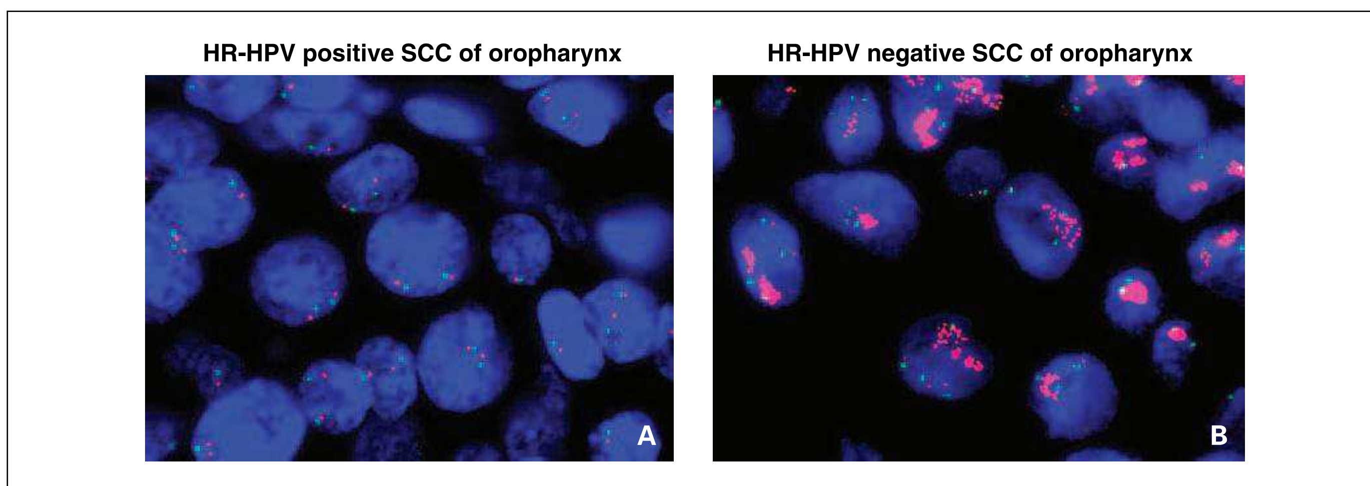

Fig. 3. FISH analysis using an *EGFR* probe to interphasic nuclei. *A,* in HPV-positive case 6, the nuclei showed a normal disomic pattern represented by two centromere 7 signals (*green spots*) coupled with two *EGFR* signals (*red spots*). *B,* in HPV-negative case 46, the nuclei showed both *EGFR* gene amplification represented by a red amplification cluster, and low-degree polysomy of chromosome 7 (trisomy) represented by three centromere 7 signals.

Kinases

Receptor thyrosine kinase: EGFR

Immunohistochemistry. The EGFR immunohistochemical analysis was done in 87 SCC cases, the majority of which (77 of 87, 88%) had an intermediate or high expression score. No significant difference in EGFR expression was found between the HPV$^+$ and HPV$^-$ cases (100% versus 86%) or between the *TP53* mut and *TP53* wt cases (90% versus 82%; Tables 3, 4, and 5).

FISH analysis. To investigate *EGFR* gene and chromosome 7 copy numbers, 86 SCCs were successfully analyzed by means of FISH, 38 (44%) of which showed an abnormal hybridization pattern: 5 of EGFR amplification, 26 of balanced polysomy, 3 of monosomy, 3 of hemizygous deletions, and 1 of homozygous deletion of chromosome 7.

There was a significant difference in the copy numbers of *EGFR* and chromosome 7 between the HPV$^+$ and HPV$^-$ cases (P < 0.01; Fig. 3). The 17 HPV$^+$ tumors showed no *EGFR* amplification; however, a single case carried high polysomy (6%; Table 3); whereas 30 of 69 HPV$^-$ cases (43%) had an increased *EGFR* or chromosome 7 copy number: 16 of 33 *TP53* mut (48%) and 14 of 36 *TP53* wt (39%; Tables 4 and 5, respectively). *EGFR* amplification occurred in 5 of 69 HPV$^-$ SCCs (7%), coupled with *TP53* mutations in all cases and with low polysomy in three cases. High polysomy was found in 9 (13%) and low polysomy (trisomy and tetrasomy) in 16 (23%) of the 69 cases. Interestingly, *EGFR* amplification or chromosome 7 polysomy was coupled with cyclin D1 overexpression in six cases.

Immunohistochemistry correlation. Despite the frequent EGFR overexpression, we did not find any correlation between the EGFR expression scores and the *EGFR*/chromosome 7 genotype. A high expression score associated with *EGFR* amplification or high polysomy as well as with low polysomy, disomy, monosomy, and hemizygous deletion, and the same associations were found for intermediate EGFR expression scores.

Mutational analysis. Forty of the 90 SCCs underwent *EGFR* mutational analysis restricted to exon 19: 15 HPV$^+$ and 25 HPV$^-$ cases (14 *TP53* mut and 11 *TP53* wt).

No *EGFR* mutation was found in the HPV$^+$ cases (Table 3), whereas 1 of 25 HPV$^-$ cases (4%) carried the Gly^{719}Glu (GGA > GAA) mutation (Table 4).

Cumulative molecular evidence of EGFR alterations. Gene amplification and chromosome 7 polysomy and mutation accounted for 32 (37%) of the 86 oropharynx SCCs, divided into the following four previously defined groups:

HPV$^+$: one case showing high polysomy;
HPV$^-$/*TP53* mut: 17 cases (5 with gene amplification, 3 with high polysomy, 8 with low polysomy, and 1 mutation);
HPV$^-$/*TP53* wt harboring cell cycle alterations: 11 cases (5 with high and 6 with low polysomy);
HPV$^-$/*TP53* wt cases lacking cell cycle alterations: 3 cases (1 with high and 2 with low polysomy).

There was a significant difference in the rate of *EGFR* alterations between the HPV$^+$ and HPV$^-$ cases (6% versus 45%; P < 0.01). Among HPV-negative cases, *EGFR* alterations did not segregate with a specific subgroup.

Serine/threonine kinase: BRAF

Mutational analysis. Thirty of the 90 SCCs were analyzed for *BRAF* mutations: 12 HPV$^+$, 8 HPV$^-$/*TP53* mut, and 10 HPV$^-$/*TP53* wt. We sequenced exon 15 of the *BRAF* gene, in which the classic V599E mutation is located, which occurs in >90% of *BRAF*-altered neoplastic diseases. No *BRAF* mutations were found, regardless of HPV or *TP53* status (data not shown).

Discussion

In a single-institution cohort of 90 surgically treated patients with oropharynx SCCs, we identified four groups of tumors that, although sharing the same histology, display different molecular/cytogenetic profiles.

The first group is represented by 17 (19%) HPV-positive oropharyngeal SCC, where the molecular/cytogenetic analyses here reported confirm our previous preliminary evidence that HPV-positive tumors are a distinct molecular entity (2). Consistently, in addition to the presence of a significant low

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occurrence of *TP53* mutations (12%; $P < 0.025$), HPV-positive SCCs show a significant reduced $p14^{ARF}$-$p15^{INK4b}$ deletion (20%; $P < 0.001$) and lack of cyclin D1 overexpression in comparison with HPV-negative tumors. Our 9p21 data are in line with the occasional allelic loss of chromosome arm 9p found in HPV-positive HNSCC by microsatellite analysis (5, 6). Moreover, the lack of *EGFR*-activating mutation and *EGFR* amplification and the unusual chromosome 7 polysomy (6%; $P < 0.01$) in comparison with HPV-negative tumors corroborate the peculiar simple profile of HPV-positive SCC characterized by low rate of genotypic alterations. Remarkably, despite of a normal disomic EGFR/chromosome 7 cytogenetic pattern in HPV-positive SCC and an amplified polysomic pattern in 43% of HPV-negative tumors, EGFR protein resulted similarly overexpressed in both tumor groups (100% versus 86%), supporting the notion that EGFR immunophenotyping does not mirror *EGFR* gene status. Cumulatively, our data strongly support that HPV-positive SCCs deserve an individualized less aggressive treatment (21), and that the neoplastic process is likely mainly due and supported by the oncogenic viral genes.

The second group (37%) consisted of 33 HPV-negative oropharyngeal SCCs carrying *TP53* mutations. The correlation between *TP53* status and the response to cisplatin/fluorouracil–based chemotherapy in HNSCC (9, 10) supports the idea that the response to cytotoxic DNA-damaging action requires an efficient p53-dependent apoptotic pathway. In this light, drugs acting through p53-independent apoptosis may be more effective in oropharyngeal SCCs carrying *TP53* mutations (22). Although the patients who may benefit from p53-independent treatment are best selected by means of mutational analysis, immunohistochemical assessments of increasing half-lives of p53 may represent a good surrogate if the cutoff point is $\geq$50% of tumoral cells with strong nuclear immunoreactivity (13). Using this cutoff level in our series, we found a significant correlation between protein stabilization and the presence of *TP53* mutations ($P < 0.001$). In this group, *TP53* alterations might represent the main oncogenic driving force.

The third group (34%) consisted of 31 HPV-negative cases carrying *TP53* wt, 9p21 ($p16^{INK4a}$ and $p15^{INK4b}$) loss, and/or cyclin D1 overexpression. The presence of these alterations offers a rationale for therapeutically target the cell cycle by means of CDK inhibition (23). Because of loss of checkpoint integrity due to $p16^{INK4a}$-$p15^{INK4b}$ inactivation or cyclin D1 overexpression, tumor cells are unable to stop at predetermined points of the cell cycle, favoring an uncontrolled proliferation. CDK inhibitors are promising new antitumor agents that suppress cell growth and then facilitate the induction of apoptosis. As this third group harbors the *TP53* wt, treatment with cytotoxic DNA-damaging drugs inducing p53-dependent apoptosis would seem to be sound. However, tumor cells treated with DNA-damaging drugs may undergo cell cycle arrest and DNA repair but not apoptosis (24), leading to a cell cycle–mediated drug resistance that may limit the effectiveness of chemotherapy. In this light, clinical observations suggest that the activity of DNA-damaging drugs in the presence of a *TP53* wt could be improved by sequentially following them with the administration of CDK inhibitors (25), which can convert a cell from cell cycle arrest to cell death by modulating the expression of antiapoptotic proteins, such as p21 (26) and cyclin D1 (27). In this group, a synergy between loss of TSGs and activation of cyclins seems to play the main role in the neoplastic process.

Cell cycle–mediated chemotherapy may also be an alternative for treating SCCs carrying *TP53* mutations (group II) because, despite the lack of G_1 arrest due a nonfunctional p53 protein, the pharmacologically induced DNA damage may counteract apoptosis by means of Chk1-mediated G_2 cell cycle arrest. Preclinical evidence suggests that chemotherapy followed by inhibitors of Chk1 or of hsp90, of which Chk1 is a client, enhances apoptosis in cells with *TP53* mutations (24). Clinical trials of these agent are currently being carried out.

It is known that $p14^{ARF}$ plays a role in the biology of oropharyngeal SCCs (28), and the tumors in our groups II and III showed evidence of $p14^{ARF}$ deletion. However, this seems to be less relevant in therapeutic terms because, in group II, the deletion simply worsens the p53 function that is already impaired by the *TP53* mutation and, in group III, the effect of the single $p14^{ARF}$ deletion on p53 function is not regarded as equivalent to a *TP53* mutation.

Because of its frequent overexpression in HNSCCs, it has recently been suggested that EGFR may be a potentially useful therapeutic target. The addition of cetuximab to radiotherapy is a new therapeutic option able to increase both the locoregional control and the survival in advanced HNSCC (29), although the mechanism underlying the selective sensitivity of HNSCC to EGFR inhibitors is still unknown. We observed EGFR overexpression in almost all of our SCCs, thus confirming that alone it is insufficient to predict the response to EGFR inhibitors (30), and, in line with previously published findings (16, 17, 31), we also found very few somatic *EGFR* mutations (4%). On the contrary, *EGFR* amplification (7%) and chromosome 7 polysomy (36%), which have previously been reported in HNSCC (31–33), accounted for 43% of our HPV-negative SCCs (groups II and III). Given the published data concerning other tumors, such as non–small cell lung carcinoma (34, 35) and colorectal cancer (36), it could be very interesting to verify the effect of these *EGFR* alterations on the response of oropharyngeal SCCs to EGFR inhibitors. The only study (to the best of our knowledge) correlating *EGFR* amplification and the response to *EGFR* inhibitors in HNSCC (31) indicates that responsive cases do not carry the amplification; however, the number of cases was very small. Moreover, we found that the *EGFR* alterations in our SCCs were generally coupled with *TP53* mutations and/or cell cycle alterations (groups II and III), and so the interaction of different pathways has to be considered when using EGFR inhibitors. In line with this, it has been found at preclinical level that cyclin D1 overexpression (which we found to be coupled with chromosome 7 polysomy) may be associated with the decreased efficacy of *EGFR* inhibitors in HNSCC (37).

In the fourth group, representing a minority of the cases, no specific alterations of the biomarkers investigated were found, with the exception of 3 cases carrying chromosome 7 polysomy; thus, further investigations are needed.

CDK4 overexpression and *BRAF* mutation do not seem to play any role at all.

In conclusion, the results of this comprehensive analysis of a cohort of surgically treated patients from a single institution support the notion that oropharyngeal SCCs are generated by at least three different mechanisms, although the inactivation of pathways regulated by TSGs like *p53* and *Rb* through viral gene

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interaction or alterations of molecular key players seems to be a common theme in the neoplastic process of these tumors. Because these three pathways may benefit from an appropriate drug combination, assessing HR-HPV status (by means of real-time PCR), *TP53* (by means of mutation analysis or immunohistochemistry), and 9p21 and *EGFR* (by means of FISH) seems to be crucial to try to individuate tumor subgroups to be treated in a more specific way.

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Correction: Article on Oropharyngeal Squamous Cell Carcinoma

In the article on oropharyngeal squamous cell carcinoma in the November 15, 2006 issue of *Clinical Cancer Research*, there was an error in the epidermal growth factor mutation reception in Table 4. The correct epidermal growth factor receptor mutation for case 33 is Glu^{746}Lys GAA>AAA.

Perrone F, Suardi S, Pastore E, et al. Molecular and cytogenetic subgroups of oropharyngeal squamous cell carcinoma. Clin Cancer Res 2006;12:6643 – 51.

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