Genetic Patterns in Head and Neck Cancers That Contain or Lack Transcriptionally Active Human Papillomavirus

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Background: **Transcriptionally active high-risk human papilloma viruses (HPVs), particularly HPV type 16 (HPV16), are found in a subset of head and neck squamous-cell carcinomas (HNSCCs). HPV16-associated carcinogenesis is mediated by expression of the viral E6 and E7 oncoproteins, which cause deregulation of the cell cycle by inactivating p53 and pRb, respectively. We tested the hypothesis that HPV-associated HNSCCs display a pattern of genetic alterations different from those of HNSCCs without HPV DNA.** ***Methods:*** **Polymerase chain reaction–based assays were used to examine 143 consecutive HNSCCs (106 of the oral cavity and 37 of the oropharynx) for the presence of HPV DNA and for viral E6 and/or E7 messenger RNA (mRNA) expression. The HPV DNA– and E6 and E7 mRNA–positive HNSCCs and an equal number of HPV DNA–negative HNSCCs were further analyzed for mutations in TP53, the gene encoding p53, and for allelic loss of 28 microsatellite markers at chromosome arms 3p, 6q, 8p, 9p, 13q, 17p, and 18q, including markers located in regions of chromosome arms 9p and 17p that harbor genes involved the p53 and pRb pathways. All statistical tests were two-sided.** ***Results:*** **Twenty-four (16.7%) of the 143 HNSCCs were positive for HPV16 DNA, and 12 of these HNSCCs (8.4% of total number) expressed E6 and E7 mRNAs. None of the HPV DNA– and E6/E7 mRNA–positive tumors had TP53 gene mutations, whereas nine (75%) of the 12 HPV DNA–negative tumors had such mutations ($P<.001$). Compared with the HPV DNA–negative HNSCCs, the E6/E7 mRNA–positive HNSCCs had statistically significantly lower levels of allelic loss for 13 of the 15 markers on 3p, 9p, and 17p.** ***Conclusions:*** **HNSCCs with transcriptionally active HPV16 DNA are characterized by occasional chromosomal loss, whereas HNSCCs lacking HPV DNA are characterized by gross deletions that involve whole or large parts of chromosomal arms and that already occur early in HNSCC development. These distinct patterns of genetic alterations suggest that HPV16 infection is an early event in HNSCC development. [J Natl Cancer Inst 2004;96:998–1006]**

Evidence implicating human papillomavirus (HPV) as an important carcinogenic agent in humans continues to accumulate. For example, HPV type 16 (HPV16) and other high-risk HPV types are recognized as the main causative factors in the development of cervical cancer *(1)*. There is also evidence that HPV plays a role in the development of head and neck squamous-cell carcinoma (HNSCC) *(2–6)*. In general, it has been established that HPV16 is etiologically involved in a subset of HNSCCs; HPV16 DNA has been detected in the tumor tissue of 10%–20% of HNSCC patients *(2–6)*. However, the frequency with which HPV DNA is detected in tumor samples depends on the tumor subsite investigated. For example, HPV DNA prevalence is low in the oral cavity *(2)* and high in the tonsil *(3,4)*. On the basis of these results, two separate pathways of head and neck squamous-cell carcinogenesis have been proposed: one in which carcinogenesis is promoted by environmental factors (e.g., tobacco smoke and alcohol consumption) and one in which carcinogenesis is promoted by infection with high-risk HPV *(5,6)*.

Expression of the viral oncoproteins E6 and E7 interferes with crucial cellular mechanisms such as cell cycle regulation and apoptosis. Therefore, expression of the E6 and/or E7 viral oncogenes in the tumor might indicate an active role of HPV in the development of HNSCC. The E6 oncoprotein can disrupt the p53 pathway, which is involved in the host cell's response to DNA damage, by targeting the p53 protein for ubiquination and degradation *(1)*. Mutations in TP53 (the gene encoding p53), which are an alternative way to inactivate the function of this tumor suppressor protein, have been observed in the majority of unselected HNSCCs but are rare in HPV-infected HNSCCs, especially those that express E6 *(5,7,8)*. The E7 oncoprotein inactivates pRb, an important regulatory molecule involved in cell cycle progression, by direct interaction *(9)*.

HNSCCs and their precursor lesions have been studied extensively with respect to their genetic alterations. Both HNSCCs *(10)* and precursor lesions *(11–13)* often show mutations in TP53, and such mutations occur at a relatively early stage of head and neck squamous-cell carcinogenesis *(14)*. In addition, loss of heterozygosity (LOH) at specific chromosomal loci has been observed in HNSCCs, and a genetic progression model that is based on patterns of LOH in HNSCCs has been proposed *(15)*. For example, multiple losses at chromosome arms 3p, 9p, and/or 17p, which are often seen in precursor lesions, are considered early markers of head and neck squamous-cell carcinogenesis because they are likely to result in loss of tumor suppressor genes that have been mapped to these loci; such losses would provide a growth advantage for the cells *(15–21)*. LOH at 17p13 is thought to involve TP53, whereas LOH at 9p21 is thought to

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involve INK4a, the tumor suppressor gene encoding p16 *(21)*. The latter gene is frequently inactivated by chromosomal loss or by hypermethylation in the majority of HNSCC precursor lesions *(21)*. In addition, among HNSCCs with tobacco-related etiology, inactivation of INK4a is the most common way to disrupt the pRb pathway *(22)*.

Because HPV E6 and E7 proteins can inactivate the p53 and pRb pathways, respectively, we hypothesized that HNSCCs that contain or lack HPV DNA would have different genetic profiles, specifically with respect to allelic losses at 9p (INK4a) and 17p (TP53). The aim of this study was to further examine the genetic profiles of HNSCCs with and without transcriptionally active HPV DNA. We also report a comparison of patterns of allelic loss at various chromosomal locations, including 9p and 17p.

Subjects and Methods

Patients and Tumor Specimens

We obtained tumor specimens and blood samples from 143 consecutive patients who underwent surgical treatment for a tumor in the head and neck region at the VU University Medical Center. Primary tumor samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C. We have previously reported *(7)* on 75 of these cases. The HNSCCs from nine of these 75 samples had transcriptionally active HPV but lacked a TP53 gene mutation *(7)*. These 75 patients were included in the present study, and we subsequently analyzed the p53 and HPV status of the carcinomas from an additional 68 patients and examined LOH patterns in 32 of the 143 tumors. The study was approved by the Institutional Review Board of the VU University Medical Center, and written informed consent was obtained from all patients.

Data Collection

Information on patients' tobacco and alcohol use was obtained from their medical files. Patients were classified as never, former (at least 1 year of tobacco abstinence), or current daily tobacco smokers. Patients were classified as never, former, or current alcohol drinkers. In addition, we calculated cumulative alcohol consumption (in unit-years) by multiplying the number of years the patient drank alcohol by the number of units of alcohol consumed per day. A unit of alcohol was defined as one alcoholic beverage (equivalent to approximately 15 mL of alcohol). Patients were stratified on the basis of tertiles of alcohol consumption.

Histopathologic examination of the tumors was performed by an experienced pathologist (CJLMM). Tumors were classified as squamous-cell carcinomas with the following categories: basaloid or poorly, moderately, or well-differentiated.

Detection of High-Risk HPV DNA and E6 and E7 mRNAs

Frozen tumor samples were used, and microdissection was performed for all tumor samples to enrich for tumor tissue. DNA and RNA were extracted as described previously *(7,18)*. We first determined the integrity of the tumor DNA samples by using the polymerase chain reaction (PCR) to amplify a 209-base-pair region of the β-globin gene as previously described *(24)*. All samples were β-globin PCR positive and were subsequently subjected to PCR analysis for HPV DNA. HPV DNA detection was performed by using a GP5+/6+ PCR enzyme immunoassay (EIA) that included an oligonucleotide probe cocktail specific for 14 high-risk HPV types (i.e., HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) as previously described *(25)*. This method targets L1 gene sequences within HPV genomes. Tumors that were positive for HPV DNA by GP5+/6+ PCR EIA according to previously described criteria for positivity *(25)* were subsequently typed for HPV by reverse line blot genotyping *(25)*. All samples that were positive for HPV DNA by PCR contained HPV16 DNA. Because disruption of the GP5+/6+ primer region within the L1 gene during integration of the viral genome could account for the failure to detect HPV DNA by GP5+/6+ PCR EIA, all samples that were HPV DNA negative by this assay were further subjected to a PCR assay that targeted the E7 region of HPV16 as previously described *(25)*. Of the 24 HPV16 DNA–positive HNSCC samples, 16 were detected by GP5+/6+ PCR EIA and eight were detected by HPV16–E7 type-specific PCR.

RNA from each of the HPV16 DNA–positive HNSCCs was subjected to a reverse transcription–polymerase chain reaction (RT–PCR) assay using a series of oligonucleotide primers that spanned nucleotides 204 to 525 of the HPV16 genome, which contain the regions encoding the viral E6 and E7 proteins, as previously described *(7)*. The primers were designed to detect full-length E6 mRNA and spliced E6*I mRNA, the latter being the major E7-encoding mRNA species. The RT–PCR products were separated on an agarose gel, transferred to a nylon filter, and hybridized with a radiolabeled full-length HPV16 DNA probe that recognized both the full-length E6 mRNA and the shorter E6*I mRNA *(26)*.

Detection of TP53 Gene Mutations

The mutation status of TP53, the gene encoding p53 in the tumors, was established by direct DNA sequencing essentially as previously described *(14)*.

LOH Analysis

DNA for microsatellite analysis was extracted from freshly frozen tumor samples as previously described *(18)*. We used DNA obtained from blood lymphocytes as a control to evaluate whether each marker was informative. For a limited number of samples (three for each group of HNSCCs), the tumor tissue had been embedded in paraffin. For these six samples, we extracted DNA from the tumor tissue and from the connective tissue, which was the source of control DNA *(18)*. For all tumor samples, microdissection was performed to enrich for tumor tissue. We used 28 microsatellite markers to evaluate LOH. Markers on chromosome arms 3p, 8p, 9p, 13q, 17p, and 18q were selected because they frequently show LOH in HNSCCs *(18,27)*. In addition, markers located at 6q were selected because they show a high frequency of LOH in HPV-related carcinomas of the cervix uteri *(28)*. LOH was determined according to established methods *(18,27)*. Primers' sequences and PCR conditions for microsatellite markers are available at http://www.ncbi.nlm.nih.gov/mapview/maps.cgi (last accessed: May 28, 2004) and also on request. In brief, one primer in each set was labeled with a fluorochrome, and the primer sets were used to amplify the microsatellite sequences by PCR (*18*). The amplification products were separated by capillary electrophoresis on a PRISM 3100-Avant Genetic Analyzer (Applied Biosystems,

Nieuwerkerk aan den IJssel, The Netherlands). Genescan analysis software (Applied Biosystems) was used for calculations. LOH was scored according to standard criteria *(18)*. Personnel performing LOH, HPV, TP53 mutation, or histological analyses were unaware of the outcome of other tests or the clinical status of the patients.

Statistical Analysis

E6 and E7 mRNA expression was considered to reflect a direct viral involvement *(7)* and was used to define the case group. This group was compared with an HPV DNA–negative control group. Cases and control subjects were stratified according to tumor site (oral cavity or oropharynx), as it has been shown that genetic alterations can depend on tumor site *(23)*. Next, cases and controls were selected in such a way that a similar distribution over the groups was ensured for those clinical parameters that might in theory confound the analysis (i.e., age, sex, smoking and drinking behavior, and tumor stage and metastasis).

Fisher's exact tests and chi-square tests were used to assess the statistical significance of frequency distributions between case and control groups and to compare allelic loss of all chromosomal regions analyzed between groups (SPSS for Windows, release 10.0.05; SPSS, Chicago, IL). Two-tailed analyses were performed, and *P* values less than .05 were considered statistically significant.

Results

Characteristics of the Study Population

We examined tumor specimens from 143 HNSCC patients for the presence of high-risk HPV DNA. The distribution of the tumors by anatomical sites was as follows: 106 in the oral cavity and 37 in the oropharynx, of which 24 were from the tonsil. The age of the patients ranged from 19 to 81 years, with a median of 57 years. Fifty-eight (40.6%) of the patients were female and 85 (59.4%) were male. Twenty-four (16.8%) of the 143 HNSCCs were positive for HPV DNA; all 24 HPV DNA–positive tumors contained HPV16 DNA. Both the full-length and the spliced E6 and E7 region mRNAs were detected in 12 HNSCC samples, all of which were positive by GP5+/6+ PCR EIA. This transcriptional pattern is consistent with simultaneous expression of both E6 and E7. HPV DNA–positive tumors that expressed E6 and E7 mRNAs were defined as the case group. The control group comprised 12 HPV DNA–negative (and E6 and/or E7 mRNA–negative) HNSCCs. The two groups of tumors did not differ statistically significantly with regard to patient age at diagnosis, patient tobacco use or alcohol consumption, tumor stage, site, or histology, or with the presence of lymphogenic tumor spread in the patient (Table 1). Both groups contained six tumors from the oral cavity and six tumors from the oropharynx. Among the 12 tumors in the case group, two had basaloid characteristics, six were poorly differentiated squamous-cell carcinomas, and four were moderately differentiated squamous-cell carcinomas. The control group tumors consisted of three poorly differentiated and nine moderately differentiated squamous-cell carcinomas.

LOH Profiles of Case and Control HNSCCs

Table 2 and Fig. 1 present the frequency of LOH in various chromosomal arms for tumors in the case and control groups. We observed less LOH among tumors that were positive for HPV DNA and E6 and E7 mRNA than among tumors that were negative for HPV DNA (and E6 and E7 mRNA). Most allelic loss among tumors in the HPV DNA–negative control group occurred at 3p, 9p, or 17p; in those chromosome arms, the frequency of LOH was 81% (i.e., at 110 of 135 informative markers). By contrast, among tumors in the case group, allelic loss in these three chromosome arms occurred at a frequency of 14% (i.e., at 16 of 116 informative markers). Large parts of chromosomal arms can be lost, making loss of a particular microsatellite marker dependent on the loss of a neighboring one. Because we do not know how dependent loss of any of the markers we analyzed is on the loss of any of the other markers, we cannot analyze the statistical significance of the overall difference in LOH frequency between the two groups of tumors. Nevertheless, we observed a statistically significant difference ($P<.05$, Fisher's exact test) between the two groups of tumors regarding loss of 13 of the 15 markers tested on 3p, 9p, and 17p (Table 2). By contrast, LOH frequencies for individual markers at 6q, 8p, 13q, and 18q did not differ statistically significantly between the case and control groups. Although all four markers tested on 8p and 18q were lost more frequently in the HPV DNA–negative group than in the HPV DNA–positive group, the difference was statistically significant for only one marker (i.e., D8S1130; $P = .029$) (Table 2).

The patterns of LOH for the individual tumors in the two groups are shown in Fig. 1. Both the frequency and the pattern of LOH differed between HNSCCs in the case and control groups. Loss of adjacent markers at 3p, 9p, or 17p was very rare in the HPV DNA– and E6/E7 mRNA–positive tumors. For example, among the 116 informative markers, we detected only five occasions in which two adjacent markers were lost and one occasion each of loss of three or four adjacent markers. By contrast, the HPV DNA–negative tumors displayed a more global pattern of loss: we observed LOH at two, three, and four adjacent markers on 19, 11, and 10 occasions, respectively, when examining the total group of 135 informative microsatellite markers.

TP53 Gene Mutation Status of Case and Control HNSCCs

We also examined the tumors for mutations in TP53, the gene encoding p53. None of the 12 tumors that were positive for HPV DNA and E6/E7 mRNA had TP53 gene mutations, whereas nine (75%) of the 12 tumors that were negative for HPV DNA (and E6/E7 mRNA) had such mutations ($P<.001$, Fisher's exact test) (Table 1). We examined whether there was an association between the presence of a mutation in TP53 and LOH patterns among tumors in the control group. We found no evidence to support such an association: the average frequency of LOH for the TP53 mutation–negative tumors in the control group (tumor numbers 22, 23, and 24) was not statistically different from that of the tumors with a TP53 mutation (tumor numbers 13, 14, 15, 16, 17, 18, 19, 20, and 21) (74.6% versus 61.8%; $P = .34$, Student's *t* test) (Fig. 1).

Pattern of LOH in HPV16 DNA HNSCCs Without E6 or E7 Expression

We observed a different pattern of LOH among HNSCCs with detectable HPV16 DNA and viral E6 and E7 expression than among HNSCCs without HPV16 DNA. We wondered whether this pattern was present in HNSCCs that had detectable

Table 1. Patient and tumor characteristics in relation to HPV status of the tumor*

Characteristic	No. (%) of case group HPV DNA$^+$/E6$^+$ (n = 12)	No. (%) of control group HPV DNA$^-$/E6$^-$ (n = 12)	P†
Sex			
Male	9 (75)	8 (67)	1.0
Female	3 (25)	4 (33)	
Age at diagnosis, y			
40–50	4 (33)	4 (33)	1.0
51–60	4 (33)	4 (33)	
61–75	4 (33)	4 (33)	
Tobacco use			
Never	1 (9)	2 (17)	.30
Former	4 (33)	1 (9)	
Current	7 (58)	9 (5)	
Alcohol use‡			
Never	1 (9)	3 (25)	.22
Former	2 (17)	0 (0)	
Current	9 (75)	9 (75)	
Total amount of alcohol consumed			
≥150 unit-years	3 (25)	3 (25)	.64
≥50 to <150 unit-years	4 (33)	6 (50)	
<50 unit-years	5 (42)	3 (25)	
Tumor site			
Oral cavity	6 (50)	6 (50)	1.0
Oropharynx	6 (50)	6 (50)	
Tonsil	5 (42)	4 (33)	1.0§
Nontonsil	1 (9)	2 (17)	
Tumor stage‖			
I and II	2 (17)	3 (25)	.64
III and IV	10 (83)	8 (67)	
Not applicable¶	0 (0)	1 (9)	
Histology			
Basaloid	2 (17)	0 (0)	.09
Poorly differentiated	6 (50)	3 (25)	
Moderately differentiated	4 (33)	9 (75)	
Lymph node metastasis			
Yes	8 (67)	7 (58)	1.0
No	4 (33)	4 (33)	
Not applicable¶	0 (0)	1 (9)	
TP53 gene mutation			
Yes	0 (0)	9 (75)	<.001
No	12 (100)	3 (25)	

*HPV = human papillomavirus; HPV DNA$^+$/E6$^+$ = positive for HPV DNA and for E6/E7 mRNA expression; HPV DNA$^-$/E6$^-$ = negative for HPV DNA and for E6/E7 mRNA expression.

†*P* values (two-sided) are for the difference between the case and control groups. For the 2 × 2 comparison with respect to the distributions of sex and TP53 mutation status, Fisher's exact test was used; for the other comparisons, the chi-square test was applied. *P* values less than .05 were considered to reflect a statistically significant difference.

‡Unit-years was taken as a measure of cumulative alcohol consumption and was calculated as the number of years drinking multiplied by the number of units per day. A unit is defined as one alcoholic beverage (equivalent to approximately 15 mL of alcohol). Cut points were based on tertiles of alcohol consumption among the HNSCC patients in this study.

§The frequency of tonsillar cancers was compared with the frequency of cancers at all other sites.

‖Staging was performed according to the classification of the International Union Against Cancer (UICC) *(29)*.

¶"Not applicable" refers to one tumor that could not be classified because it was a recurrent tumor.

HPV16 DNA but no detectable E6 or E7 mRNA. Among the 143 HNSCCs analyzed in this study, 12 were positive for HPV16 DNA but lacked detectable E6 and E7 mRNA expression. Eight of these tumors were available for further genetic analysis (Fig. 1). Three of the eight HNSCCs had a mutation in the TP53 gene. Among the eight tumors, LOH in chromosome arms 3p, 9p, and 17p was relatively high (i.e., 75%; 66 of 88 informative markers). We observed a statistically significant difference ($P<.05$, Fisher's exact test) between these HPV DNA–positive, E6/E7 mRNA–negative HNSCCs and the 12 case HNSCCs (HPV DNA positive, E6/E7 mRNA positive) in LOH for seven of the 15 markers tested. By contrast, there was no statistically significant difference between the HPV DNA–positive, E6/E7 mRNA–negative HNSCCs and the 12 HPV DNA–negative control tumors in LOH at any marker (Table 3).

Discussion

We tested tumors obtained from a cohort of 143 HNSCC patients for the presence of HPV DNA and transcription of E6 and E7 viral oncogenes. Approximately 17% of the HNSCCs had detectable HPV16 DNA; half of the HPV DNA–positive tumors expressed E6/E7 mRNA. Our most important finding was that the tumors harboring transcriptionally active HPV16 had a distinct genetic pattern; i.e., most of the individual markers at 3p, 9p, and 17p showed a statistically significantly lower frequency of loss in the HPV DNA– and E6/E7 mRNA–positive tumors than in the HPV DNA–negative (and E6/E7 mRNA negative) tumors. The HPV DNA–negative tumors had a substantial number of losses at these chromosomal sites, consistent with the findings of others who have studied larger series of HNSCCs by using a variety of genetic techniques *(10,18,30–32)*. Allelic losses at 3p, 9p, and/or 17p are considered early events in head and neck squamous-cell carcinogenesis because they have frequently been detected in premalignant lesions in the squamous epithelium of the head and neck (*2,15,16,17,20,33–35)*. For example, we previously reported that among 31 dysplastic lesions in the mucosa of the oral cavity and oropharynx, 24 lesions (77%) had LOH at 3p, 9p, and/or 17p, and 13 lesions (42%) had LOH at all three chromosomal arms. The absence of LOH at these chromosome arms in HPV DNA–positive and E6/E7 mRNA–positive tumors was therefore remarkable. Viral E6 and E7 expression is apparently associated with an exceptionally low level of LOH at these loci, whereas LOH changes at these loci are common in early carcinogenesis when no HPV is involved. This low level of LOH at chromosomes that usually show changes early in HNSCC carcinogenesis can be considered indirect evidence for the concept that HPV16 is actively involved in the early steps of the development of a subgroup of HNSCC.

Other lines of evidence implicate HPV in the early steps of HNSCC development. For example, none of the HPV DNA– and E6/E7 mRNA–positive HNSCCs in our study had a mutation in the TP53 gene, and all these HNSCCs had retained both alleles at the TP53 locus, at least in the informative cases for which LOH could be assessed (Fig. 1). This absence of a TP53 mutation confirmed the results of previous studies *(5,8)* and was previously reported by us for a subset of the tumors in the present study *(7)*. The viral E6 protein inactivates the p53 protein *(1)*, and therefore the absence of TP53 gene mutations in HPV DNA–positive HNSCCs seems biologically logical. In

Table 2. LOH frequencies for 28 microsatellites in relation to the HPV DNA status of the tumor*

Chromosome arm	Microsatellite†	Case group (HPV DNA$^+$/E6$^+$) Frequency (No. of tumors with loss/no. of tumors with informative marker)	Case group (HPV DNA$^+$/E6$^+$) %	Control group (HPV DNA$^-$/E6$^-$) Frequency (No. of tumors with loss/no. of tumors with informative marker)	Control group (HPV DNA$^-$/E6$^-$) %	*P*‡
3p	D3S1284	1/7	14	8/9	89	.008
	D3S1274	1/6	17	9/10	90	.007
	D3S1217	2/8	25	9/9	100	.002
	D3S1766	1/7	14	5/6	83	.029
	D3S1029	1/8	13	7/9	78	.015
	D3S1293	0/7	0	7/11	63	.013
	≥1 marker§	4/12	33	11/12	92	.009
9p	D9S171	2/8	25	7/10	70	.154
	D9S1748	0/10	0	7/10	70	.003
	D9S1751	1/6	14	9/9	100	.002
	IFNA	1/9	11	9/9	100	<.001
	D9S162	2/7	29	10/11	91	.013
	D9S157	2/11	18	7/7	100	.002
	≥1 marker	5/12	42	12/12	100	.004
17p	CHRNB1	1/6	17	4/6	67	.242
	TP53	0/9	0	6/11	54	.014
	D17S1866	1/7	14	6/8	75	.040
	≥1 marker	1/12	8	8/12	67	.009
13q	D13S294	4/10	40	5/10	50	1.000
	D13S168	5/10	50	6/9	67	.650
	D13S170	4/7	57	3/10	30	.350
	D13S158	2/8	25	6/8	75	.132
	≥1 marker	8/12	67	8/12	67	1.000
18q	D18S34	0/7	0	4/9	44	.088
	D18S57	1/10	10	5/10	50	.141
	≥1 marker	1/10	10	6/12	50	.074
8p	LPL-GZ	1/8	13	2/4	50	.236
	D8S1130	1/7	14	5/6	83	.029
	≥1 marker	2/11	18	6/9	67	.065
6q	D6S251	1/6	17	0/5	0	1.000
	D6S278	3/9	33	2/8	25	1.000
	D6S287	1/9	11	0/6	0	1.000
	D6S281	2/7	29	1/8	13	.570
	D6S305	2/4	50	2/7	29	1.000
	≥1 marker	7/12	58	4/11	36	.413

*LOH = loss of heterozygosity; HPV = human papillomavirus; HPV DNA$^+$/E6$^+$ = positive for HPV DNA and for E6/E7 mRNA expression; HPV DNA$^-$/E6$^-$ = negative for HPV DNA and for E6/E7 mRNA expression.

†Microsatellite markers are listed in the order they exist on the chromosome, from centromere to telomere.

‡Fisher's exact test was used to determine the statistical significance of the difference in each frequency between groups.

§≥1 marker = frequency of HNSCC with loss of at least one microsatellite on that chromosomal arm.

HNSCCs that lack HPV DNA, mutation of the TP53 gene is a frequent event, as shown in the present study as well as in other studies *(10,11,19).* Precursor lesions also often have TP53 mutations *(11,19).* Thus, our results from the TP53 gene mutation analysis suggest, albeit in an indirect way, that HPV16 is involved early in head and neck squamous-cell carcinogenesis. A previous study found that HPV16 infection, as detected by measuring antibodies against HPV16 in serum, is an independent risk factor for the development of HNSCC *(36).* Importantly, in that longitudinal cohort study *(36),* the interval between the detection of the virus and the development of the tumor was more than 15 years, which also suggests that HPV may be etiologically involved in the early phase of HNSCC development. Together, these and the present results provide supportive (albeit circumstantial) evidence for the concept that HPV infection is involved in the early phases of development of a subset of HNSCCs *(5).*

Besides the involvement of HPV in carcinogenesis *(37,38),* other clinicopathologic variables might influence the pattern of genetic alterations *(37,38).* Results of a comparative genomic hybridization analysis *(23)* suggest that the site of the tumor in the head and neck region may influence the genetic patterns, and we therefore ensured that the two groups of tumors had an equal distribution with respect to site. In addition to tumor site, smoking status might also influence the pattern of genetic changes. Koch et al. *(32)* reported that the frequencies of LOH at 6p, 8p, 9p, 14q, and 17p in HNSCCs did not differ between patients who smoked and patients who did not smoke. They observed less LOH at 3p (33% less), 4q, and 11q for nonsmokers than for smokers. However, there was no statistically significant difference in the extent of tobacco use between the two groups of patients in our study.

Our results are consistent with the hypothesis that HNSCCs develop by two different etiologies *(5,6):* one driven by expo-

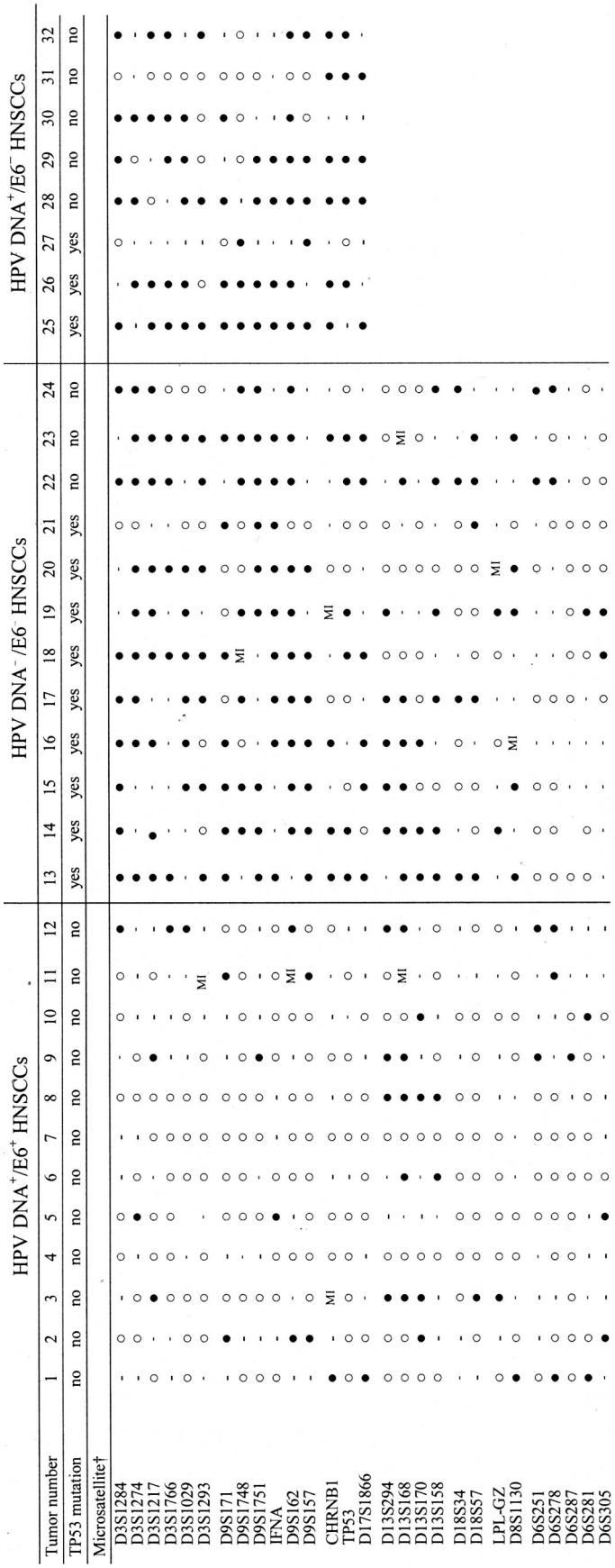

Fig. 1. Summary of TP53 gene mutation status and microsatellite analysis of HPV DNA$^+$/E6$^+$, HPV DNA$^-$/E6$^-$, and HPV DNA$^+$/E6$^-$ HNSCCs. HPV = human papillomavirus; HPV DNA$^+$/E6$^+$ = positive for HPV DNA and for E6/E7 mRNA expression; HPV DNA$^-$/E6$^-$ = negative for HPV DNA and for E6/E7 mRNA expression; HPV DNA$^+$/E6$^-$ = positive for HPV DNA and negative for E6/E7 mRNA expression; HNSCCs = head and neck squamous-cell carcinomas; – = not informative; ○ = retained alleles (i.e., no loss of heterozygosity); ● = loss of heterozygosity; MI = microsatellite instability, a frameshift mutation (insertion or deletion of one or a small number of bases) in a microsatellite sequence resulting in a larger or shorter microsatellite fragment length; † = microsatellite markers are listed in the order in which they exist on the chromosome, from centromere to telomere.

sure to environmental carcinogens (i.e., tobacco and alcohol) without HPV involvement and the other involving infection with transcriptionally active HPV16. Although these carcinogenic routes may overlap, our results suggest that they differ with respect to at least two genetic parameters: the mutation status of TP53 and allelic loss in three chromosome arms. We found that all HNSCCs that harbored transcriptionally active HPV had wild-type TP53 genes and low levels of LOH at 3p, 9p, and 17p. The question arises as to if there is evidence whether the two carcinogenic routes—one with HPV involvement and one tobacco exposure related—show overlap. At present, no clear answer is available. Controversy exists about the extent to which tobacco consumption is involved in HPV-mediated carcinogenesis. Although several studies have reported that tobacco consumption is lower among HNSCC patients with HPV-infected tumors than among HNSCC patients with uninfected tumors *(4,5,32,36,39)*, other studies did not find such a connection *(37,40,41)*.

We began this study with the hypothesis that the genetic profiles of HNSCCs that express HPV E6 and E7 would differ from HNSCCs that lack such expression, especially with respect to LOH at 9p and 17p, but we were not certain what to expect at the other chromosomal locations. By expressing E6 and E7, the virus is capable of disrupting two molecular pathways important in HNSCC development: the p53 pathway and the pRb pathway, respectively *(1)*. In the absence of viral infection, these pathways are often disrupted by allelic loss and epigenetic changes at 9p (INK4a locus) and allelic loss and mutation of the TP53 tumor suppressor gene at 17p *(15–21)*. We observed comparable alterations in HPV DNA–negative tumors: seven of 10 informative HNSCCs showed loss of marker D9S1748, and six of 11 informative HNSCCs showed loss of the microsatellite marker TP53. These findings suggest that, when HPV is actively involved in HNSCC development, mutation of TP53 and loss of 17p are not necessary events. A similar reasoning can be followed with respect to loss of 9p. Viral E7 is capable of disrupting the pRb pathway by interaction with pRb itself *(1)*. Loss at 9p21 is thought to involve INK4a, the tumor suppressor gene encoding p16 *(21)*. Inactivation of p16 is an alternative way to disrupt the pRb pathway. Our results suggest that when HPV is active in head and neck squamous-cell carcinogenesis, loss at 9p21 is not a necessary event. It is intriguing that loss at 3p is also almost absent in HPV DNA–positive and E6/E7 mRNA–positive HNSCCs but is frequent in HPV DNA–negative HNSCCs *(30)*. This difference suggests that HPV proteins might interact with a protein encoded by a gene at 3p *(31)*. We also observed that both the HPV DNA–positive and the HPV DNA–negative HNSCCs in our study had similar high frequencies of LOH at 13q, which suggests that the viral and nonviral routes also might share common genetic events in head and neck squamous-cell carcinogenesis.

Table 3. LOH frequencies among HPV DNA$^+$/E6$^-$ HNSCCs*

Chromosome arm	Microsatellite†	LOH		*P*‡	*P*§
		Frequency (No. of tumors with loss/no. of tumors with informative marker)	%		
3p	D3S1284	5/7	71	.103	.550
	D3S1274	3/4	75	.190	.501
	D3S1217	4/6	67	.277	.143
	D3S1766	5/6	83	.029	1.000
	D3S1029	5/6	83	.025	1.000
	D3S1293	3/7	43	.192	.630
	≥1 marker	6/8	75	.170	.537
9p	D9S171	4/6	67	.277	1.000
	D9S1748	3/7	43	.051	.350
	D9S1751	4/5	80	.080	.357
	IFNA	4/4	100	.007	1.000
	D9S162	6/7	86	.103	1.000
	D9S157	5/7	71	.049	.461
	≥1 marker	7/8	88	.067	.400
17p	CHRNB1	6/6	100	.015	.454
	TP53	5/6	83	.002	.333
	D17S1866	4/4	100	.015	.515
	≥1 marker	6/7	86	.002	.603

*LOH = loss of heterozygosity; HPV = human papillomavirus; HPV DNA$^+$/E6$^-$ = positive for HPV DNA and negative for E6/E7 mRNA expression; HNSCCs = head and neck squamous = cell carcinomas.

†Microsatellite markers are listed in the order in which they exist on the chromosome, from centromere to telomere. ≥1 marker = frequency of HNSCC with loss of at least one microsatellite at that chromosomal arm.

‡Two-sided *P* values (Fisher's exact test) for comparison with LOH frequencies among case group HNSCCs (i.e., HPV DNA$^+$/E6$^+$ HNSCCs; specific data in Table 2).

§Two-sided *P* values (Fisher's exact test) for comparison with LOH frequencies among control group HNSCCs (i.e., HPV DNA–negative HNSCCs; specific data in Table 2).

As can be deduced from our data, HPV infection is associated with relatively subtle genetic alterations in HNSCCs. By contrast, most HNSCCs with no evidence of HPV infection are characterized by gross chromosomal alterations that often involve the loss of whole or large parts of chromosome arms, and they are frequently associated with isochromosome formation *(10,30)*. Our results show that etiologic involvement of HPV results in a subgroup of HNSCCs with a unique pattern of genetic alterations, characterized by a low level of LOH at 3p, 9p, and/or 17p. The presence of HPV DNA in a tumor might thus be an important parameter when correlating a genetic profile with a clinical outcome *(42)* or the progression of dysplasia into cancer *(20)*. The unique pattern of allelic loss in HPV16 DNA–positive HNSCCs might confound correlations of genetic changes with outcome, and it is advisable to use HPV analysis to exclude these cases.

The present study also addresses the biologic significance of HPV DNA detection by very sensitive methods. Detectable HPV DNA in a tumor does not necessarily mean that the viral DNA is transcriptionally active (i.e., functional). HPV16 is likely to play a role in carcinogenesis only in tumors bearing transcriptionally active HPV DNA (i.e., tumors that express E6 and/or E7 mRNA). These tumors generally show relatively high levels of HPV DNA, on average approximately 10 times higher than those in E6 and E7 mRNA–negative tumors *(7)*. Low levels of HPV DNA and the absence of viral transcriptional activity are likely to have no or limited biologic significance, and hence it is plausible to argue that tumors with these characteristics should be considered HPV negative from the biologic point of view *(43)*. The lack of a consistent definition for active involvement of HPV in the development of HNSCC is probably the major explanation for conflicting results in the literature about the relationship between HPV infection and patient survival. Many studies have reported an association between HPV infection and improved survival *(5,39,44–48)*, whereas other studies have not found such an association *(7,37,40,49,50)*. It is our opinion that the presence of E6 and E7 mRNA in a tumor may be indicative of an active viral infection. When no good-quality RNA is available—for instance, when only paraffin-embedded material can be analyzed—an assessment of the genetic pattern may allow the tumor to be classified as biologically HPV positive or biologically HPV negative.

In conclusion, our data suggest that two genetic routes of multistep head and neck carcinogenesis exist. The first and most frequent route is characterized by losses of whole or large parts of chromosome arms at 3p, 9p, and 17p without HPV involvement. The second route is associated with HPV infection and is characterized by a low level of chromosomal loss at these hot-spots.

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