Inverse Relationship between Human Papillomavirus-16 Infection and Disruptive *p53* Gene Mutations in Squamous Cell Carcinoma of the Head and Neck

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Abstract

Purpose: Squamous cell carcinomas of the head and neck (HNSCC) often harbor p53 mutations, but p53 protein degradation by the viral oncoprotein E6 may supercede *p53* mutations in human papillomavirus 16 (HPV16) – positive tumors. The prevalence of *p53* mutations in HPV-positive HNSCCs is indeed lower, but in some tumors these alterations coexist. The purpose of this study was to discern whether HNSCCs differ in the type of *p53* mutations as a function of HPV16 status.

Experimental Design: The study was nested within a prospective multicenter study (ECOGE 4393/RTOG R9614) of patients with HNSCC treated surgically with curative intent. Tumors from one study center were used to construct a tissue microarray. The tumors were well characterized with respect to p53 mutational status. The tissue microarray was evaluated by HPV16 *in situ* hybridization. HPV16 analysis was also done on a select group of tonsillar carcinomas known to harbor disruptive *p53* mutations defined as stop mutations or nonconservative mutations within the DNA binding domain.

Results: HPV16 was detected in 12 of 89 (13%) HNSCCs. By tumor site, HPV16 was detected in 12 of 21 (57%) tumors from the palatine/lingual tonsils, but in none of 68 tumors from nontonsillar sites ($P < 0.00001$). Both HPV16-positive and HPV16-negative HNSCCs harbored *p53* mutations (25% versus 52%), but disruptive mutations were only encountered in HPV16-negative carcinomas. Of seven tonsillar carcinomas with disruptive *p53* mutations, none were HPV16 positive, in contrast to HPV16-positive tonsillar carcinomas without disruptive *p53* mutations (0% versus 57%; $P = 0.008$).

Conclusions: Although HPV16 and mutated *p53* may coexist in a subset of HNSCCs, HPV16 and disruptive *p53* mutations seem to be nonoverlapping events. A less calamitous genetic profile, including the absence of disruptive *p53* mutations, may underlie the emerging clinical profile of HPV16-positive HNSCC such as improved patient outcome.

The oncogenic virus human papillomavirus 16 (HPV16) is detected in a subset of squamous cell carcinomas of the head and neck (HNSCC). These HPV-positive HNSCCs typically arise in the oropharynx, are less commonly associated with tobacco or alcohol exposure, show enhanced sensitivity to radiation therapy, and are consistently associated with favorable patient outcomes compared with non–HPV-related HNSCCs (1–4). This emerging clinical profile of HPV16-positive HNSCC likely reflects a pattern of molecular genetic alterations that is distinct from its HPV-negative counterpart.

The *p53* tumor suppressor gene plays a critical role in regulating key cellular pathways including those involving apoptosis and cell cycle control, and it is a frequent target of inactivation during the development of HNSCC (5). Abrogation of p53 function can be mediated by a variety of mechanisms. Mutations and loss of heterozygosity directly target the *p53* gene, whereas expression of the HPV oncoprotein E6 binds and degrades wild-type p53 protein product (6–8). Unlike cervical carcinomas where HPV infection and p53 mutations are mutually exclusive events, HPV infection and *p53* mutations sometimes occur together in HNSCC (1, 9–13). The apparent superfluous presence of HPV16 in *p53*-mutated HNSCCs has raised the suspicion that HPV is incidental, not causal, in the development of these carcinomas.

Not all inactivating events are equivalent in their ability to knock out p53 function. Depending on where they occur, assorted *p53* mutations are highly divergent in their effect on p53 protein structure, stability, and DNA binding properties. Those that occur within the core domain affecting p53 protein interaction with sequence-specific DNA completely block DNA binding and entirely abrogate p53 function (14). Those that occur outside of these DNA contact points may affect p53 function in a more limited manner. Furthermore, E6 degradation of p53 protein is not functionally equivalent to a p53 mutation. Even in HPV-infected cells expressing E6 oncoprotein, endogenous wild-type p53 can

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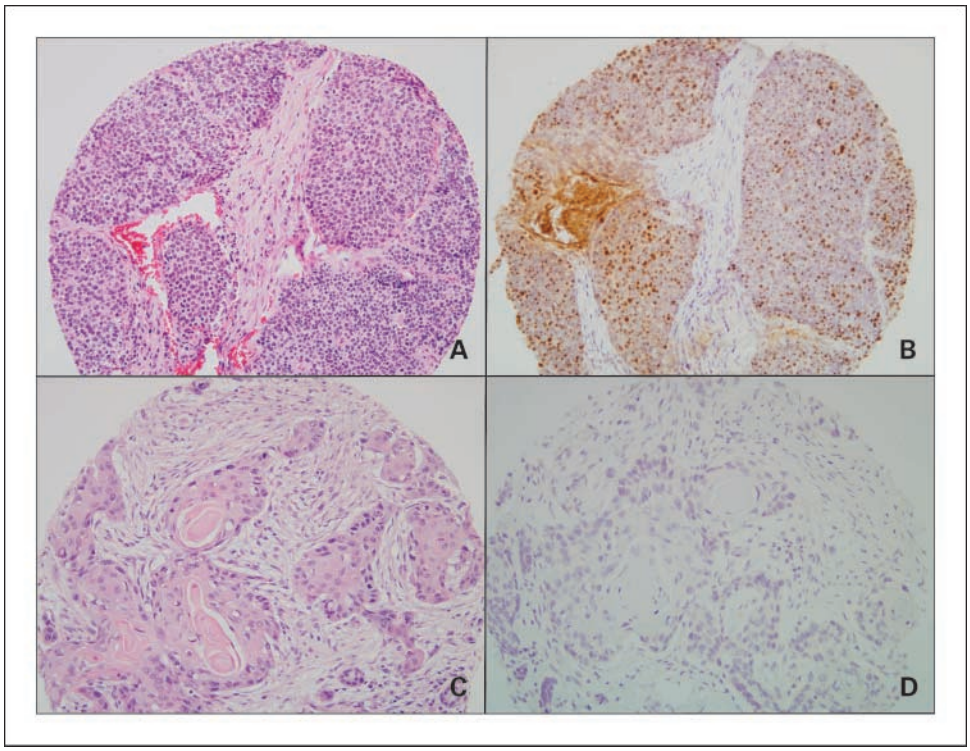

Fig. 1. HPV16 analysis of two representative HNSCCs. Top, HPV16-positive carcinoma (*A,* routine H&E staining; *B,* HPV16 *in situ* hybridization). Bottom, HPV16-negative carcinoma (*C,* routine H&E staining; *D,* HPV16 *in situ* hybridization).

activate some cellular target genes (15), and the apoptotic response to radiation remains intact (16). These observations would seemingly account for the dual presence of inactivating p53 events, particularly when the effect of any single event on p53 function is incomplete. The purpose of this study was to determine the relationship between HPV16 and the types of *p53* mutations in HNSCC.

Patients and Methods

Patients. The study was nested within a prospective multicenter study (Eastern Cooperative Oncology Group E4393 and Radiation Therapy Oncology Group R9614) of patients with HNSCC treated surgically between 1996 and 2002 with curative intent. Formalin-fixed and paraffin-embedded tumor samples were obtained from a subset of those HNSCCs (i.e., those resected at the Johns Hopkins Hospital), and these tumors were used to construct a tissue microarray. A second highly selected group of tumors consisted of tonsillar carcinomas that were known to carry disruptive p53 mutations. These selected carcinomas were pooled from other study sites.

p53 mutation analysis. *p53* analysis had been done on tumor samples that were rapidly frozen at -80°C. Tumor purity was assessed from microscopic analysis of the frozen tumor block. Only samples with at least 70% tumor cells were eligible for *p53* analysis. In some cases, samples with a low concentration of tumor cells were microdissected to obtain enriched samples. Mutation status of exons 2 to 11 of the *p53* gene was evaluated using the GeneChip *p53* assay (Affymetrix) as previously described (17). All mutations detected by GeneChip *p53* assay analysis were identified and confirmed by automatic (ABI BigDye cycle sequencing kit) or direct dideoxynucleotide sequencing (17). Based on available information about the functional differences of various *p53* mutations, *p53* mutations were grouped as "disruptive" and "nondisruptive." Disruptive mutations were defined as stop mutations, frameshift mutations, or nonconservative mutations occurring within the key DNA binding domain L2/L3. All other mutations were defined as nondisruptive mutations. DNA from blood lymphocytes was also evaluated for *p53* status to help discern true allelic differences between tumor DNA and germ line DNA.

HPV16* in situ *hybridization. HPV16 detection was done using the *in situ* hybridization catalyzed signal amplification method for biotinylated probes (DAKO GenPoint). This catalyzed signal amplification system permits visualization of single copies of HPV16 in infected cells (18). Briefly, 5-μm tissue sections underwent deparaffinization, heat-induced target retrieval in citrate buffer, and digestion with Proteinase K (Roche Diagnostics). Slides were subsequently hybridized with a biotinylated HPV16 type–specific probe (DAKO). Signal amplification was done by consecutive application of streptavidin-horseradish peroxidase complex, biotinyl tyramide, and streptavidin-horseradish peroxidase complex. Visualization of positive hybridization signals was done by incubation with the chromogenic substrate diaminobenzidine. Cases were considered positive if hybridization signals visualized as nuclear dots were present within tumor nuclei (Fig. 1). HPV16-positive and HPV16-negative tonsillar carcinomas served as positive and negative controls, respectively. In these control samples, HPV16 status had been rigorously determined using a combination of HPV detection techniques including consensus L1 PCR, E7 type-specific PCR, and Southern blot hybridization of unamplified tumor DNA (1).

Statistical evaluation. The associations between HPV16 status and p53 mutational status were evaluated by use of the Fisher exact test. *P* values are two sided unless otherwise specified. Statistical analysis was conducted using STATA software, version 7 (STATA).

Results

The tissue microarray contained tumors from 89 patients with squamous cell carcinomas from various anatomic sites of the head and neck including the oral cavity (n = 38), larynx (n = 20), palatine/lingual tonsils (n = 21), hypopharynx (n = 6), and palate (n = 4; Table 1). Of the 89 tumors, 43 (48%) harbored a *p53* mutation. Tumors arising from the tonsils were less likely to harbor a *p53* mutation than

Table 1. p53 mutations and HPV16 positivity in HNSCCs by anatomic site

Site	*p53* mutation (%)	HPV16-positive (%)
Palatine/lingual tonsils	8/21 (38) Disruptive: 1 (13) Nondisruptive: 7 (88)	12/21 (57)
Nontonsillar	37/68 (54) Disruptive: 14 (38) Nondisruptive 23 (62)	0/68 (0)
Oral cavity	22/38 (58) Disruptive: 10 (45) Nondisruptive: 12 (55)	0/38 (0)
Larynx	11/20 (55) Disruptive: 3 (27) Nondisruptive: 8 (73)	0/20 (0)
Hypopharynx	4/6 (67) Disruptive: 1 (25) Nondisruptive: 3 (75)	0/6 (0)
Palate	0/4 (0) Disruptive: 0 Nondisruptive: 0	0/4 (0)

carcinomas arising from nontonsillar sites, but the difference was not statistically significant (38% versus 54%; $P = 0.19$). For cases with *p53* mutations, disruptive mutations were less likely to occur in carcinomas arising from tonsillar than nontonsillar sites, but the difference was not statistically significant (13% versus 38%; $P = 0.24$).

HPV16 was detected in 12 of 89 (13%) HNSCCs. When stratified by site of origin, HPV16 was detected in 12 of 21 (57%) HNSCCs of the lingual/palatine tonsils, but in none of 68 carcinomas from nontonsillar sites (55% versus 0%; $P < 0.00001$; Table 1). HPV16-positive tumors were less likely to harbor *p53* mutations than HPV16-negative tumors (25% versus 52%; $P = 0.12$). Disruptive *p53* mutations were not identified in any of the HPV16-positive cancers (Table 2). To determine whether the inverse correlation between HPV16 and disruptive *p53* mutations persisted in a larger group of tonsillar carcinomas, tonsillar carcinomas known to harbor disruptive *p53* mutations were pooled from multiple study sites. Although HPV16 was present in 12 of 20 (60%) tonsillar carcinomas that did not harbor disruptive *p53* mutations, HPV16 was not detected in the 7 tonsillar carcinomas with disruptive *p53* mutations ($P = 0.008$).

Discussion

The presence of transcriptionally active HPV genome in the nuclei of clonally expanded transformed cells has helped establish an etiologic link between HPV16 and a subset of HNSCC, but the occurrence of *p53* mutations in a subset of HPV-positive HNSCCs has prompted a resurgent skepticism over the strength of this link. Mutation of the *p53* gene is accepted as an important mechanism of *p53* pathway inactivation, and its presence in a HPV-positive HNSCC would seemingly diminish the role of HPV in tumor development. Most studies of HNSCC have noted a reduced but persistent prevalence of *p53* mutations in HPV-positive tumors with the dual presence of HPV DNA and *p53* mutations ranging from 0% to 42% (1, 9–13). We noted the paradoxical overlap of these *p53* pathway–inactivating events in 25% of the HPV16-positive HNSCCs.

A detailed understanding of p53 protein structure indicates that variant *p53* mutations are not equivalent in their capacity to disrupt p53 function. Nonconservative mutations located within the L2/L3 region—the region of the p53 protein directly involved in DNA contact—have limited consequences on protein structure but profoundly disrupt binding to DNA (14). Mutations in the β sandwich of the core domain, on the other hand, generally lead to an extended denaturation of the p53 protein. The effect of these variant mutations goes well beyond differences in protein structure. In contrast to mutations that do not disrupt DNA binding, disruptive mutations both (*a*) selectively eliminate the wild-type *p53* allele (19) and (*b*) transactivate cellular proliferation genes by mechanisms independent of direct DNA binding (20, 21), and thereby confer "gain-of-function" characteristics as characterized by enhanced tumor growth and increased resistance to antitumor therapies (20–22). For HNSCCs with disruptive *p53* mutations, acquisition of a malignant phenotype seems to be complete. In these tumors, the coexistence of other events targeting the *p53* pathway is neither required nor anticipated. Indeed, we did not identify HPV16 in any of the HNSCCs with disruptive *p53* mutations, including those carcinomas most likely to be associated with HPV16 infection (i.e., tonsillar carcinomas). For HNSCCs with nondisruptive *p53* mutations, the additive effect of other inactivating events such as transcriptionally active HPV could heighten the overall effect of a p53 functional abrogation. Of the HNSCCs that showed

Table 2. HPV16 status of palatine/lingual tonsillar carcinomas harboring *p53* mutations

Tumor	Exon	Codon	Nucldeotide Δ	Amino acid Δ	Disruptive vs nondisruptive	HPV16 status
1	8	273	CGT > CAT	Arginine > histidine	ND	-
2	4	205	TAT > TGT	Tyrosine > cysteine	ND	+
3	5	273	CGT > CAT	Arginine > histidine	ND	-
4	2	138	GCC > CCC	Alanine > proline	ND	-
5	5	273	CGT > CTT	Arginine > leucine	ND	-
6	4	244	GGC > AGC	Glycine > serine	ND	+
7	5	278	CCT > ACT	Proline > threonine	ND	+
8	7	248	CGG > CAG	Arginine > glutamine	D	-
9	6	193	CAT > CTT	Histidine > leucine	D	-
10	6	196	CGA > TGA	Arginine > stop	D	-
11	5	167	CAG > GAG	Glutamine > glutamic acid	D	-
12	7	250	CCC > -CC	Frameshift	D	-
13	7	237	ATG > -TG	Frameshift	D	-
14	10	342	CGA > TGA	Arginine > stop	D	-

the overlapping presence of HPV16 and *p53* mutations, the *p53* mutations were all of the nondisruptive type.

Retrospective case-series have consistently shown that patients with HPV-positive HNSCCs have an improved prognosis when compared with patients with HPV-negative tumors (1, 23–25). Several hypotheses have been offered to account for this difference. The immune system likely plays a pivotal role in modulating the behavior of HPV-related cancer of the head and neck, and strategies designed to up-regulate the immune response hold promise for further improving patient outcomes. The effects of "field cancerization" are diminished in patients with HPV-positive HNSCCs. Whereas chronic exposure to chemical carcinogens induce genetic alterations that tend to be distributed across large tracts of epithelium (26–28), HPV16 specifically targets the tonsillar epithelium resulting in a more restricted distribution of HPV-positive cancers. In effect, patients with HPV16-positive HNSCCs are less likely to succumb to synchronous and metachronous tumors (24, 29). Like other studies, we did not detect the presence of HPV16 in any cancers arising outside of the oropharynx (1, 30, 31).

HPV16 infection does not track with certain genetic alterations that characterize most HNSCCs, and the segregation of genetic profiles as a function of HPV16 status may also contribute to differences in clinical outcomes. For example, Braakhuis et al. (32) observed that HPV16-positive tumors are not associated with widespread allelic loss that characterizes most HNSCCs. Our results indicate that the presence of HPV16 occurs at the exclusion of disruptive *p53* mutations. Importantly, disruptive *p53* mutations greatly affect survival outcomes. In breast cancer, *p53* mutations that reside within DNA binding sites are associated with treatment resistance and early cancer relapse compared with those *p53* mutations that reside outside of these DNA binding sites (33). In HNSCC, disruptive p53 mutations likewise have been correlated with accelerated tumor progression and reduced therapeutic responsiveness (19). We do not report clinical follow-up in our small nested study population, but in the complete cohort of study patients with HNSCC, disruptive *p53* mutations were found to be a significant predictor of survival independent of stage and treatment (34).

HPV16 infection and *p53* mutation may coexist in a subset of HNSCC, but there is an inverse correlation between HPV16 and the types of *p53* mutations that most severely disrupt DNA binding. A less calamitous genetic profile such as the absence of disruptive *p53* gene mutations may underlie the emerging clinical profile of HPV16-positive HNSCC including improved response to therapy and patient outcome.

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