

Second Esophageal Tumors in Patients with Head and Neck Squamous Cell Carcinoma: An Assessment of Clonal Relationships

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ABSTRACT

Patients with squamous cell carcinoma of the head and neck (HNSCC) often develop second carcinomas elsewhere in the upper aerodigestive tract. Some of these paired tumors share a common origin, reflecting the ability of a single progenitor cell to replicate, expand, and populate contiguous regions of the upper aerodigestive tract—a process referred to as clonal expansion. The geographical limitations of clonal expansion, however, have not been adequately addressed. For example, it is not known whether a neoplastic clone from the oral cavity, pharynx, or larynx can migrate to the esophagus. We compared paired tumors from 16 patients with HNSCC and a second squamous cell carcinoma of the esophagus (ESCC) for patterns of allelic loss on chromosomal arms 3p, 9p, and 17p. Losses at these loci occur early during neoplastic transformation of the respiratory tract. In 14 cases (87%), the paired tumors had discordant patterns of allelic loss, suggesting that these tumors were not clonally related. Conversely, two (13%) of the 16 paired tumors had identical genetic alterations, which suggests clonal expansion as the mechanism underlying tumor multifocality. One clone spread from the hypopharynx into the cervical esophagus, and the other spread from the tonsil to the distal esophagus. Although most second ESCCs appear to arise as independent neoplasms, a clonal population of neoplastic cells is capable of traveling across substantial distances to give rise to second tumors at different anatomical sites.

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INTRODUCTION

Second tumors of the aerodigestive tract have a sobering effect on the outlook for patients with HNSCC.² They are often fatal, and they will develop in the head and neck, lungs, or esophagus of 10–40% of patients with HNSCC (1–4). As treatment of the index HNSCC improves, the threat of developing a second tumor only intensifies (5). Consequently, the overall survival for patients with HNSCC has not significantly improved over the past several decades (6).

Advances in the prevention, treatment, and surveillance of HNSCC await a more complete understanding of the mechanistic underpinning of tumor multifocality. One theory to explain multifocal tumor origin in patients with HNSCC was proposed over 4 decades ago by Slaughter *et al.* (7). According to this “field cancerization” concept, multiple cell groups independently undergo neoplastic transformation under the stress of regional carcinogenic activity. Molecular genetic approaches to the study of human cancer have recently challenged the notion that independent transforming events are commonplace in the respiratory tract of patients with HNSCC. Indeed, when a primary HNSCC is compared with second tumors elsewhere in the respiratory tract, the paired tumors often harbor identical patterns of genetic alterations (8–11). Understanding the clonal relationships of multifocal tumors has clarified certain practical clinical issues regarding tumor spread. For example, most second solitary lung tumors in patients with HNSCC are now felt to be metastases rather than independent primary tumors (11); and many “skip lesions” in the head and neck result from the expansion of neoplastic cells from a primary site to nearby locations (8–10).

The scope of clonal expansion is not well understood, but several observations suggest that the process may account for the extension of neoplastic cells well beyond the microscopic boundaries of a tumor mass. In the lung, the presence of specific genetic alterations throughout the airways of smokers suggests that surface migration of clonal cells can be widespread even in the absence of overt histopathological changes of malignancy (12). In the head and neck, the epithelium may harbor numerous genetic alterations but may lack any histopathological evidence of dysplasia (13, 14). For example, certain genetic alterations that are present in a resected HNSCC can also be detected at the margins of resection, even when these margins are histologically free of tumor (15). In these instances, clonal expansion has been implicated as an important mechanism underlying local tumor recurrence after “complete” surgical removal. Discerning the

² The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; ESCC, squamous cell carcinoma of the esophagus; LOH, loss of heterozygosity.

presence and extent of clonal spread could ultimately generate novel strategies for assessing cancer risk, margin status, tumor recurrence, and response to chemopreventive agents (16, 17).

We attempted to discern the relationship of HNSCCs and second ESCCs by comparing tumors for genetic alterations that occur frequently and early during neoplastic transformation of the upper aerodigestive tract. Previous work has shown that comparative analysis of microsatellite markers located on chromosomal arms 3p, 9p, and 17p is a good method for discerning the relationship of multifocal tumors of the aerodigestive tract (8, 10, 11). Our objective was to determine whether clonal expansion is a common mechanism of tumor multifocality in this specific setting, and to explore the distance limitations of clonal expansion.

PATIENTS AND METHODS

Patients. The surgical pathology files of The Johns Hopkins Hospital were searched for all of the patients with HNSCC who also developed ESCCs. After this initial patient identification, all of the original histological slides and medical records were reviewed. Criteria for inclusion included: (a) the HNSCCs and the ESCCs must have been noncontiguous tumors that were anatomically separated by normal appearing mucosa based on the clinical, surgical, and pathological findings; (b) the head and neck and esophageal tumors must have been of the squamous cell type; and (c) sufficient material must have been available for DNA extraction and subsequent analysis.

Microsatellite Analysis. To determine the relationship between HNSCC and ESCC of the same patient, we examined the status of chromosomes 3p, 9p, and 17p using a total of 10 polymorphic microsatellite markers. The specific markers used in this study were selected because they identify a minimal area of loss at putative tumor suppresser gene loci and because they are lost frequently and early during neoplastic progression of squamous cell carcinomas of the head and neck (13). Chromosomal arm 3p has been shown to contain at least three putative HNSCC tumor suppresser loci (18, 19), the minimal region of loss on chromosome 9 includes the *INK4a* locus encoding *p16^{INK4a}* and *p19^{ARF}* (20–22), and chromosomal arm 17p13 contains the *p53* tumor suppresser gene (21, 23).

Archival formalin-fixed and paraffin-embedded tissues were sectioned, and tumor sections were carefully microdissected to obtain greater than 75% neoplastic cells. The nonneoplastic tissues used for the study were obtained from uninvolved lymph nodes or adjacent nonepithelial tissues. DNA was extracted and subjected to PCR amplification as described previously (13). The microsatellite markers used in this study included four markers from chromosomal arm 3p (D3S1067, D3S1274, D3S1038 and D3S1766), four markers from chromosomal arm 9p (D9S157, *IFN- α* , D9S1748 and D9S171), and two markers from chromosomal arm 17p (*TP53* and *CHRNB-1*). Before amplification, 50 ng of one primer (Research Genetics, Huntsville, AL) was end-labeled with [γ -³²P]ATP (20 mCi; Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (New England Biolaboratories, Beverly, MA) in a total volume of 50 μ l. PCR reactions were carried out in a total volume of 12.5 μ l containing 10 ng of genomic DNA, 0.2 ng of labeled primer, and 15 ng of each unlabeled primer as described

previously (13). PCR amplifications of each primer set were performed for 30–35 cycles consisting of denaturation at 95°C for 30 s, annealing at 50–60°C for 60 s, and extension at 70°C for 60 s. The products were separated by denaturing gel electrophoresis and visualized by autoradiography. Alleles were considered to be lost when the PCR assay of nonneoplastic tissue showed heterozygosity of the microsatellite markers, and the relative intensity of one allele in the tumor DNA differed from the relative intensity in the nonneoplastic tissue DNA by at least 30% based on visual inspection. Allelic status was confirmed by at least two independent observers (J. C. and D. S.).

Determination of Clonal Relationship. Establishing the clonal relationship of multiple tumors from the same patient is not always straightforward. Unrelated tumors can lose the same allele as a chance event. Assessing LOH at multiple loci can minimize the impact of chance events. Conversely, related tumors can demonstrate discordant patterns of allelic loss as a result of the accumulation of new alterations during tumor progression. In these instances, the presence of shared novel microsatellite shifts or common breakpoints (*i.e.*, a common boundary of loss and retention between closely spaced microsatellite markers) is helpful in establishing clonal origin. In this study, tumor pairs were judged to be clonally related if they demonstrated: (a) an identical pattern of LOH at all of the three chromosomal arms; (b) an identical breakpoint between microsatellite markers that are less than 10 cM apart; and/or (c) an identical unique microsatellite shift. Although it is possible that an undetermined genetic event preceded loss at the tested markers, tumor pairs that did not meet these criteria were considered to be clonally unrelated.

RESULTS

Clinical Parameters of Patients with HNSCC and ESCC. Sixteen patients with HNSCC and ESCC formed the basis of this study (Table 1). In all of the 16 cases, the tumors in the head and neck and the esophagus were clinically, radiographically, and/or pathologically judged to be noncontiguous tumors that were separated by normal intervening esophagus. Five of the ESCCs arose in the upper esophagus, seven arose in the mid-esophagus, and four arose in the lower esophagus. Twelve of the ESCCs were synchronous tumors that were discovered during evaluation of the HNSCC. The other four ESCCs were metachronous tumors that were discovered during or after treatment of the index HNSCC. The mean duration from treatment of the index HNSCC to the diagnosis of these metachronous ESCCs was 15 months.

Only the tumors from patients 5 and 9 were found to be clonally related (see below). Patient 5 developed a stage T₃ N₀ M₀ squamous cell carcinoma of the hypopharynx 25 years after a laryngectomy for squamous cell carcinoma of the vocal cord. The vocal cord tumor was not available for analysis. She underwent a total pharyngectomy with a pectoralis flap reconstruction. The tumor was completely resected, and the margins were microscopically free of dysplasia and infiltrating carcinoma. Sixteen months later during evaluation for dysphagia, a squamous cell carcinoma was found in the cervical esophagus near the myocutaneous flap junction, and an esophagectomy with gastric pull up was performed. Histopathological evaluation of

Table 1 Clinical features and clonal relationships of paired tumors

Patient no.	HNSCC Site	ESCC		Clonal relationship (by molecular analysis)
		Interval ^a	Location in esophagus ^b	
1	Hypopharynx	0	Lower	Not clonal
2	Hypopharynx	0	Lower	Not clonal
3	Hypopharynx	14	Upper	Not clonal
4	Glottic larynx	0	Middle	Not clonal
5	Hypopharynx	16	Upper	Clonal
6	Floor of mouth	0	Middle	Not clonal
7	Supraglottic larynx	0	Middle	Not clonal
8	Base of tongue	14	Upper	Not clonal
9	Tonsil	0	Lower	Clonal
10	Glottic larynx	49	Middle	Not clonal
11	Hypopharynx	0	Lower	Not clonal
12	Floor of mouth	0	Middle	Not clonal
13	Base of tongue	0	Upper	Not clonal
14	Tonsil	0	Middle	Not clonal
15	Hypopharynx	0	Middle	Not clonal
16	Lateral tongue	0	Upper	Not clonal

^a Months between treatment of head and neck cancer and diagnosis of esophageal cancer; an interval of 0 months indicates diagnosis was made during the evaluation of head and neck cancer.

^b Upper, cervical portion extending approximately 18 cm from the upper incisor teeth; Middle, upper thoracic portion extending from the thoracic inlet to the level of the tracheal bifurcation; Lower, lower thoracic portion from the level of the tracheal bifurcation to the esophago-gastric junction.

both tumors showed *in situ* and infiltrating moderately differentiated squamous cell carcinomas. The resection margins were free of dysplasia and infiltrating carcinoma.

Patient 9 had a stage T₃ N_{2c} M₀ squamous carcinoma arising in the tonsil and a synchronous squamous cell carcinoma arising in the distal esophagus. The ESCC was a circumferential and exophytic mass that was clinically thought to arise from the surface epithelium. It was separated from the tonsillar carcinoma by 40 cm of normal appearing mucosa. The lesions were biopsied and both the tonsillar and esophageal tumors were found to be *in situ* and infiltrating moderately differentiated and keratinizing squamous cell carcinomas. The tumors were not surgically resectable.

Genetic Analysis of Paired HNSCCs and ESCCs.

Comparison of specific genetic alterations is now a feasible method for addressing the clonal relationships of multifocal neoplasms of the aerodigestive tract. We compared HNSCCs and their paired esophageal tumors for patterns of allelic loss on chromosomal arms 3p, 9p, and 17p (Fig. 1). Of the 16 paired tumors, only 2 (13%; from patients 5 and 9) were judged to be clonally related according to the criteria used for this study ("Patients and Methods"; Fig. 2). The paired tumors from patient 5 demonstrated identical patterns of allelic loss at all of the three chromosomal arms. The probability that this pattern of loss occurred by chance between two tumors is low, even when correcting for linkage of all of the markers tested for a particular chromosomal region (*i.e.*, $P = 0.5^3 = 0.125$). Moreover, the paired tumors shared breakpoints between markers *IFN-α* and D9S167 (a distance of 4 cM), and between markers *IFN-α* and D9S1748 (a distance of 1–2 cM). This pattern of retention of *IFN-α* surrounded by LOH at closely spaced markers is indic-

ative of homozygous deletion at the *p16 (CDKN2A/MTS-1/INK4a)* locus. Thus, the northern and southern boundary of this homozygous deletion is identical in both tumors. The paired tumors from patient 9 also demonstrate the same pattern of LOH at all of the informative loci, and shared an identical breakpoint between markers D9S1748 and D9S171 (a distance of 1 cM). The other 14 tumor pairs (87%) demonstrated discordant patterns of allelic loss at one or more chromosomal arms, and they did not share novel microsatellite alterations or breakpoints between closely spaced markers.

DISCUSSION

Individuals with HNSCC have a high likelihood of developing a second tumor in their upper aerodigestive tract including the esophagus (1–4). Despite the clinical impression that these multifocal tumors are distinct and independent, genetic analysis has shown that closely spaced tumors in the head and neck are often derived from the same neoplastic clone (8–10). Presumably, a critical genetic alteration in a single cell provides a growth advantage over its neighboring cells. All of the subsequent daughter cells share this initiating genetic event. At some point after transformation, cells harboring these early genetic alterations migrate to populate contiguous tracts of mucosa. As the process evolves further, the accumulation of other independent genetic alterations confers an additional growth advantage to subpopulations of cells. Ultimately, dominant outgrowths of more aggressive phenotypes give rise to multiple tumors at discontinuous sites along the respiratory tract. Thus, the clonal origin of tumors can be recognized by the presence of shared early genetic events that occurred before the initial migration, even in those tumors separated both in time and space (24).

The limits of clonal spread are not well defined, but the detection of tumor-specific genetic alterations far beyond the histological confines of an overt malignancy has suggested that it can be widespread. We, therefore, compared patterns of allelic loss on chromosomes 3p, 9p, and 17p to assess whether clonal expansion could account for the development of second primary ESCCs in patients with HNSCC, even when the tumors are separated by considerable distances.

Only 2 (13%) of the 16 paired tumors (from patient 5 and patient 9) demonstrated compelling evidence of a clonal relationship. The other 14 (87%) tumor pairs did not meet criteria for a common clonal origin, including three tumor pairs from the pharynx and upper esophagus (from patients 3, 8, and 13). Four of these tumor pairs (from patients 2, 3, 6, and 7) did not share any common allelic losses at informative loci, and 10 tumor pairs (from patients 1, 4, 8, 10, 11, 12, 13, 14, 15, and 16) shared common allelic losses at some but not all of the informative loci. Thus, clonal spread of neoplastic cells can account for the development of second esophageal carcinomas in a subgroup of patients with HNSCC, but this does not represent the prevailing pathway underlying tumor multifocality in the head and neck and esophagus. Most second ESCCs seem to arise as independent neoplasms even when they are anatomically juxtaposed.

Admittedly, assessing clonal origin in the 10 tumor pairs sharing some, but not all, of the genetic alterations is not straightforward. Allelic loss of 3p, 9p, and 17p is common in squamous cell carcinomas of both the esophagus and the upper

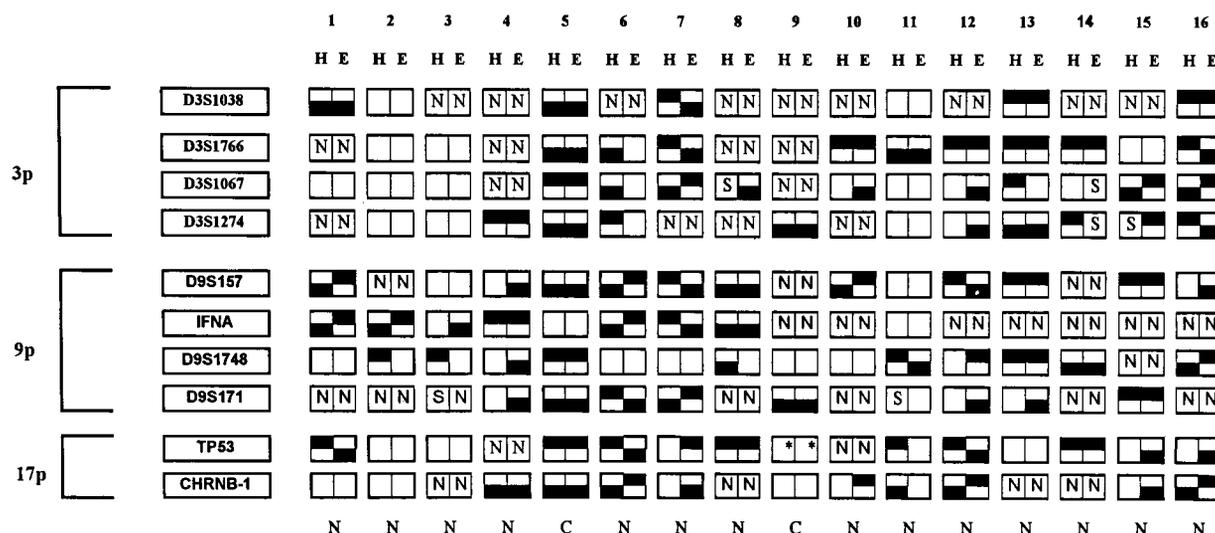


Fig. 1 Results of comparative microsatellite analysis. *Top row*, patient numbers. The paired tumors from the head and neck (*H*) and esophagus (*E*) for patients 5 and 9 were judged to be clonally related based on the concordant patterns of microsatellite loss at all of the loci tested. The presence of common “breakpoints” between closely spaced markers provided further evidence of clonality. *On the left*, the 3p, 9p, and 17p markers are listed in sequential order, proceeding from the most telomeric (*top*) to the most centromeric (*bottom*). □, allelic retention; ■, allelic loss; *the position of the black boxes (upper or lower)*, the relative position (upper or lower) of the lost alleles; S, shifted microsatellite allele; N, not clonal; C, clonal; □, noninformative; □, equivocal result.

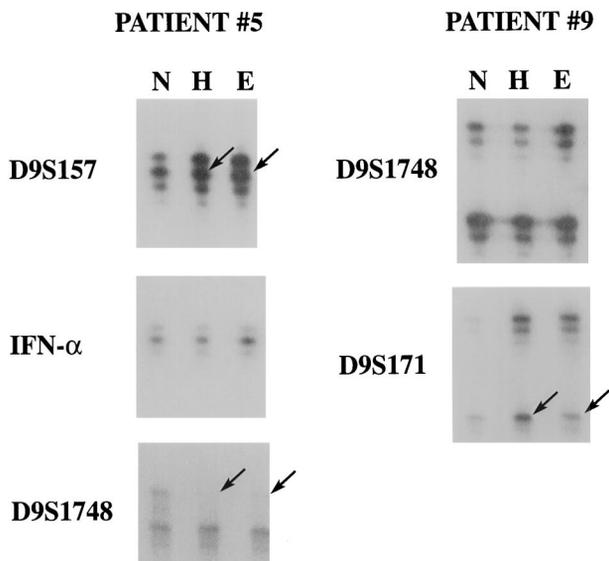


Fig. 2 Analysis of allelic status in paired normal tissue (*N*), head and neck tumor (*H*), and esophageal tumor (*E*) from two patients with metachronous or synchronous squamous cell carcinomas. *On the left*, selected markers from chromosomal arms 9p (*D9S157*, *IFN- α* , *D9S1748*, and *D9S171*). *Arrows*, the lost alleles in each of the informative tissue samples. Both tumor pairs show a common boundary between allelic loss and retention (breakpoint).

respiratory tract (25–27) such that shared allelic loss can occur as a coincident event. To minimize the impact of coincident events, we stringently required identical patterns of LOH at three different chromosomal arms. On the other hand, the order of genetic alterations during tumor progression is not absolute

and may vary for individual tumors. Consequently, loss of specific genetic loci may not occur until a later stage of tumor progression such that certain tumor pairs may be clonally related, although they display discordant allelic losses. To minimize the impact of clonal progression, we selected chromosomal loci that are consistently lost early in the progression of HNSCC and ESCC (24, 26). From our previous studies, in which molecular genetic assessment was compared with clinical assessment, these specific loci were found to be reliable in ascertaining the clonal relationships between HNSCC and second squamous cell carcinomas in the lung (11). Shared novel microsatellite alterations and/or chromosomal breakpoints between closely spaced microsatellite markers also help establish clonal origin, particularly in tumor pairs that are not entirely concordant at all of the chromosomal loci evaluated. Of note, none of the tumor pairs showing discordant patterns of LOH demonstrated such evidence of common clonal origin.

Although our results indicate that most ESCCs do not seem to be clonally related to the index HNSCC, they do demonstrate the extremes to which neoplastic cells can migrate from one site to another. At one extreme, a clonal population of cells can focally extend beyond the histological boundaries of a HNSCC to give rise to local tumor recurrence. The ESCC from patient 5 developed in the cervical esophagus after the resection of a primary carcinoma in the hypopharynx. This pattern of spread may account for the frustrating occurrence of local tumor failure of a HNSCC, even after “complete” surgical removal with histologically clear margins. Just because tumors are closely related anatomically, however, does not necessarily indicate that they are clonally related. The three other esophagus/pharyngeal tumor pairs (in patients 3, 8, and 13) seemed to represent independent primary tumors rather than local tumor recurrences.

At the other extreme, a clonal population of cells can be

widely dispersed across relatively vast distances. The ESCC from patient 9 arose in the distal esophagus in a patient with a primary oropharyngeal carcinoma. Remarkably, these tumors were separated by 40 cm of normal appearing mucosa. The mode by which neoplastic clonal cells traveled from the oropharynx to the distal esophagus is unclear. Conventional metastatic spread by way of lymphatics or blood is possible. Many presumed-primary second lung cancers, for example, have been found to actually represent solitary lung metastases (11). This mechanism, however, was not likely because: (a) clinically, the ESCC was an exophytic lesion that involved the esophagus circumferentially in a manner typical of ESCC arising from the surface epithelium; (b) histologically, both the HNSCC and the ESCC appeared to arise from an area of overlying squamous carcinoma *in situ*; (c) anatomically, lymphatics draining the oropharynx flow to the deep cervical lymph nodes and not the esophagus; and (d) the esophagus was never observed as a site of metastatic implantation in two large series that evaluated patterns of metastatic spread in a combined total of 1001 patients with HNSCC (28, 29). As an alternative mechanism, neoplastic clonal cells could exfoliate from their primary site and then implant elsewhere in the aerodigestive tract in an area of mucosal erosion. This mechanism has been proposed for multifocal tumor growth seen in certain types of lung cancer (30). Most investigators, however, believe that a clonal migration represents expansion of a single progenitor cell to the point at which cells harboring certain specific gene alterations populate contiguous tracts of the upper aerodigestive tract, either by gradual replacement of the normal mucosa or by intramucosal migration of single cells (*i.e.*, pagetoid extension). Sometimes these genetically damaged cells may be widespread throughout the epithelium of the aerodigestive tract, even when they are not apparent histologically. Because only small biopsies of the tumors were obtained, we were not able to confirm the presence of genetic alterations in the intervening mucosa. However, recent studies have confirmed the presence of large clonal patches of epithelium involving the distal esophagus in patients with Barrett's esophagus (31, 32).

Molecular genetic strategies for the detection of specific genetic alterations are becoming increasingly important for monitoring the progression and spread of HNSCC. In patients with premalignant lesions of the upper respiratory tract, the detection of genetic alterations may serve as useful biomarkers of disease response to chemoprevention (16). After apparently complete surgical resection of HNSCC, the presence of specific genetic alterations at the histologically benign resection margins is an indicator of inadequate excision and a predictor of local failure (15). In the present study, we used a molecular genetic approach to assess the clonal relationship of synchronous/metachronous tumors of the head and neck and esophagus. Although most HNSCCs and ESCCs were found to be entirely independent tumors, our observations confirm that the extent of clonal migration is not always restricted to certain anatomical compartments. Adequately identifying the extent of clonal expansion by molecular analysis, including extension to remote sites, may have profound implications for patient management.

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