

Multiple Head and Neck Tumors: Evidence for a Common Clonal Origin¹

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Abstract

Patients with head and neck cancers have a high (2–3%/year) incidence of second primary lesions. Clinically, these new lesions are identified either simultaneously with the primary lesion (synchronous) or after a period of time (metachronous). This observation has been attributed to the concept of “field carcinogenesis,” which is based on the hypothesis that prolonged exposure to carcinogens leads to the independent transformation of multiple epithelial cells at several sites. An alternative theory is based on the premise that any transforming event is rare; following initial transformation, the progeny of the transformed clone spread through the mucosa and give rise to geographically distinct but genetically related tumors. We analyzed the pattern of X-chromosome inactivation in multiple primary tumors from eight female patients with head and neck cancer. In addition, we used microsatellite analysis to examine the pattern of allelic loss on chromosomes 9p and 3p, identified as early events in the progression of head and neck malignancies. In four of four cases, multiple tumors demonstrated the same pattern of X-chromosome inactivation. In the remaining four cases, X-chromosome deletions prevented interpretation ($n = 3$), or the androgen receptor locus was noninformative ($n = 1$). In three of nine patients, multiple tumors displayed the same pattern of loss of heterozygosity, two with identical breakpoints on chromosome 9p. In one patient, there was an identical microsatellite alteration at a 3p locus, definitive evidence that these tumors arose from the same clone. Our findings suggest that in at least a proportion of patients with head and neck cancers, multiple primary tumors arise from a single clone.

Introduction

In 1953, Slaughter *et al.* (1) reported a high incidence of second primary cancers in patients with HNSCC³ and proposed the concept of field cancerization to explain this phenomenon. Since then, there have been numerous clinical reports confirming the observation of Slaughter *et al.* (1), with some noting that patients with HNSCC have a 2–3% chance of developing a second primary cancer each year (2, 3). The occurrence of multiple tumors can be explained by two competing hypotheses: (a) multiple transforming events give rise to genetically unrelated multiple tumors, or (b) a single cell is transformed and through mucosal spread gives rise to genetically related multiple tumors. The distinction between the origins of multiple tumors is not possible based on the microscopic histological appearance of the lesions. However, it is possible to test these hypotheses by modern molecular techniques. To establish the relationship between multiple tumors at a molecular level, it is necessary to study early genetic events, preferably the first transforming event. In one report, discordant *p53* mutations in multiple tumors of the upper aerodigestive tract were presented as evidence that they arise as independent

events (4). However, *p53* mutations follow other events in the genetic progression of head and neck tumors, and could still be distinct if the tumors arose from the same clone, but migrated to different sites before the *p53* event. In our study of bladder cancer, multiple tumors from female patients were established to arise from a single clone based on the pattern of X-chromosome inactivation and chromosome 9 loss (5). There has been a recent report of two topographically distinct head and neck tumors that shared a clonal Y marker, providing definitive evidence that these tumors arose from the same clone (6).

To determine whether multiple head and neck tumors arise from a single clone or are independent events arising in a field barraged by carcinogenic insults, we analyzed multiple topographically distinct tumors from eight female patients. We studied their clonal origin by analyzing the pattern of X-chromosome inactivation, an event that occurs during embryogenesis, and therefore definitely precedes tumorigenesis. We also examined the patterns of allelic loss on chromosomes 9p and 3p, events which are believed to occur early in the genetic progression of head and neck tumors (7–9). In several patients, we obtained molecular evidence establishing the identical clonal origin of multiple tumors.

Materials and Methods

Patients with two or more topographically distinct synchronous tumors were identified, and tissue specimens were obtained. Metachronous lesions were identified retrospectively, and the possibility of recurrence was excluded by the location of the lesions at geographically distinctly sites (Table 1). All of the specimens were archival formalin-fixed, paraffin-embedded tissues, with the exception of one sample (patient 8, tumor T₂) that was obtained as fresh frozen tissue. Sections (8 μ m) were cut, and neoplastic cells were microdissected away from nonneoplastic tissue. Corresponding normal (control) samples were obtained from muscle, blood, or a distant solid organ site. After deparaffinization, the microdissected samples were incubated in SDS/proteinase K at 48°C for 48 h, with additional proteinase K being added every 12 h. DNA was then extracted as described previously (10).

X-chromosome inactivation was tested using the PCR-based androgen receptor assay. Five μ l of the resuspended DNA were incubated overnight at 37°C with 10 units *Hha*I (Life Technologies, Inc.) in a 10- μ l reaction volume. Simultaneously, in a mock reaction, 5 μ l of the same DNA were incubated with 5 μ l 1 \times reaction buffer. The enzyme was inactivated by heating the reaction mixture at 60°C for 10 min. Five μ l of the reaction mixture were used in each case as the template for PCR. The oligonucleotide primers used for PCR have been described previously (11). One of the primers was end labeled with [γ -³²P]ATP using T4 polynucleotide kinase (New England Biolabs). Reactions were carried out at 95°C for 30 s, 60°C for 60 s, and 70°C for 60 s, for 35 cycles. All reactions were repeated by redigestion and reamplification. Products were separated by denaturing gel electrophoresis, followed by autoradiography.

Dinucleotide microsatellite markers on chromosome 9p21 (*D9S156*, *D9S157*, *D9S162*, *IFNA*, *D9S1751*, *D9S1747*, *PKY9*, *D9S1748*, and *D9S171*), 3p (*D3S1284* on 3p12, *D3S1038* on 3p25), and the X-chromosome flanking the androgen receptor gene on Xq12 (*DXS1212*, *DXS1216*, and *DXS453*) were tested. The conditions for PCR amplification and denaturing gel electrophoresis have been described previously (12).

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³ The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity.

Table 1 Clinical characteristics and X-chromosome inactivation status of multiple head and neck tumors

Patient	Lesion	Date	Site	Histology	X-chromosome inactivation ^a
1	T ₁	11/92	Tongue/FOM ^b	Invasive	2
	T ₂	9/93	L buccal	Invasive	2
2	T ₁	3/88	L tonsil	Invasive	1
	T ₂	10/91	Nasal/premax.	Invasive	1
3	T ₁	11/83	Hard palate	Invasive	2
	T ₂	11/88	FOM	Invasive	2
4	T ₁	11/87	L BOT/tonsil	Invasive	LOH
	T ₂	9/92	Ant. tongue	Invasive	LOH
5	T ₁	12/92	R RMT	Invasive	2
	T ₂	12/92	R post. alv.	Invasive	LOH
6	T ₁	12/92	Ant. FOM	Dysplasia	LOH
	T ₂	1/95	R tongue	Invasive	2
7	T ₁	1/95	L tongue	CIS	2
	T ₂	1/95	L RMT	Dysplasia	LOH
8	T ₁	7/86	Ant. FOM	Invasive	NI
	T ₂	7/94	Mid-L tongue	Invasive	NI
8	T ₁	2/94	Ant. tongue	Invasive	LOH
	T ₂	2/95	Larynx	Invasive	2

^a 1, upper allele active and unmethylated; 2, lower allele active and unmethylated.
^b FOM, floor of mouth; BOT, base of tongue; RMT, retromolar trigone; Post. alv., posterior alveolus; L, left; R, right; CIS, carcinoma *in situ*; NI, noninformative; Ant., anterior; Premax, premaxillary.

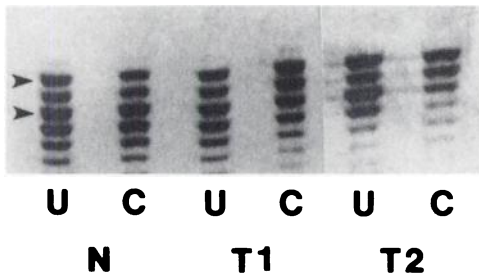


Fig. 1. Representative example of analysis of X-chromosome inactivation at the androgen receptor locus. Patient 6 had three synchronous multiple head and neck tumors, of which the results of X-chromosome inactivation could be interpreted in tumors T₁ and T₂. DNA samples from normal tissue (N) and tumors T₁ and T₂ were digested with *HhaI* [cut (C)] or subjected to a mock reaction [uncut (U)] followed by PCR amplification. Arrowheads, parental alleles, which are of similar intensity in the uncut samples of the normal and tumor specimens. After digestion with *HhaI*, the normal specimen revealed no change in relative intensity of the two alleles, a normal polyclonal pattern. On the other hand, both tumor specimens displayed a significant reduction in the intensity of the lower allele as compared to the upper allele, thus displaying similar monoclonal patterns of X-chromosome inactivation.

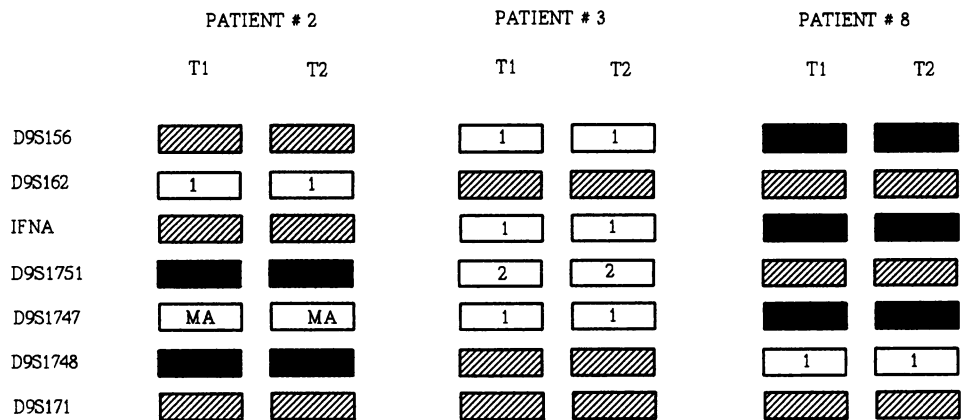
Results

We used X-chromosome inactivation to determine the clonal origin of multiple tumors in eight female patients with HNSCC (total of 18 neoplastic lesions). In females, the inactivation of one X-chromosome

occurs randomly early in embryogenesis, subsequently remains stable throughout the lifetime of the cell, and the same pattern is passed on to its daughter cells (13). The human androgen receptor assay is a PCR-based method that utilizes a highly variable region of trinucleotide repeats in the first exon of the androgen receptor gene, with a number of CpG restriction sites located close to the repeats (14). X-chromosome inactivation is manifested by CpG island methylation, thus these restriction sites are methylated in the inactive allele (15). After digestion with a methyl-sensitive restriction enzyme and amplification using PCR, it is possible to distinguish between the active and inactive alleles in an informative (heterozygous) female. There were four sets of multiple tumors (patients 1–3 and 6) in which the results of the X-chromosome inactivation could be interpreted, and all four demonstrated the same pattern of X-chromosome inactivation (Fig. 1). As X-chromosome inactivation is a random event, each tumor pair has a 50% chance of displaying the same pattern of inactivation if the tumors are independent. Thus, the probability that these events occurred by chance alone is $(0.5)^4 = 0.0625$. One patient (patient 7) was noninformative at the androgen receptor locus (*i.e.*, homozygous), and in the three remaining patients (and tumor T₃ from patient 6), one or both tumors displayed LOH at the androgen receptor locus. To confirm that there was indeed allelic loss of the X-chromosome in this region, we tested several dinucleotide markers flanking the androgen receptor gene. Allelic losses at these loci (data not shown) confirmed the findings at the androgen receptor locus. X-chromosome inactivation could not be interpreted in these cases, since we do not know whether the methylated or the unmethylated allele is lost in this region of the X-chromosome.

As noted earlier, shared genetic events that occur early in progression can also point to a common clonal origin. We tested several microsatellite markers on chromosome 9p21, and LOH in at least one marker was seen in all of the tumors. Two sets of tumors (patients 2 and 8) had the same patterns of loss, with identical breakpoints between markers *D9S1747* and *D9S1748* in patient 8 (a distance of 150 kb) and *D9S162* and *D9S1751* in patient 2 (a distance of <2 cM) (Fig. 2). These results provide strong evidence for a common clonal origin of these multiple tumors, and were in concordance with the same pattern of X-chromosome inactivation seen previously. Both tumors from patient 3 had identical losses on all of the informative markers tested on 9p. However, we did not identify any breakpoints, and the likelihood of this pattern occurring by chance is 50%. Two markers on chromosome 3p were tested, and the patterns of loss were different in most sets of tumors. However, an identical and unique microsatellite alteration was identified in the two tumor samples from patient 8 at the locus *D3S1284* (Fig. 3), confirming a common clonal origin for these two tumors.

Fig. 2. Pattern of allelic loss on chromosome 9p: results of microsatellite analysis. Tumors from patients 2, 3, and 8 displayed identical patterns of loss. The two sets of tumors from patients 2 and 8 had identical breakpoints, whereas the tumors from patient 3 had LOH of all informative markers tested. There were microsatellite expansions seen in both tumors from patient 2 at *D9S1747*, but they were of different sizes. 1, upper allele lost; 2, lower allele lost; MA, microsatellite alteration; ■, retention; ▨, noninformative.



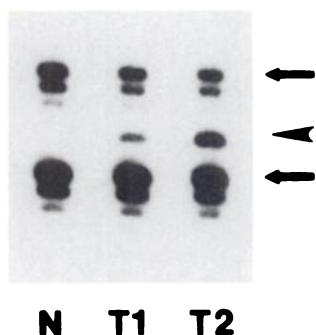


Fig. 3. Identical microsatellite alteration observed in both tumors T₁ and T₂ from patient 8 at the microsatellite locus *D3S1284*. The novel band (arrowhead) here lies between the two parental alleles (arrows) and is seen in the two tumors but not the normal specimen (N).

Discussion

An important unresolved question regarding head and neck neoplasms is the genesis of multiple tumors in this region. Modern molecular techniques can help us understand the biology of these lesions, and may have an impact on designing effective diagnostic and therapeutic strategies to deal with these tumors which have a significant adverse effect on survival (16, 17).

Neoplastic transformation is believed to occur in a single cell as a result of a critical genetic alteration that provides a growth advantage over its neighboring cells (15, 18, 19). All subsequent daughter cells in the tumor arise from this transformed cell and share the initiating genetic event. As the tumor grows, subclones develop, which are populations with additional genetic changes, and this leads to heterogeneity (20–22). Those subclones that confer an additional significant growth advantage, gradually take over as the dominant population in the tumor. This cycle continues, with cells continuing to accumulate genetic changes as the tumor develops a more aggressive phenotype. When multiple tumors arise from a single clone, the hypothesis is that at some point after transformation, some cells break away and migrate to another site. They may gradually replace normal mucosa in some fashion, migrate, or be shed into the saliva and settle down in an area where there is a small mucosal erosion. Whatever the mode of transfer, the cell that has moved away carries with it the genetic changes from the progenitor cell in the initial lesion. From then on, this cell continues to divide, grow, and accumulate additional genetic changes independent of the parent clone. To test whether two tumors have the same clonal origin, it would need to be determined whether they shared an early genetic event, one that occurred before the initial migration. This migration may occur even before a tumor becomes invasive, perhaps after acquiring just a few early genetic changes. We tested the clonal origin of our multiple head and neck tumors using X-chromosome inactivation, an event which occurs during embryogenesis, and thus before transformation and migration. Unfortunately, a number of tumors had to be excluded from our analysis due to LOH at the androgen receptor locus on the X-chromosome. Deletions of portions of the X-chromosome have been previously identified in a significant fraction of head and neck tumors by cytogenetic analysis (23, 24). The pattern of inactivation in the four cases that we could evaluate was identical and approached statistical significance ($P = 0.0625$).

To complement these data, we studied the pattern of allelic loss at loci that are frequently lost early in the progression of HNSCC. The minimal region of loss on chromosome 9p includes the *p16* gene on 9p21 (7, 25, 26), and has been established as a common area of loss in early precursor lesions (27). The pattern of loss was different in the tumor pairs in five of eight patients and identical in three patients. In

two of these three cases, we identified identical patterns of loss (breakpoints), confirming the common clonal origin of these tumors. However, differences in the pattern of loss on 9p is not sufficient to ascertain a distinct and independent origin in the remaining five cases. The genetic progression of neoplasms is due to an accumulation of a number of events; however, the exact order of these events can vary considerably in individual tumors (21). Thus, in those tumors exhibiting different patterns of loss, the 9p event may have occurred after migration. An earlier genetic event that we did not test may still be shared. Alternatively, it is possible that although some tumors arise from the same clone, others arise independently.

Occasional microsatellite alterations at dinucleotide repeats occur infrequently in a large variety of different neoplasms. This rate of “background” shifts in dinucleotide markers is about 0.5–1%, and, when present, they are useful as clonal markers (28, 29). We found an identical alteration in both tumors from one patient at one of the markers on 3p. With the low rate of alterations seen with dinucleotide microsatellite markers, this is very unlikely to be a chance occurrence and presents definitive evidence that these two tumors share a common clonal origin.

We have demonstrated that multiple head and neck tumors in some patients definitely arise from the same clone. We have no evidence that any of these tumors arose independently. Although it is possible that some second primaries arise as independent lesions, most probably share a common origin. These results are in concordance with the observations reported in the accompanying article, in which “skip lesions” around a primary lesion (also described by Slaughter *et al.* (1) as an effect of “field cancerization”) share a common clonal origin (27). Thus, it appears that neoplastic transformation is indeed a rare event. Although it is possible that in some patients more than one initial event occurs independently, in a significant proportion, all tumors seen—distant synchronous or metachronous tumors, recurrences,⁴ and skip lesions—arise from a common progenitor.

These observations have significant clinical implications. If the initial transforming event can be identified, diagnostic studies that detect residual cells with these changes could predict recurrence. Moreover, additional studies that can identify the initial transforming genetic event may generate novel chemopreventive and therapeutic approaches.

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⁴ J. Califano and D. Sidransky, unpublished observations.

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