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Synchronous Oral Carcinomas: Independent or Common Clonal Origin?[1]

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ABSTRACT

Second primary tumors in patients with head and neck cancer have a detrimental impact on long-term survival; at least 15% of patients develop additional tumors. Originally, it was hypothesized that multiple tumors developed independently after widespread epithelial exposure to carcinogens (the field cancerization theory), but recent molecular studies now support the alternative theory of a common clonal origin. If multiple tumors originate from the same clone, early genetic alterations in these cells should be common to all of the tumors. We have compared the pattern of allelic imbalance in paired tumors from five male patients with two synchronous oral squamous cell carcinomas and in peripheral dysplasia using microsatellite markers on chromosomes 3p, 9p, and 17p. Discordance, usually through loss of alternate alleles at the same microsatellite loci, was detected in two patients. The remaining three patients had identical alterations in their tumors. The changes identified occurred early in tumorigenesis, because, with only one exception, these were also present in the associated dysplasia. Thus, we provide evidence that synchronous oral squamous cell carcinomas are of independent origin in some patients but may be of common clonal origin in others.

INTRODUCTION

Long-term survival of head and neck cancer patients has not significantly improved in the last 20 years, despite advances in therapy. An important reason for this lack of progress is the development of second primary tumors in the upper aerodigestive tract (1, 2). The reported incidence of these multiple primary tumors varies, depending in part on the length of patient follow-up, but on average, 15% of head and neck cancer patients are affected (3). Patients at highest risk are those with early-stage disease, when control of the first (index) tumor, and therefore survival, is greatest (2, 3). Second primary tumors may occur simultaneously or within 6 months of the index tumor (synchronous), but in the majority of cases, they occur more than 6 months apart (metachronous; Ref. 4).

To account for the development of multiple (multicentric) primary tumors in the oral cavity, Slaughter *et al.* (5) proposed the theory of field cancerization, whereby multiple tumors could originate independently in an area of epithelium preconditioned to cancer development by long-term exposure to carcinogens. Initial molecular studies showing discordant *p53* mutations between index and second primary tumors in the head and neck seemed to support this theory of independent tumor origins (6). However, the alternate possibility, that multiple tumors in the head and neck are of common clonal origin, has recently gained support as a result of the detection of identical genetic alteration in these tumors (7, 8). It has been proposed that the previous findings of differences in *p53* mutations between multiple tumors may simply reflect a divergence in genetic changes later in tumor pathogenesis (8). Thus, it is only by comparison of the earliest genetic events that reliable determination of the dependent or independent origins of multiple primary tumors can be achieved.

In this study, we compared microsatellite alterations on chromosomes 3p, 9p, and 17p in five male patients with two synchronous OSCCs.[3] Dysplasia was microdissected from the margins of an OSCC in each patient, thereby allowing determination of whether the genetic alterations detected in the tumor occurred in the early stages of tumor development.

MATERIALS AND METHODS

Five male patients with two synchronous OSCCs formed the basis of the present study. The patients underwent radical resection of the index tumor and areas of erythroleukoplakia with simultaneous neck dissection as the primary mode of treatment (Table 1). In all cases, a second tumor was identified, separated from the first by nonneoplastic epithelium (4). Dysplastic epithelium of at least moderate severity was present at the periphery of one tumor in each patient. Tissue was formalin-fixed and paraffin-embedded. For experimental purposes, one 5-μm section from each block was stained with H&E to confirm histology; four adjacent 10-μm sections were deparaffinized, stained with 0.1% toluidine blue, and microdissected using a three-dimensional Narishige micromanipulator to provide tumor, dysplasia, and histologically normal epithelium. Submandibular salivary gland from these patients was used as a source of normal DNA. Microdissected samples were incubated in 40–100 μl of buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 0.5% Tween 20] containing 200 μg/ml proteinase K at 42°C overnight, followed by 95°C for 10 min to inactivate the enzyme. Samples were centrifuged briefly, and the supernatant was used for PCR-based microsatellite analysis.

Microsatellite markers (Research Genetics) were analyzed at loci on chromosomes 3, 9, and 17: (*a*) 3p25–24.2 (*D3S1293* and *D3S1283*); (*b*) 3p21.3–21 (*D3S1029*, *D3S1217*, and *D3S1210*); (*c*) 3p14.2–14.1 (*D3S1233*); (*d*) 9p23–21 (*D9S157* and *D9S171*); and (*e*) 17p13–11 (*D17S804*, *D17S520*, *D17S805* and *CHRNB-1*). DNA (5 μl) was amplified by PCR in a final reaction volume of 50 μl, containing 200 μM each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 16 mM $(NH4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.1% Tween-20, 10 pmol of each primer, and 0.5 unit BIOPRO DNA polymerase (Bioline, London, United Kingdom). An initial DNA denaturation step of 94°C for 3 min was followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 5 min. The PCR product (10 μl) was electrophoresed through a 8–10% native polyacrylamide gel followed by silver staining. Allelic imbalance (LOH) was recorded for informative markers if allele intensity in tumor DNA was reduced by at least 50% compared with normal DNA.

RESULTS

In this study, a comparison was made of genetic alterations in synchronous OSCCs from five male patients using microsatellite analysis on chromosomes 3p, 9p, and 17p. Allelic imbalance was present on chromosomes 3p, 9p, and 17p in all informative patients, but two distinct patterns emerged: (*a*) two patients showed discrepancy with the majority of markers; and (*b*) in the remaining three patients, the microsatellite changes were identical (Fig. 1).

Discordant results were obtained with patients A and B. In patient

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[3] The abbreviations used are: OSCC, oral squamous cell carcinoma; LOH, loss of heterozygosity; SCCHN, squamous cell carcinoma(s) of the head and neck.

Table 1 *Clinicopathological details of synchronous OSCCs*

Patient	Index SCC (SCC1)			Second SCC (SCC2)		
	Site[a]	Clinical appearance	Dimensions[b] (mm)	Site[a]	Clinical appearance	Dimensions[b] (mm)
A	R FOM	Ulcerated mass	14 × 5.5	L FOM	White papillary lesion	10 × 2.5
B	R LBT	Indurated ulcer	10 × 10	R FOM	Indurated white plaque	4 × 3
C	Ventral tongue	Indurated ulcer	25 × 6	L ant. FOM	Leukoplakia	5 × 2
D	R lower alveolus	Ulcerated lesion	60 × 12	Midline ventral tongue	Leukoplakia	5 × 1.2
E	R FOM	Ulcerated mass	20 × 17	L FOM	White papillary lesion	5 × 2

[a] FOM, floor of mouth; LBT, lateral border of tongue; R, right; L, left; ant., anterior. SCC1 and SCC2 were separated by >1.5 cm of histologically normal epithelium.
[b] Diameter × thickness of SCC.

A, losses on chromosome 3p were identical, but divergence between tumors occurred on chromosome 9p, where different parental alleles were lost at markers *D9S157* and *D9S171*, and on chromosome 17p, where only SCC2 showed imbalance at markers *D17S520* and *CHRNB-1* (Figs. 1 and 2). Dysplastic epithelium microdissected from the margins of SCC2 showed identical changes to this tumor (Fig. 2), suggesting that these alterations were early events in tumorigenesis. In patient B, imbalance was identified at the same microsatellite loci on chromosomes 3p, 9p, and 17p in SCC1 and SCC2, however, alternate alleles were lost at each marker (Fig. 1). All alterations detected in SCC2 were also present in dysplasia contiguous to this tumor.

An identical pattern of allele loss was present in the tumors from the remaining three patients (Fig. 1, patients C, D, and E). For each discrete locus showing imbalance in both tumors, there is a 50% chance that the same parental allele will be lost even if the tumors are of independent origin. In each of the three patients, five markers showed loss of the same parental allele; if these were all separate areas of loss within 3p, 9p, and 17p, then it is highly improbable that this occurred by chance alone ($0.5^5 = 0.031$). Alternatively, it is possible that losses identified on a particular chromosome arm, for example, on chromosome 3p in patient C, may represent loss of the whole chromosome arm, because intervening heterozygous markers were not identified. If one assumes that loss of an individual marker represents loss of the whole chromosome arm on which it is located (*i.e.*, 3p, 9p, or 17p), then the probability of this occurring by chance in an individual patient is greater ($0.5^3 = 0.125$). However, combining the results of the three patients for each chromosome arm is highly significant; the probability that these identical results were obtained by chance alone is only $0.5^7 = 0.0078$. With one exception (marker *D3S1293* in patient D), the allele losses identified in SCC2 from each patient were also present in adjacent dysplasia.

DISCUSSION

Second primary tumors are a major problem in head and neck oncology, because their development has a profound impact on long-term survival, particularly of patients with early disease. Current understanding of the origin of these second tumors is limited. Two possibilities exist: (*a*) either the tumors arise independently after transforming events in separate cells (the field cancerization theory); or (*b*) alternatively, the tumors develop from a single clone, with cells migrating to different sites. Elucidation of whether tumors are of independent or common clonal origin requires determination of whether separate tumors have developed along similar or disparate genetic pathways. Analysis should focus on early genetic events, which conceivably occur before the migration of cells forming tumors

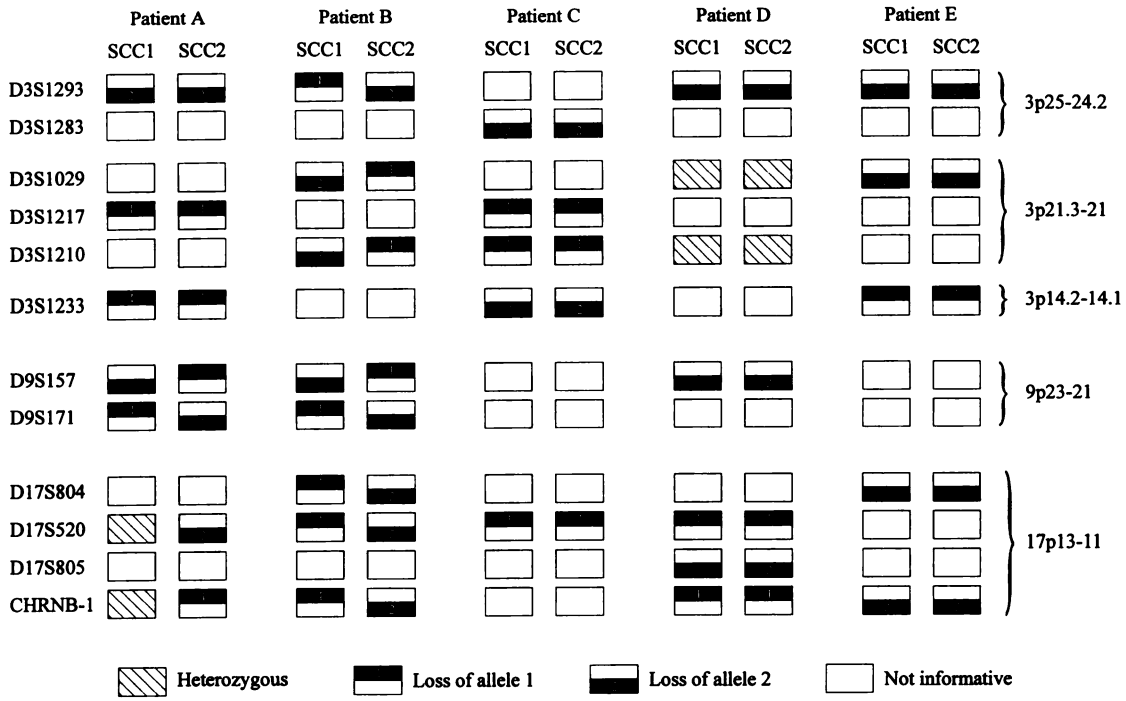

Fig. 1. Pattern of allelic imbalance (LOH) on chromosomes 3p, 9p, and 17p in patients with synchronous oral carcinomas (*SCC1* and *SCC2*). Carcinomas in patients A and B showed a discordant pattern of allele loss, whereas those in patients C, D, and E showed identical changes. In patient A, divergence between tumors occurred on chromosome 9p, where different parental alleles were lost at markers *D9S157* and *D9S171*, and on chromosome 17p, where only SCC2 showed imbalance at markers *D17S520* and *CHRNB-1*. In patient B, alternate alleles were lost at each marker showing imbalance.

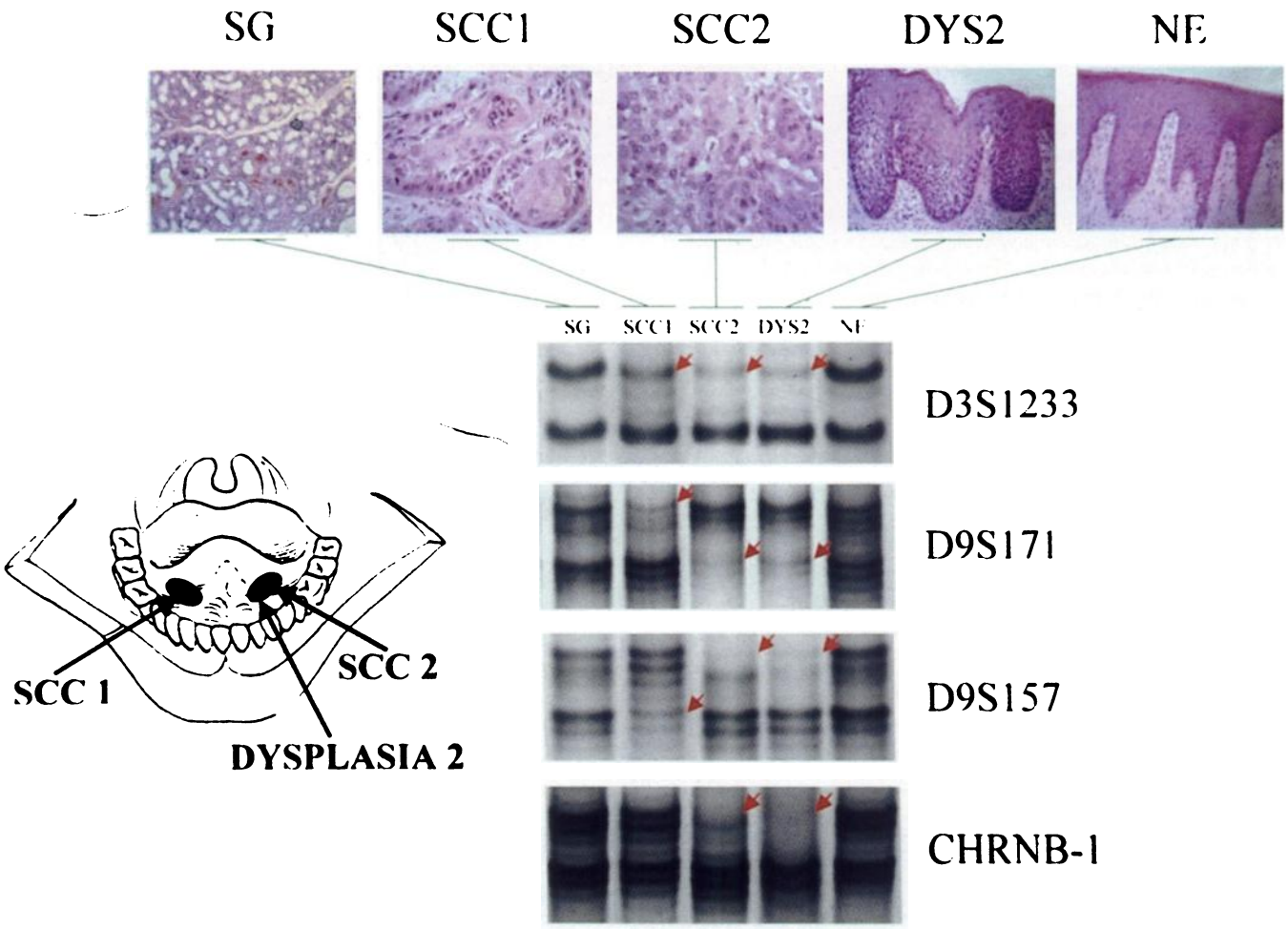

Fig. 2. Representative results of histology and microsatellite analysis of microdissected tissue from patient A. *SCC1* and *SCC2*, synchronous OSCCs; *DYS2*, dysplasia adjacent to SCC2; *NE*, histologically normal oral epithelium; *SG*, salivary gland (used to provide normal DNA). *Arrows*, loss of alleles. SCC1, SCC2, and DYS2 all showed loss of the upper allele at *D3S1233*. However, divergence occurred at markers *D9S171*, *D9S157*, and *CHRNB-1*: (*a*) at *D9S171*, SCC1 lost the upper allele, whereas SCC2 and DYS2 lost the lower allele; (*b*) at *D9S157*, SCC1 lost the lower allele, whereas SCC2 and DYS2 lost the upper allele; and (*c*) at *CHRNB-1*, SCC1 was heterozygous, whereas SCC2 and DYS2 showed allele loss.

of common clonal origin; analysis of later events may result in discordance due to the molecular evolution of tumors of common clonal origin once in their separate sites.

In this study, we used microsatellite analysis at sites of known or putative tumor suppressor genes (on chromosomes 3p, 9p, and 17p) involved in the pathogenesis of SCCHN (9–14) and determined whether alterations detected in tumors were early events by analysis of dysplastic tissue microdissected from tumor margins. Because the analyzed tumors frequently showed imbalance, recording which parental allele was lost at a particular locus became an important factor in determining tumor origin.

Our results from patients A and B suggest that synchronous OSCCs can be of independent origin. Differences between tumors in patient B were particularly striking, because the same markers showed imbalance in each tumor, but different parental alleles were lost. Loss of alternate alleles was also detected on chromosome 9p in patient A, whereas on chromosome 3, findings were identical. The finding of identical changes on chromosome 3 in OSCCs from patient A also raises the possibility that these tumors originated from a common clone but diverged genetically later in their pathogenesis. However, the findings on chromosome 3p in this patient may represent the loss of the entire chromosome arm, because all informative markers tested showed imbalance. Support for a common clonal origin was obtained from the remaining three patients, because paired OSCCs showed identical genetic changes; statistically, these results were highly significant ($P = 0.0078$).

In a previous study, Bedi *et al.* (8), examined tumors from two patients with synchronous OSCCs using X chromosome inactivation and microsatellite analysis on chromosomes 3p and 9p, but their findings with these two patients were inconclusive. Unfortunately, LOH confounded the results of X chromosome inactivation in one patient. In the second patient, a carcinoma and a carcinoma *in situ* showed inactivation of the same chromosome, but the probability of this occurring by chance is high (50%), and a third sample from this patient showed LOH. The study did, however, provide evidence for a common clonal origin for metachronous head and neck tumors in two patients through identification in each patient of a unique microsatellite alteration (instability) common to both tumors. Further evidence for a common clonal origin of multiple tumors reported by Worsham *et al.* (7) was the presence of a Y chromosome rearrangement and similar aneuploidy pattern in two distinct SCCHN.

Apparent support for an independent origin for multiple tumors in the aerodigestive tract derives from reports of discordance in *p53* mutations between primary SCCHN and secondary tumors (6) and also between synchronous lung cancers (15). As proposed by Bedi *et al.* (8), it is possible that differences in *p53* in multiple SCCHN represent divergence during the genetic evolution of tumors that originated from a common clone. It seems unlikely that this could

account for all of the discrepancies; although the prevalence of *p53* mutations has been found to increase with the progression of SCCHN, histologically normal epithelium and preinvasive lesions of the head and neck and lung have been found to carry mutations (15–18). A further possibility is that dysplasia surrounding a tumor could harbor late as well as early genetic changes due to the lateral spread of tumor cells; however, the frequency of this is unknown.

The results of our study indicate that synchronous OSCCs can be of common clonal origin in some patients but also raise the possibility of an independent origin in others. The finding that multiple OSCCs can originate from the same clone has important implications for the early detection of second tumors and gene therapy. However, if further research provides support for the alternative model of independent origin, different strategies may be required.

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