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Advances in Brief

The Use of Exfoliative Cell Samples to Map Clonal Genetic Alterations in the Oral Epithelium of High-Risk Patients[1]

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Abstract

Although it is widely accepted that clonal genetic alterations are an essential component of tumor progression, little is known of the distribution of such changes in high-risk lesions or how such clones are altered over time. We explored the feasibility of using exfoliative cells collected by scraping the mucosal surface to detect allelic loss in oral lesions of 22 patients (14 squamous cell carcinomas, 2 carcinomas *in situ*, and 6 dysplasias). The data show that the patterns of allelic loss observed in these samples closely represent those observed in biopsies of the same region. Furthermore, early indications are that this approach can be used to detect recurrent outgrowth of clones of altered cells in patients after therapy.

Introduction

Nowell's model holds that tumor progression is characterized by the onset of genetic instability in a tissue, leading to rapid mutation and yielding cells with a selective capacity to proliferate or survive in specific microenvironments (1). The tissue enters a period characterized by the evolution of clones of genetically altered cells. Through new molecular technology, many of these genetic changes are now being identified. For example, recent studies have shown that allele-specific loss is associated with the histopathological progression of oral cancers and that such markers might be valuable indicators of clonal change (2, 3). Less is known about the dynamics of clonal change in a progressing lesion, largely because serial observations are rare due to ethical and technical limitations.

Whereas most studies of genetic change in premalignant and malignant lesions involve the analysis of biopsies, a few recent studies have used exfoliative cells isolated from sputum, urine, or saliva (4–6). Such approaches have the advantage of being less invasive than a biopsy, making them a better-suited tool for repeated analysis of a lesion and for screening high-risk populations. However, none of these studies has examined the possibility of following the evolution of genetic clones across a mucosal surface by the repeated collection of exfoliative cells from scrapes of the mucosa. In this study, we explored the feasibility of monitoring clonal genetic changes using this approach by assaying exfoliative cell samples and their matching biopsy samples for allelic loss using PCR-based microsatellite analysis.

Materials and Methods

Sample Collection. Twenty-two patients were chosen for study (14 with SCCs,[3] 2 with CIS, and 6 with dysplasia). The patients were undergoing diagnostic biopsy at either the British Columbia Cancer Agency or Vancouver General Hospital. Of the 22 cases, 20 exfoliative samples had concurrent biopsies from the same lesion (Table 1). Before biopsy, a scrape was collected with a metal spatula from the lesion site. The whole area that was to be biopsied (approximately 0.6–2 cm^2) was scraped. As a control, a second exfoliative cell sample was taken of clinically normal mucosa contralateral to the lesion site (*e.g.*, opposite cheek) and similar in total area. Each scrape was transferred to a cryovial containing Tris buffer and stored in liquid nitrogen. In a few cases, multiple exfoliative cell samples were taken from a previously biopsied site during follow-up to determine whether genetic clones present in the original biopsy were present after surgical removal of the lesion.

Serial sections were cut from each biopsy and stained with hematoxylin. Microscopic areas on each section that contained the dysplastic or malignant cells were microdissected and placed in an Eppendorf tube. At the same time, connective tissue was dissected from each specimen and placed into a second Eppendorf tube. The connective tissue contained normal cells and served as a source of normal control DNA for each patient.

Digestion and Extraction of Samples. The scrapings were thawed and centrifuged, and the pellets were resuspended in 50 mM Tris-HCl (pH 8.0) containing 1% SDS and proteinase K (0.5 mg/ml). The same enzyme solution was added to the microdissected cells. All samples were incubated at 48°C for 72 h or more with repeated spiking of the sample with fresh concentrated proteinase K (20 mg/ml). The DNA was then extracted two times with PC-9, a phenol-chloroform mixture, and precipitated with 100% ethanol in the presence of glycogen. DNA was resuspended in Tris buffer and quantified fluorometrically (Picogreen kit; Molecular Probes).

LOH Analysis. LOH was assayed on three chromosomal arms (3p, 9p, and 17p) selected because allelic loss occurs most frequently on these arms in oral tumors (2, 3, 7). Furthermore, loss of loci on these arms has been associated with risk of progression of premalignant lesions to cancer. The microsatellite markers used for LOH analysis were obtained from Research Genetics (Huntsville, AL) and mapped to the following regions: 3p14.2, *D3S1234* and *D3S1300*; 3p25.3–25.1, *D3S1110*; 9p21, *IFNA*, *D9S171*, and *D9S1751*; 17p13.1, *TP53*; and 17p11.1–12, *CHRNB1*. The protocol used for LOH analysis is described in Zhang *et al.* (3). After PCR amplification, PCR products were separated on 8% urea-formamide-polyacrylamide gels and visualized by autoradiography. For informative cases, allelic loss was inferred when the signal intensity of one allele was at least 50% decreased in the DNA sample from a lesion as compared to the corresponding allele in the matching connective tissue DNA.

In several cases, DNA concentrations were too low to perform multiple LOH assays. To increase the amount of available DNA, these samples were subjected to total genomic DNA amplification before LOH assay using a PCR-based technique developed by Zhang *et al.* (8). This procedure uses a mixture of degenerate 15-mers to prime the reaction in a 60-μl volume. Aliquots from the reaction were then assayed for allelic loss using the same LOH procedure as described above (3). We have shown that the patterns of

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[3] The abbreviations used are: SCC, squamous cell carcinoma; CIS, carcinoma *in situ*; LOH, loss of heterozygosity.

Table 1 *Microsatellite analysis of exfoliated cells and concurrent biopsies*

Patient	Age (yr)/sex	Diagnosis	Site	Chromosomal arms showing LOH	
				Exfoliated cells from lesion[a]	Biopsy
1	80/F	Moderate dysplasia	Gingiva	9p	9p
2	64/F	SCC	Tongue	3p	3p, 9p
3	64/M	SCC	Tongue	9p, 17p	9p, 17p
4	74/F	SCC	Gingiva	3p	3p
5	60/M	Severe dysplasia	Tongue	No loss	No loss
6	46/M	SCC	Tongue	3p, 9p, 17p	3p, 9p, 17p
7	55/F	SCC	Tongue	3p, 9p, 17p	3p, 9p, 17p
8	79/F	CIS	Floor of mouth	9p	9p
9	54/F	SCC	Tongue	9p, 17p	9p, 17p
10	41/M	SCC	Tongue	9p	9p
11	89/F	SCC	Tongue	3p, 9p, 17p	3p, 9p, 17p
12	47/F	SCC	Floor of mouth	9p, 17p	9p, 17p, 3p
13	70/M	SCC	Gingiva	3p	3p, 9p, 17p
14	37/F	Severe dysplasia	Tongue	3p, 9p, 17p	3p, 9p, 17p
15	53/M	SCC	Tongue	3p, 17p	3p, 17p
16	63/F	SCC	Retromolar pad	3p, 9p, 17p	3p, 9p, 17p
17	75/M	Mild dysplasia	Maxillary vestibule	No loss	No loss
18	44/F	Mild dysplasia	Tongue	No loss	No loss
19	58/F	Moderate dysplasia	Tongue	9p	9p
20	30/F	SCC	Tongue	3p, 17p	3p, 9p, 17p

[a] LOH was not seen in any samples from clinically normal mucosa.

allelic loss are identical for DNA tested directly and after total genomic amplification (data not shown).

Results and Discussion

As shown in Table 1, when a scrape and a biopsy from the same lesion were compared, there was a close match in patterns of allelic loss (Fig. 1, *a* and *b*). LOH did not occur in any of the exfoliative cell samples collected from clinically healthy normal mucosa. In contrast, allelic loss was present in 17 of the 20 exfoliative cell samples obtained from clinical lesions; this included SCCs (13 of 13 cases), CIS (1 case), and 3 of 6 dysplastic lesions (2 moderate and 1 severe dysplasia). Ten of the 17 cases, all SCCs, showed loss on more than 1 arm. These results were compared to those obtained with DNA isolated from microdissected cells in the concurrent biopsy. All 17 patients with LOH in exfoliative cell samples demonstrated identical LOH in the concurrent biopsies. Furthermore, the three cases (two mild and one severe dysplasia) that showed retention of all examined markers in exfoliative cell samples displayed a similar lack of allelic loss in their corresponding biopsies. Four of the cases (cases 2, 12, 13, and 20), all with SCC, had additional allelic losses in their biopsies that were not observed in their exfoliative cells (Table 1). Because tumors are genetically heterogeneous, it is possible that these data reflect a difference in the cell population being studied in biopsies and scrapes. Another possible explanation is that the scrape may involve a larger area than the dissected biopsy area and only early genetic alterations occupying a large area could be detected. Clones of cells with more advanced genetic changes may occupy a relatively smaller region and be missed in the analyzed exfoliative samples.

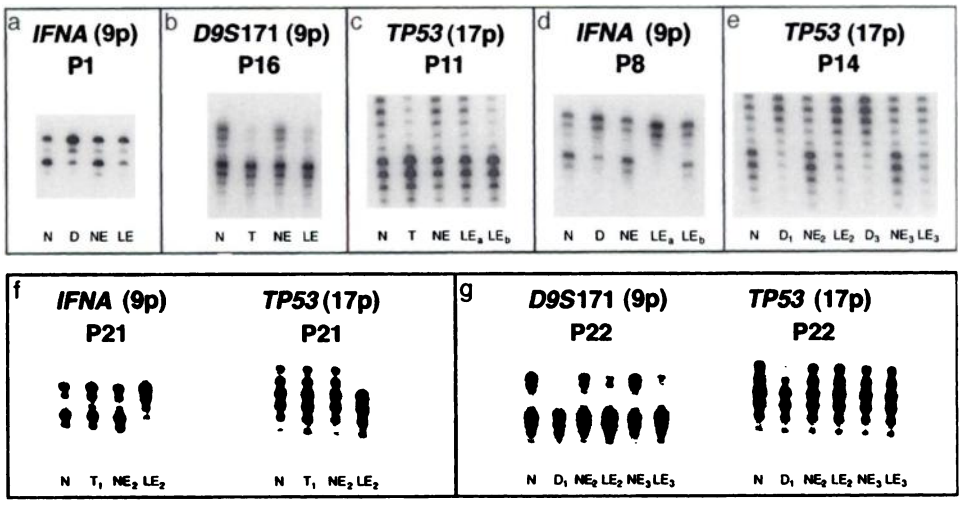

Fig. 1. LOH analysis of seven patients (*a–g*). DNA was isolated from stromal (*N*), dysplastic (*D*), or tumor (*T*) cells microdissected from the lesion biopsy and from exfoliative cells from clinically normal oral mucosa (*NE*) and the lesion (*LE*). Microsatellite markers, the chromosomal arm being assayed, and patient numbers are indicated *above* each block. Allelic loss was not present in any sample of exfoliated cells from normal mucosa (*NE*). *a*, loss of lower allele at *IFNA* was noted in both biopsy (*D*) and lesion scrape (*LE*). *b*, loss of upper allele at *D9S171* was noted in both biopsy (*T*) and lesion scrape (*LE*). *c*, loss of upper allele at *TP53* was noted in biopsy (*T*) and lesion scrape taken from site *b* (LE_b) but was less obvious in the scrape taken from site *a* (LE_a). *d*, loss of lower allele at *IFNA* was noted in biopsy (*D*) and lesion scrape taken from site *a* (LE_a; the loss was even more prominent than the biopsy) but was not as obvious in lesion scrape taken from site *b* (LE_b) of the same large diffuse lesion. *e*, loss of the lower allele at *TP53* was noted in the original biopsy (D_1; *subscripts* identify samples taken on the same day and are in temporal sequence). Follow-up lesion scrapes (LE_2 and LE_3) and another excision from the site (D_3) showed a similar loss. *f*, a partial loss of lower allele at *IFNA* was noted in the tumor biopsy (T_1). Follow-up showed that 10 months later, the same allelic loss was evident in exfoliated cells collected from the site (LE_2), and this loss was more pronounced. Furthermore, loss of the upper allele of *TP53* was noted at this time. *g*, loss of upper allele at *D9S171* and at *TP53* were noted in the initial biopsy (D_1). Exfoliative cells collected at 6 and 14 months (LE_2 and LE_3), showed the same loss at 9p but no loss at 17p.

The noninvasive nature of collecting exfoliative cells means that multiple samples can be taken easily, which allows investigation of whether different genetic clones are emerging in different sites of the oral cavity or in different areas of a large lesion. As shown in Fig. 1, *c* and *d*, exfoliative cells were taken from two different sites in two large diffuse oral lesions. The results showed different patterns of allelic loss in the two sample regions. This suggests that it may be possible to map the position of genetic clones within lesions by collecting exfoliative cells from specific sites.

We have begun to examine clinical situations in which exfoliative cell sampling could assist in patient management. One use lies in the monitoring of patients after treatment of head and neck SCC. These patients are prone to local tumor recurrence as well as the development of second primary tumors in the aerodigestive tract. The latter phenomenon, termed field cancerization, is usually attributed to the multifocal development of independent lesions within tobacco-exposed epithelium (9).

The local recurrence of an oral lesion is generally felt to be a result of reemergence of a small population of residual dysplastic or tumor cells that is left in the tissue after treatment. Our data show that exfoliative cells can be used to monitor such cases. In an illustrative case (Fig. 1*e*) involving the tongue, an organ in which the preservation of tissue is critical to retain function, a severely dysplastic lesion was excised. Follow-up scrapes showed the same LOH, and reexcision of the scraped region confirmed the presence of a lesion that was similar to the original lesion both in degree of dysplasia and LOH. In two other cases (patients 21 and 22), also involving the tongue, the follow-up exfoliative samples seemed to detect further genetic changes in patient 21 and partial persistence of genetic changes in patient 22. In patient 21 (Fig. 1*f*), a SCC was completely removed, and the tumor showed partial loss of 9p. Ten months later, an exfoliative cell sample showed not only persistence of 9p loss, but the loss actually became more prominent. Furthermore, there was an additional loss at 17p. This loss was confirmed with two separate markers (*CHRNB1* and *TP53*). In patient 22 (Fig. 1*g*), a CIS was completely removed and demonstrated LOH at both 9p and 17p. Although the subsequent exfoliative cells sampled from the site 6 and 14 months later showed a lack of 17p loss, there was still a persistent loss at 9p.

Presumably, patients with LOH in scrapes subsequent to treatment that resemble those in the original tumor would be more likely to have local-regional recurrence. As understanding of the significance of specific genetic changes in cancer development improves, so should interpretation. For example, there are indications that 17p loss may be a later event than 3p and 9p loss in head and neck carcinogenesis (3). Thus, lesions in which exfoliative cells show LOH at 17p may have a greater tendency to recur than lesions that demonstrate some LOH, but not at 17p. Additional studies are needed to determine which clones of genetically altered cells have prognostic significance. If the detection of these clones accurately predicts recurrence, a variety of options would be possible, including additional surgical excision or possibly the use of adjuvant therapy with chemopreventive agents.

In summary, we have shown that allelic loss can be identified in exfoliative cells and that the profile of change in these cells is similar to that observed in biopsies. The immediate value of using this approach to monitor patients for tumor development or recurrence has yet to be established, but results are sufficiently encouraging that it may be of value to begin archiving such samples. As predictive indicators of progression, risk of invasion, or metastases are identified, the samples could become a critical resource for testing specific clonal changes.

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