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Microsatellite Analyses of Recurrence or Second Primary Tumor in Head and Neck Cancer

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Abstract. *Background: In head and neck squamous cell carcinoma, distinguishing second primary tumours and recurrences may help to orient clinical decisions concerning therapy. Patients and Methods: A panel of eight microsatellite markers was used to analyse the loss of heterozygosity and genomic instability in a selected group of 32 patients experiencing a recurrence after having undergone surgery for oral or oropharyngeal carcinoma, in order to establish the clonality and origin of the recurrence. Results: Twenty-three patients showed genetic changes in primary and/or relapsing tumour DNA: clonally-related patterns were detected in six cases, whereas the different patterns between paired tumours indicated the presence of a second primary tumour in 17 cases. None of the markers was informative in nine cases. Conclusion: Our observations suggest that only a small proportion of patients have primary and secondary tumours developing from a single contiguous altered field (thus indicating a common clonal origin), whereas the metachronous tumour arises in unrelated fields in the majority of cases.*

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common solid tumour worldwide: it accounts for 2% of all neoplasms in the West (1), and affects approximately 500,000 patients per year (2). Despite advances in treatments and traditional diagnostic methods, no significant improvement in survival has been reported over the last 20 years (2) because of the high incidence of second primary tumours of the aerodigestive tract or a relapsing primary tumour (3-5).

Frequent chromosomal losses and imbalances have recently been identified during the development and progression of HNSCC, including specific alterations in proto-oncogenes and tumour suppressor genes (6-8). It is worth noting that loss of heterozygosity (LOH) at multiple chromosomal loci and instability in repeat DNA sequences (MSI) occur with various frequencies in HNSCC (9), and current theories of tumour progression have focused on the emergence of clonal cell populations that undergo multiple genetic alterations with a selective growth advantage (10). A number of authors have demonstrated the feasibility of PCR-based microsatellite analyses of DNA in detecting clonal tumour-derived cell populations in different samples (11-14). Clonality analyses may help to identify whether two cell populations are genetically "similar" or different (15) and, therefore, determine whether a relapse is genetically related to the primary tumour or has an independent origin. In HNSCC, distinguishing second primary tumours from recurrences may help to orient clinical decisions concerning therapy.

For this study, a panel of eight microsatellite markers that are frequently altered in HNSCC and other solid tumours was created in order to analyse LOH and MSI in a selected group of 32 patients experiencing a recurrence after having undergone surgery for oral or oropharyngeal carcinoma and to establish the clonality and origin of the recurrence.

Patients and Methods

Patients. Thirty-two consecutive patients with oral and oropharyngeal carcinoma (29 males and three females) were selected from the 1997-2002 files of the ENT Department of Milan University's School of Medicine, Italy, all of whom gave their written informed consent to the study. Their mean age at the time

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Table I. *Clinico-pathological characteristics.*

Characteristics	Number
Total number of patients	32
Age (years)	
Mean	61.2
Range	43 - 78
Gender	
Male	29
Female	3
Tumor site	
Oral cavity	21
Oropharynx	11
Tumor stage	
I	5
II	21
III	6
IV	0
Tumor degree of differentation	
Well-differentiated	4
Moderately-differentiated	18
Poorly-differentiated	10

of diagnosis was 61.2 years (range 43-78), and their clinical characteristics are provided in Table I.

All of the subjects were enrolled at the time of the appearance of a recurrent tumour after previous surgical treatment of a primary neoplasm. The tumours were staged (pTNM Union International Cancer 1997) on the basis of the findings of a physical examination, panendoscopy and head and neck tomography. The staging was subsequently modified on the basis of any additional evidence acquired after surgery and of a histopathological examination.

All of the patients underwent unilateral or bilateral neck dissection depending on the tumour site(s) and node extension. All of the primary tumours had to have been completely resected, as assessed by means of a histopathological examination of the resection margins. The local recurrence developed within three years of the original surgery and within 2 cm of the treated area. During the follow-up, all of the patients underwent clinical and endoscopic examinations every three months during the first two years, and then every six months until five years after the end of treatment. A chest X-ray was performed every year, and a head and neck tomography scan 6-8 months after the end of surgical treatment. For each patient, one sample of the primary tumour and one of the second were examined; venous blood for the extraction of normal DNA was used as a control.

DNA preparation and analysis of microsatellite repeat polymorphisms. Serial paraffin sections (12 μm thick) of tumoral and normal tissues immediately adjacent to those used for the histopathological evaluation were collected for molecular analysis.

After being put into 1.5 ml polypropylene tubes containing 1 ml of xylene, the samples were incubated at 37˚C for 15 min and then spun down for 15 min before the xylene was removed, and the pellet was washed twice in absolute ethanol (1 ml). The samples were dried, and DNA was extracted using the QIAamp DNA mini-kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA); subsequently. 1 ml of a 1:10 dilution was used for each PCR reaction.

Table II. *Characteristics of the microsatellite markers.*

Marker	Chromosome localisation	Repeat motif	Annealing temperature	Ref
BAT25	4q12	(A)n	50˚C	(20)
D9S156	9p21	(CA)n	59˚C	(20)
D9S171	9p21	(CA)n	60˚C	(20)
D16S260	16q22.1	(CA)n	56˚C	(20)
TP53	17p12	(CA)n	60˚C	(20)
D17S250	17q12	(CA)n	59˚C	(33)
D17S261	17p12	(CA)n	52˚C	(20)
D21S1245	21q2	(AAAG)n	54˚C	(20)

We selected a panel of eight microsatellite markers, including TP53 and D17S261, to analyse the locus of the *p53* tumour suppressor gene on chromosomal arm 17p13, which corresponds to an area of frequent LOH in HNSCC (16); D9S156 and D9S171 to analyse the 9p21 chromosomal band containing the *p16* (MTS1) gene, a cyclin/cyclin-dependent kinase inhibitor involved in cell cycle regulation, which corresponds to an area of genetic loss common to many solid tumours (17) and is currently the region with the most frequent LOH in HNSCC (the p16 protein is not expressed in more than 80% of cases) (16); and four repeat polymorphisms (D21S1245, BAT25, D17S250 and D16S260), which are recognised as being prone to microsatellite instability in many solid tumours (see Table II for chromosomal locations, type of polymorphism, annealing temperatures and references).

The PCR reactions were carried out in a volume of 30 μl with 1.5 mM $MgCl_2$; the amplified PCR fragments were analysed by means of 10% polyacrylamide gel electrophoresis run at 10 W for 3-4 h; and the bands were detected by means of silver staining as previously described (18). MSI was defined as an upward or downward shift in the PCR products from the tumour in comparison with the band pattern of normal tissue; LOH was defined as a more than 50% visual reduction in the signal intensity of one allele.

Results

Thirty-two samples of paired primary tumours and recurrrences obtained from patients with oral or oropharyngeal carcinomas were analysed in order to determine whether microsatellite abnormalities can be used as reliable markers to distinguish a real recurrence from a second primary tumour.

Using our microsatellite panel, 23 patients (71.9%) showed genetic changes in primary and/or relapsing tumour DNA in comparison with normal peripheral blood DNA (LOH and MSI); the other nine cases (28.1%) did not show any microsatellite abnormalities.

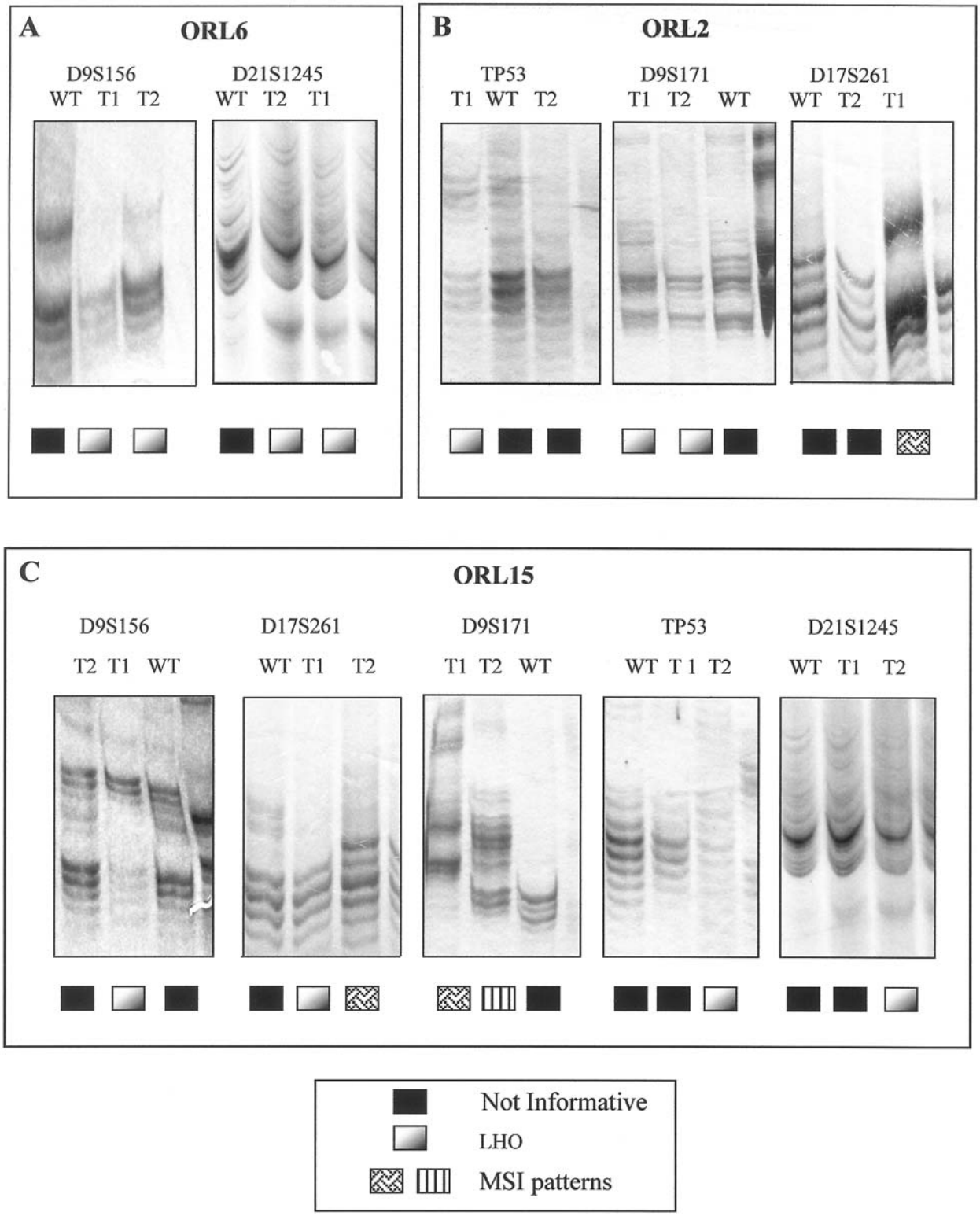

Figure 1. *Three examples of microsatellite analyses for clonality in metachronous tumours showing informative silver-stained gel electrophoresis (WT=normal; T1=primary tumour; T2=second primary tumour or recurrence); the examined microsatellite is specified above each gel. Cases ORL 6 and 2 are considered recurrences because the identical or similar pattern of microsatellite abnormalities between the tumours indicates a clonal relationship; case ORL 15 is considered a second primary tumour.*

In order to assess the clonal relationship between the paired tumours, the patterns were considered "identical" when the same combination of parental alleles were lost (LOH) or the same shifted band was present (MSI), or "similar" when one of the two tumours showed additional microsatellite abnormalities (19). The paired samples with completely different patterns in all informative microsatellites were considered clonally-unrelated tumours.

Clonally-related patterns were found in six of the 23 cases with genetic changes (26.1%): four with "identical" and two with "similar" patterns (see Figure 1). In the remaining 17 cases, the difference in the patterns of the paired tumours indicated the presence of a second primary tumour (Figure 1). The number of microsatellite markers necessary to establish a clonally-related pattern between primary tumours and metastases ranged from one to five, as reported by other authors (19).

Discussion

The aim of this study was to verify whether microsatellite abnormalities can be reliably used to distinguish real recurrences from second primary tumours and, to this end, the frequency and type of microsatellite alterations in 32 patients affected by oral or oropharyngeal carcinomas were investigated. Using our panel of eight microsatellite markers, abnormalities were identified in 23 cases (71.9%), a finding that is consistent with that reported in other head and neck carcinoma series (20-22). The paired tumours of only six (26.1%) of these patients had "identical" or similar" clonal patterns supporting the concept of clonal expansion and tumour recurrence (19, 23, 24); in the two with a "similar" pattern between the primary tumour and recurrence, the additional alterations probably arose during tumour progression.

It is now generally agreed that progression probably involves the clonal outgrowth of a subpopulation of cells (25) with additional genetic alterations (10, 26, 27), and so it can be hypothesised that, when the primary tumour and recurrence are genetically "similar", the recurrence may have developed from a few cancer cells left behind after surgery or from the same field (28).

Another important finding of this study was that the pattern of microsatellite changes had a totally different genetic arrangement, indicating an independent origin, in about three-quarters of the cases (73.9% of the informative samples). This finding suggests the presence of a second primary tumour that is not clonally-related to the first, and supports the hypothesis of widespread genetic changes in the field at risk for the same carcinogen exposure, which is in line with the concept of field cancerisation (26, 29).

The development of second primary tumours negatively affects the prognosis of head and neck carcinoma (4, 30-32). However, our knowledge of the origin of such tumours is limited and thus, when a metachronous tumour develops in the same area as a primary cancer, the molecular distinction of second primary and recurrent tumours may orient subsequent therapy (33). Finally, the clinical implications of the presence of mucosal areas at risk of cancer progression in HNSSC may help to select subgroups of patients who require closer follow-up.

Our results suggest that only a small proportion of patients develop primary and secondary tumours from a single contiguous altered field that therefore have a common clonal origin, and that the majority of metachronous tumors arise independently from unrelated fields. Given the various prognostic and therapeutic implications, the genetic profiling of second tumours should be used to support traditional clinico-pathological methods as a means of establishing a true diagnosis and appropriate subsequent therapy.

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