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Frequent Deletion of Chromosome 3p in Oral Squamous Cell Carcinoma

Maxine Partridge, S. Kiguwa and J.D. Langdon

Evidence suggests that the accumulation of genetic lesions in dominant oncogenes and tumour suppressor genes is involved in the development of human cancers. We have used restriction fragment length polymorphism analysis to identify chromosomal deletions which may indicate the location of potential tumour suppressor genes. Deletion of chromosome 3p was found in 17/21 (81%) of informative primary oral squamous cell carcinoma (SCC) using a polymorphic probe recognising the D3F15S2 locus (3p21). Loss of heterozygosity (LOH) was not confined to patients exposed to recognised risk factors such as heavy smoking or alcohol consumption and was present at an early stage in the disease suggesting that this genetic alteration may be a fundamental event for oral cancer.

Keywords: chromosome deletion, genes, suppressor, tumour, mouth neoplasms
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INTRODUCTION

CURRENT KNOWLEDGE of the origin of cancer suggests that it arises from a highly complicated process involving the accumulation of genetic events in a single cell. These genetic alterations include the activation of oncogenes and inactivation of tumour suppressor genes. Over the past decade attention has focused on the role of dominantly acting oncogenes in the aetiology of cancer, however, recent studies suggest that loss or alteration of tumour suppressor genes also plays a critical role.

Identification of tumour suppressor genes has been difficult as they are normal genes of unknown function. The best characterised tumour suppressor genes are the retinoblastoma gene (Rb), p53 and a gene deleted in colorectal carcinoma (DCC, for review see Marshall [1]). The loss of a single copy of a tumour suppressor gene is not generally sufficient for tumorigenesis and these genes are referred to as recessive. When a single copy of a tumour suppressor gene is lost by a chromosomal deletion other events including point mutation, genomic rearrangement or deletion inactivate the tumour suppressor gene on the homologous chromosome.

Two types of tumour suppressor gene are now recognised and have been termed class 1 and 11 [2]. For class 1 genes loss of function results from mutation and/or deletion of DNA as described above, resulting in an altered or lost gene product. Class 11 genes are those for which loss of function arises as a result of a mutation or deletion within a regulatory gene, which blocks the expression of a tumour suppressor gene.

As part of our studies to investigate the pathogenesis of oral cancer we have looked for chromosomal deletions which may indicate the location of potential tumour suppressor genes. DNA from normal cells contains two copies (alleles) of every sequence. If these can be distinguished the sequence is described as polymorphic in the population and heterozygous in the individual. When DNA is digested with restriction enzymes a characteristic banding pattern results following Southern analysis of DNA from a heterozygous patient. Some of the bands are due to one allele and the others represent the second. When loss of one allele occurs in a tumour the bands corresponding to the lost chromosomal region will be missing. This change from a heterozygous to a hemizygous state is termed loss of heterozygosity (LOH).

There have been recent reports of LOH at specific chromosomal sites in several tumours including colorectal cancer (characterised by deletions by deletions at chromosomes 5q, 17p, 18q), renal cancer (3p), small cell lung cancer (3p, 13q, 17p) and bladder cancer (6p, 9q 11p, 17p, see Weinberg *et al.* [3]). In some cases the identity of the tumour suppressor gene within the deleted regions is known, for example, p53 is the tumour suppressor gene at 17p, Rb the deleted gene at 13q and DCC is deleted at 18q.

At the present time there are no reported LOH studies for oral cancer. Several recent investigations have focused on determining cytogenetic abnormalities present in head and neck squamous cell carcinoma (SCC) and a complex pattern of deletions, translocations and aneuploidy has been found consistent with the view that these cancers arise as the result of the accumulation of genetic alterations [4–6]. However, in these series the majority of samples analysed were cell lines derived from tumours or samples obtained from patients who had received radiotherapy. Thus, in order to study chromosome loss for a well defined group of oral tumours this investigation has focused on the examination of primary oral SCC. Our preliminary analysis involved screening for deletions of the short arms (p) of chromosomes 1, 3 and 17 and we report the subsequent findings of frequent deletion of chromosome 3p.

Correspondence to M. Partridge.
The authors are at the Epithelial Cell Biology Unit, Dept Oral and Maxillofacial Surgery, King's College School of Medicine and Dentistry, Denmark Hill, London SE5 8RX, U.K.
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Table 1. Clinicopathological profile of patients with deletions of chromosome 3p

Case no.	Age/sex	Site	Size (mm)	Stage	Histology	Tobacco usage	Alcohol consumption	Prognosis after surgery (months)
1	M/55	Retromolar	40	3	Well	Nil	Nil	D oca (5)
2	F/35	Buccal	20	2	Moderately	Nil	Nil	Q (36)
3	M/68	Alveolus	15	1	Well	Nil	Nil	AR (16)
4	M/52	Floor of mouth	40	4	Well	25/day	4–8 pints/day	A (8)
5	M/43	Alveolus	50	4	Poor	<10/day/betel	Social	AR (9)
6	M/70	Alveolus	25	3	Well	10/day*	Nil	D oca (36)
7	M/58	Antra	nd	na	Poor	>40/day	4 pints/day	D oca (19)
8	M/55	Floor of mouth	20	1	Well	>40/day	8 pints/day	D oca (22)
9	M/65	Post third tongue	25	2	Well	40/day	>7 pints/day	A (96)
10	M/48	Alveolus	20	2	Well	40/day	7 pints/day	A (24)
11	F/64	Lateral tongue	10	3	Well	Nil	Nil	AR (4)
12	F/43	Dorsum tongue	3	1	Well	Nil	Nil	A (13)
13	M/35	Floor of mouth	20	2	Well	20/day	Regular	A (15)
14	M/71	Post third tongue	30	2	Poor	20/day	3 pints/day	D oca (8)
15	F/45	Buccal	20	2	Well	Nil	Nil	A (12)
16	M/62	Alveolar	25	2	Verrucous	<10/day	2 pints/day	D unoca (14)
17	M/58	Retromolar	40	4	Moderate	<10/day	Social	D oca (34)

M, male; F, female; D oca, oral cancer-related death; D unrel, death unrelated to oral cancer; A, alive; AR, alive but treated for recurrence. Social alcohol consumption <2 units/week, regular 2 units/day. *Stopped smoking more than 8 years before cancer developed. nd, not determined; na, not appropriate.

PATIENTS AND METHODS

Tumour tissue and venous blood were collected at surgery from patients with primary oral SCC. Samples were selected from a viable area of tumour, frozen and stored in liquid nitrogen. Prior to extraction of DNA cryostat sections were examined microscopically to ensure that the sample contained at least 60% tumour cells. Tumours which fell below this threshold were not analysed. Venous blood samples were stored at −20°C in tubes containing EDTA. Patient profiles including age, sex, smoking and alcohol habits, tumour site, clinical stage and histology were recorded, see Table 1. The median follow-up time was 15 months.

Genomic DNA was prepared from blood by lysis of cells in water (4 × blood volume). Triton-X-100 was added to a final concentration of 2% and samples centrifuged at 3400 rpm for 20 min at 4°C. The pellet was resuspended in 2.5 ml 75 mM NaCl, 25 mM EDTA at a pH of 8.4 and 200 µl of 10% sodium dodecyl sulphate (SDS) added followed by 100 µl of proteinase K (10 mg/ml) prior to digestion for 2 hr at 56°C with gentle shaking. Samples were extracted twice with phenol/chloroform and DNA precipitated, washed and dissolved in Tris-EDTA (TE). Tumour tissue was powdered under liquid nitrogen and lysed in 1.2 ml of digestion buffer (100 mM NaCl, 10 mM Tris HCl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS, 0.1 mg/ml proteinase K) for each 100 mg of tissue at 37°C for 18 h with gentle shaking. Samples were extracted twice with phenol/chloroform before DNA was precipitated.

Fractionation of 15 µg of paired samples of tumour and blood DNA digested with the appropriate enzymes was performed by electrophoresis in 0.8% agarose gels (1.4% for analysis of chromosome 1p deletion). Gels were stained with EtBr to check the amount of DNA loaded, denatured and transferred to Hybond N nylon membrane (Amersham, Bucks, U.K.) in 20 × SSC. Radioactive cDNA probes were prepared by random primer extension [7]. Samples were hybridised to three recombinant probes mapped to the short arms of chromosome 1 (MCT58, D1S77 which detects polymorphic sequences in Pvu 11 digested DNA, 8); chromosome 3 (pH3H2, 3p21 which detects polymorphic sequences in *Hind* 111 digested DNA, 9) and chromosome 17 (pYNZ22 which recognises sequences in Taq 1 digested DNA, 10). After hybridisation and stringent washing according to the manufacturers protocols radioactive bands were visualised by autoradiography. Patients were classified as uninformative (one band in the tumour and blood) or informative (two bands in the blood DNA). Any samples yielding equivocal results were reanalysed. Allelic loss was scored if a restriction fragment length polymorphism (RFLP) present in normal DNA was lost in at least 50% of the neoplastic cells as assessed by comparison of the autoradiograph from the tumour and blood samples.

RESULTS

We have examined 28 DNA paired samples isolated from primary oral SCC and venous blood obtained from the same patients by Southern blot analysis for possible loss of heterozygosity at chromosome 3p. 12 paired samples were also examined for possible loss at chromosomes 1p and 17p.

Sixty-six per cent of patients examined were heterozygous (informative) at the 3p locus D3F15S2. The probe used, pH3H2 detects a RFLP with alleles of 2.0 and 2.3 Kb and also detects constant bands of 7.5 and 4.0 Kb derived from a related locus on chromosome 1, see Fig. 1 which shows a representative analysis of six paired samples. The informative cases are 1, 2, 3, 5 and 6. An example of paired samples obtained from a patient who remains heterozygous at this locus (two bands in the tumour and blood) is shown, case 6. A non-informative homozygous patient (1 band in the tumour and blood) is shown, case 4. Loss of heterozygosity (loss of one allele) is seen in the tumour tissue from cases 1, 2, 3 and 5. In cases 1 and 5 a second weak band present in the tumour samples reflects either contamination with normal heterozygous DNA or indicates that the tumour is a mixture of heterozygous and hemizygous malignant cells.

Partial or complete deletion of chromosome 3p was found in 17/21 (81%) of informative cases. The upper and lower alleles were lost with equal frequency. Analysis of the data in relation to patients clinicopathological profile and known risk factors for this disease (see Table 1), shows that LOH at this locus may be a fundamental event for oral SCC. Deletion of chromosome 3 was found in samples from patients with early-stage disease and was not confined to patients exposed to known risk factors such as heavy smoking or alcohol consump-

tion. The deletion did not appear to provide useful prognostic information being present in tumours from patients who experienced early local or nodal recurrence and in lesions from patients who were alive and well, without recurrence, 5 years after treatment. However, only 28 cases have been examined in this study and further analysis is required.

The studies involving loci at chromosome 1p and 17p revealed chromosomal loss or rearrangement at only low frequency. 1/12 (8%) of informative patients showed a rearrangement at chromosome 1p and 2/12 (16%) of informative cases deletion and rearrangement of 17p (data not shown).

DISCUSSION

Oral carcinomas occur frequently as the result of genetic damage following exposure to agents present in tobacco products and alcohol. However, tumours also arise in individuals not exposed to these risk factors. Other aetiological factors, and the mechanisms by which environmental or inherited factors exert their effects have yet to be determined. There is now considerable experimental support for the concept that the inactivation of specific genes plays a role in tumour development. We have used RFLP to examine LOH at a locus on chromosome 3p and report the first association between loss of sequences on chromosome 3p and primary oral SCC. The finding of this deletion in such a high percentage of informative patients (81%) suggests that loss of a gene in this region may be a fundamental event in the development of this tumour type. The results are not due to random chromosomal losses as we did not find a high percentage of deletions at loci on chromosome 1p and 17p.

In the present study no association was found between the presence of a deletion of 3p and recognised risk factors however, studies involving a larger number of patients are required before firm conclusions can be made.

Our findings are consistent with published karyotypic studies which show loss of a portion of chromosome 3 in cell lines derived from oral cancer [11, 12]. However, deletion of chromosome 3p has not been reported in all cytogenetic studies of oral cancer cell lines. This discrepancy may occur as RFLP analysis is able to reveal cytogenetically undetectable chromosome changes. A recent report also indicates that the karyotype may depend on the culture conditions used to maintain cell lines (Dr Y. Jin, University Hospital, Lund).

Evidence from examination of other tumour types strongly suggests that one or more tumour suppressor genes is located on chromosome 3p and that there may be clustering of genes which influence epithelial proliferation or differentiation. Deletion of chromosome 3p14-23 was first reported following cytogenetic analysis of small cell lung cancer (SCLC) in 1982 by Whang-Peng *et al.* [14] and gene deletion confirmed by several studies using RFLP probes (see Carritt *et al.* [15]). LOH at 3p has also been reported for adenocarcinoma [16, 17] and SCC of the lung [16], breast [18], uterine cervix [19], ovary [20] and kidney [21].

However, deletions associated with the loss of tumour suppressor genes are usually large with the areas of loss being much greater than the actual tumour suppressor gene(s) themselves. As an initial step towards cloning of putative tumour suppressor genes detailed analysis of the minimum deleted regions in each tumour is required using probes recognising adjacent loci.

A number of studies have attempted to characterise the minimum deleted region at 3p. For example, in renal cell carcinoma a region located between 3p14-21 has been identified as the location of a putative tumour suppressor gene [21]. Large chromosomal deletions are usually found in SCLC and the pattern of allele loss suggests that a lung tumour suppressor gene lies close to 3p21 with the locus most consistently deleted being D3F15S2 [22, 23], which is part of the coding region of a mRNA expressed in lung. However, two studies [24, 25] have identified individuals with SCLC who have an interstitial deletion proximal to D3F15S2 extending to 3p13-14. In each case the D3F15S2 locus was retained

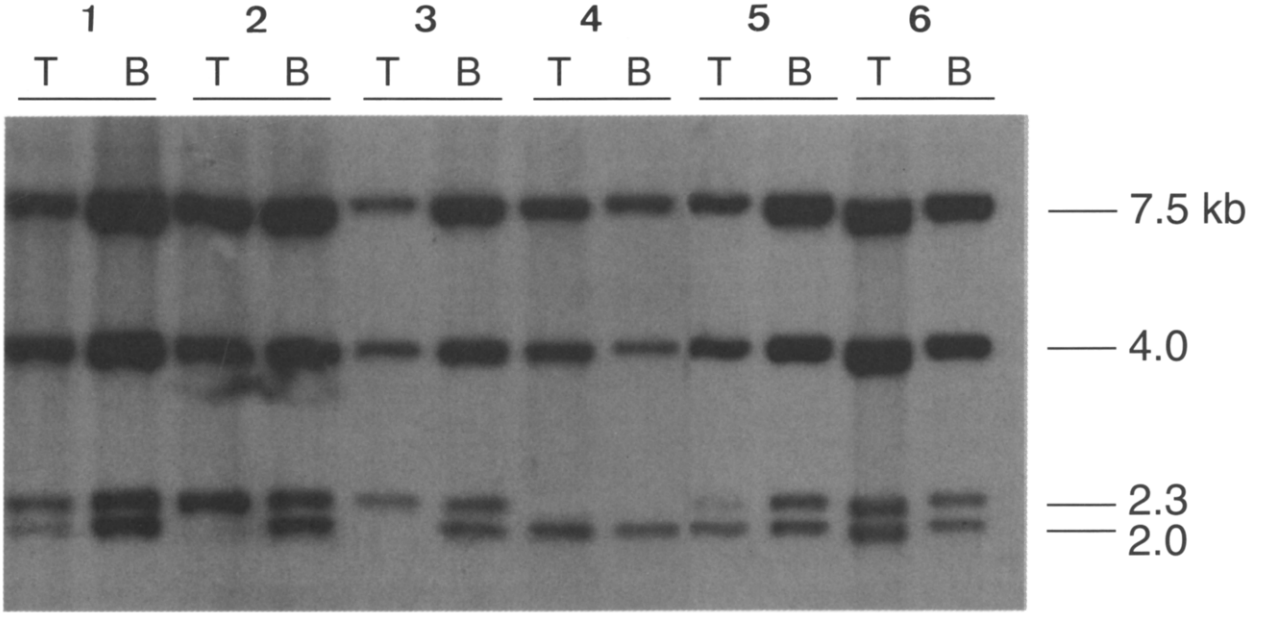

Fig. 1. Representative Southern blot analysis of six pairs of matched tumour (T) and blood (B) DNA using the pH3H2 probe which detects a RFLP with *Hind* 111 fragments (alleles) of 2.3 or 2.0 Kb. Informative cases are 1, 2, 3, 5 and 6. Case 6 retains heterozygosity in the tumour, case 4 is non-informative.

indicating that the SCLC tumour suppressor gene is likely to lie proximal to D3F15S2 or that there may be a second tumour suppressor gene in this region. An additional study has suggested an even more complex pattern of deletions identifying three regions, 3p25, 3p21.3 and 3p14, which are frequently deleted in lung cancer [17], loci also implicated following classic cytogenetics [26].

In a recent study involving analysis of cell lines derived from head and neck cancer [13], 3p deletions were detected using RFLP markers and comparing the average heterozygosity computed for 24 loci in randomly chosen individuals with the heterozygosity for DNA obtained from tumour cell lines. The authors showed that the commonly deleted region, at which a tumour suppressor gene is likely to be located, probably lies telomeric to D3S3 (3p14) and centromeric to RAF1 (3p25). The identification of a deletion as proximal as 3p14 corresponds with the fragile site (a chromosome breakpoint occurring in response to exposure to mutagens) which is found most frequently when chromosomes from normal individuals, both smokers and non-smokers, are analysed [27]. Yunis and Soreng [28] have postulated that chromosomal deletions and or gene rearrangements may be facilitated by the presence of these heritable and constitutive sites.

Limited information is available concerning the identity of tumour suppressor genes at these loci. Four genes assigned to 3p21 identified as candidate tumour suppressor genes for SCLC and have been termed D8, ACY 1, APEH and PTPγ (see Carritt *et al.* [15]). In order to be recognised as a class 1 tumour suppressor gene evidence of deletion and/or mutation in the residual allele is required. Although some of these candidate genes show reduced expression in lung tumours they do not meet these strict requirements. They may be class 11 tumour suppressor genes, however, it is more likely that the relevant sequences have yet to be identified.

At the present time the commonly deleted region(s) on chromosome 3p has yet to be determined for oral SCC to help identify the location of genes whose function is required to suppress the development of this tumour type. The finding of this deletion in T_1 lesions implies that it may be a relatively early event in tumorigenesis. Future studies will examine dysplastic oral lesions and carcinoma *in situ* to determine if this is the case and if so whether deletion of 3p may serve as an indicator of possible progression to malignancy. However, it must be remembered that although a very high percentage of oral tumours show deletions of 3p they also show loss of other chromosomal loci, reflecting the multiple genetic abnormalities occurring during the development of tumours. Studies to identify other genetic abnormalities which play a role in the initiation and/or progression of this in this disease are essential.

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