Altered *p53* in microdissected, metachronous, premalignant and malignant oral lesions from the same patients

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Abstract

Aims—To determine whether mutant *p53* alleles harboured by malignant tumours of the oral cavity were also present in previous premalignant lesions at the same site.

Methods—Paraffin embedded tumour specimens along with their premalignant counterparts were analysed for *p53* alterations using immunohistochemistry, microdissection, polymerase chain reaction amplification, and DNA sequencing.

Results—Malignant lesions from five of eight patients showed overexpression of *p53* protein by immunohistochemistry. Upon DNA sequencing, two of these five specimens had *p53* mutations. Of the five patients whose cancers showed *p53* overexpression by immunohistochemistry, three had previous premalignant lesions that also had immunohistochemically detectable *p53* protein. However, DNA sequencing showed that none of these three had mutations in the *p53* gene. The remaining five premalignant lesions had no immunohistochemically detectable *p53* protein.

Conclusions—Some premalignant lesions have increased *p53* protein which can be detected by staining with antibody to *p53*. This staining is not caused by mutations in *p53* that are found in subsequent tumours at the same site.

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Keywords: Oral cancer, *p53* gene, mutations, loss of heterozygosity.

Premalignant lesions in the oral cavity and upper aerodigestive tract are at risk of progressing to invasive cancers.^{1,2} The molecular basis of facilitated progression in these tumours is not well understood. Several proto-oncogenes, including *c-myc*, *N-ras*, *bcl-1*, and *int-2*, among others, have been reported to be amplified, overexpressed, or both in a large percentage of head and neck cancers.³⁻⁶ Furthermore, mutation of the *p53* gene occurs in many different types of human cancers,⁷⁻⁹ including squamous cell carcinoma of the head and neck.^{10,11} Using immunohistochemical

techniques, increased *p53* protein has been reported in 50–60% of head and neck cancers.¹⁰⁻¹³ Stabilisation of *p53* protein, allowing immunohistochemical detection, frequently accompanies *p53* mutation.^{14,15} Mutation at *p53* also is commonly associated with loss of the normal allele on the short arm of chromosome 17, where *p53* is located.^{16,17} In colorectal cancer, mutation and loss of heterozygosity at *p53* is a late event in tumorigenesis.^{18,19} In contrast, *p53* mutation appears to be an early event in oesophageal cancers,²⁰ lung cancers,²¹ and other types of tumour.^{22,23}

To gain a better understanding of the role and timing of *p53* gene overexpression and mutation in head and neck cancer, biopsies of premalignant lesions and malignant tumours that developed at the same sites at a later time were analysed by immunohistochemistry and DNA sequencing from polymerase chain reaction (PCR) amplified DNA from microdissected slide preparations.

Methods

SAMPLE SELECTION

Eight paraffin embedded tumour sections, along with their premalignant counterparts (leukoplakia and dysplasia), were collected from the Karolinska Hospital, Stockholm, Sweden, and the University Hospital of Zagreb, Croatia.

IMMUNOHISTOCHEMISTRY

The premalignant and malignant pathology was confirmed by ZPP and JSM. Tissue sections 5 µm thick were mounted on polylysine coated slides. A method for revealing previously inaccessible epitopes in paraffin embedded tissues was recently described by Shi *et al*²⁴ and Pavelic *et al*.²⁵ Immunostaining was done with the mouse antihuman *p53* specific monoclonal antibody (PAb 1801, a gift from Oncogene Science Inc, Manhasset, New York, USA) using the PAP technique described by Pavelic *et al*.²⁶ The intensity scores (0, none; +, weak; ++ intermediate; +++ strong) were evaluated by two independent pathologists in a blinded fashion (ZPP, JSM).

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Immunohistochemical detection of p53 and mutation at p53 in metachronous premalignant and cancerous lesions

Patient	Age (years)/sex	Premalignant lesions			Mutation	Cancer		Mutation		Sequence change
		Type	Year of appearance	p53 staining		Year of appearance	p53 status	exon	codon	
1	72/M	L	1985	+	—	1993	++	—	—	—
2	49/M	L	1983	—	—	1988	+	—	—	—
3	71/M	L	1988	+	—	1990	+++	8	273	CGT→CAT
4	64/F	L	1984	—	—	1991	—	5	164	AAG→AAT
5	60/M	L	1991	—	—	1992	—	—	—	—
6	58/M	L	1978	—	—	1987	+	—	—	—
7	79/M	D	1986	+	—	1987	+++	5	143	GTG→TTG
8	73/M	L	6/1987	—	—	11/1987	—	—	—	—

L = leukoplakia; D = dysplasia.

DNA ISOLATION

Genomic DNA was prepared directly from microdissected tissue. In the specimens that had moderate to intense p53 staining, tissue was selected from reactive regions. The tissue samples were dewaxed by two washes in xylene and one wash in 95% ethanol, then digested with proteinase K (0.2 mg/ml) in a buffer containing 50 mM Tris, 1 mM EDTA, and 0.5% Tween 20 at 37°C for 3 h or overnight.

PCR AMPLIFICATION

PCR primers were synthesised based on the published sequences.²⁶ The reaction was performed in a volume of 50 µl in 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (wt/vol) gelatin, and 2.5 units of Taq DNA polymerase, with the deoxynucleotide triphosphates (dNTP) at a final concentration of 200 µM each, primers at 10 pmol, and template DNA for 50 cycles under the following condition: 20 s at 94°C (denature); 30 s at 55°C (annealing); 30 s at 72°C (extension) and with final step extension at 72°C for 7 min. PCR products were fractionated by 1.5% Seaplaque agarose gel. The corresponding DNA bands were excised and purified with Gene Clean Kit (Bio 101). Purified DNA samples were subjected to thermal cycles of DNA sequencing.

SEQUENCING OF PCR PRODUCTS

The sequencing primer was one of a pair of PCR primers used for exon amplification.²⁷ Sequencing was done in accordance with the manufacturer's protocol (VentT[™] thermal cycles dideoxy DNA sequencing kit, New England Biolab)²⁸ for 20 cycles under the following conditions: 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s. The sequencing products were resolved by 7 M urea-6% PAGE.

LOSS OF HETEROZYGOSITY

The DNA from premalignant and malignant lesions was evaluated for loss of heterozygosity. A highly polymorphic dinucleotide repeat polymorphism marker at the human p53 locus was used to detect loss of heterozygosity.²⁹ Primers were synthesised flanking a (CA)₂₅ repeat to give a 116 bp product: GT strand primer: AGGGATACTATTTCAGCCCCGAGGTG; AC strand primer: ACTGCCACTCCTTGCC-

CCATTC. PCR was performed at an initial temperature of 94°C for 3 min for 35 cycles under the following conditions: 30 s at 94°C, 1 min at 62°C, and a final extension for 5 min at 72°C. Reaction products were resolved with a 10% polyacrylamide gel, stained with ethidium bromide, and photographed. The results were analysed to determine changes in allele intensity between normal and tumour tissue.

Results

Immunohistochemical staining with anti-p53 monoclonal antibody produced positive staining in three of eight of premalignant lesions and five of eight of malignant lesions. Three of the five patients whose oral cancer was p53-positive by immunohistochemistry also had immunohistochemically detectable p53 in their previous premalignant lesions. These lesions had been removed 1 year (patient 7), 2 years (patient 3), and 8 years (patient 1), respectively, before a tumour developed. These data, summarised in the table, show that stabilisation of p53 protein can occur in the very early stage of oral cavity tumorigenesis. Adjacent normal tissue did not stain with the anti-p53 antibody.

To determine whether or not the increased cellular level of p53 protein was due in each case to mutation in p53, we performed direct genomic sequencing of PCR amplified product of exons 5, 6, 7, and 8, which include the majority of known mutated sites. None of the premalignant lesions showed p53 mutations within this region (table). Two of the eight cancer patients (patient 3 and patient 7) had two missense mutations in p53 (table). One patient (P3) had two missense mutations, one at the second base of codon 273 (CGT to CAT) causing an amino acid change from arginine to histidine, and the other at codon 164 (AAG to AAT) causing a lysine to asparagine change (figure). The other (P7) had mutation at codon 143 (GTG to TTG) causing an amino acid change from valine to leucine (figure).

In all three cases, the sequencing gels displayed a wild type band in the same position as the mutant band (figure), indicative of two alleles. That there was no loss of heterozygosity was confirmed by microsatellite analysis (data not shown). The other three malignant tumours and the three premalignant lesions which stained positive with antibody to p53 did not reveal mutations within the regions sequenced.

Representative sequence analysis of p53 in samples from patients 3 and 7. Each exon and its splice junctions were sequenced in both directions as detailed in Methods. Panels A and B depict sequence extended from an antisense primer, and panel C from a sense primer. All three gels show a wild-type band of equal intensity to the mutant band, suggesting that the tumours are heterozygous for mutant and normal p53 at those codons.

Discussion

The important finding in this study was that three of five patients whose cancer tissue had immunodetectable p53 also manifested p53 staining in the premalignant lesion at the same site 1–8 years before a tumour developed. These results suggest that immunohistochemical detection of p53 can occur very early in head and neck tumorigenesis. The number of patients presented here is necessarily small because of the scarcity of cohorts in which metachronous lesions are available for study. This report is the first to describe p53 staining in head and neck cancer and in a corresponding earlier premalignant sample from the same patient.

It is possible that mutations may have occurred outside the region sequenced (that is, exons 5–8). However, other studies in which the complete cDNA mutations were screened found very few mutations outside this region.³⁰ Alternatively, staining could be facilitated by activating expression of other genes encoding proteins that might interact with or stabilise p53, such as that which encodes the murine

double minute 2 (MDM2) gene product.³¹ Staining of cells in the preneoplastic lesions could also be a result of exposure to xenobiotics and localised DNA damage, which in turn can induce a rise in p53.³²

To determine whether or not p53 immunostaining or p53 mutation are related to loss of heterozygosity, we used a highly polymorphic marker to detect loss of heterozygosity at p53. No evidence of loss of heterozygosity was found in either the premalignant or the malignant lesions, including two specimens with mutations. This was in contrast to other reports of frequent loss of heterozygosity seen in colorectal carcinoma,³³ osteosarcoma,³⁴ and other cancers.³⁵ The observation of mutation at p53 with maintenance of heterozygosity seen in our patients has also been observed by others.³⁶ It is possible that loss of heterozygosity occurred distal to the region screened since a second locus, distal to p53, at 17p 13.3 appears to be involved in loss of heterozygosity in breast cancers,^{37,38} astrocytomas,³⁹ neuroectodermal tumours,⁴⁰ and hepatocellular carcinoma.⁴¹ In preliminary studies by us (unpublished data) using a highly polymorphic probe (p144D6 and P68RS2.0) with Southern blotting, loss of heterozygosity was found at loci on chromosome 17p13.3 and 13q14, leaving the p53 gene itself unaffected.

Our findings that p53 status differs in premalignant and malignant tissues are in contrast to earlier reports in which the same p53 mutation was found in premalignant and malignant tissue from the same patient.^{42,43} However, it is important to recognise that the experimental paradigm was not the same. In the earlier reports, the premalignant specimen was obtained from tissue in proximity to the tumour, and may have arisen from the same progenitor cell that gave rise to the tumour proper. In the present report, the premalignant and malignant samples were obtained from the same site but at different times. Even though in two cases both premalignant and counterpart malignant lesions stained with antibody to p53, only the malignant specimen had a mutation in p53 within the region assayed.

These results show that p53 protein accumulation, but not gene mutation, may occur at a very early stage of oral cavity tumorigenesis, suggesting that the accumulation of p53 protein was mutated by a p53 mutation independent mechanism.

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