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ras Gene Point Mutation is a Rare Event in Premalignant Tissues and Malignant Cells and Tissues from Oral Mucosal Lesions

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Three series of biopsy specimens of premalignant and malignant oral lesions, together with seven human keratinocyte cultures, previously established from oral squamous cell carcinomas, were analysed for point mutation in exons 1 and 2 of the *c*-Ha-*ras*, *c*-Ki-*ras* and N-*ras* genes by direct nucleotide sequencing of DNAs amplified in the polymerase chain reaction (PCR). Only one out of 12 biopsy samples (8.3%), a well-differentiated carcinoma which was the latest in a series of floor of mouth lesions from 1 of the 3 patients studied, harboured a mutant *c*-Ha-*ras* gene, being heterozygous at codon 12 for a GGA-GTA change. One cell line (H357) showed heterozygosity in both exons 1 and 2 of *c*-Ha-*ras*, harbouring a GGT to AGT mutation over codon 13 and a CAG to CAA mutation over codon 61. The remaining six oral carcinoma cell lines (85.7%) were homozygous normal at both exons 1 and 2 of *c*-Ha-*ras*. All cell lines showed normal *c*-Ki-*ras* and N-*ras* loci. We conclude that *ras* gene mutation is an infrequent occurrence in the malignant progression of oral epithelial cells, despite the probable importance of chemical carcinogens in the aetiology of the disease. We emphasise the need to search for other cellular sequences which may be targets for chemical or viral carcinogens.
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INTRODUCTION

THE DEVELOPMENT of oral squamous cell carcinoma has traditionally been linked with exposure to mutagens in tobacco and alcohol. Members of the *ras* gene family are thought to be common targets for mutagenesis in a wide range of animal and human cancers [1]. Activation of *ras* genes frequently involves a single point mutation at specific sites within exon one (codons 11–13) or exon two (codon 61) and results in the expression of an abnormal p21 protein harbouring a single amino acid substitution at areas important for guanine nucleotide binding, favouring the active GTP-bound state [2, 3].

Point mutations in *ras* genes have been detected in various human cancers, although the incidence varies with tumour type (reviewed in ref. 1). Some 90% of pancreatic malignancies, for example, harbour mutations within codon 12 of Ki-*ras* [4], whereas only 50% of cases of colon adenocarcinoma harbour mutant *ras* alleles [5, 6] These findings may reflect the fact that *ras* point mutations appear to be carcinogen-specific [7, 8]. Intriguingly, comparable findings have been noted in head and neck carcinomas, where *c*-Ha-*ras* mutations are common in betel quid-induced carcinomas in an Indian population [9], but are less frequent in similar tumours in Western populations [10–12].

There is some controversy regarding the stage at which *ras* mutations appear in tumour development. In certain animal model systems the point mutation appears to be an initiating event [13] but, in others, *ras* mutations are only detected in overt carcinomas [14]. Similarly, in human malignancy, there is evidence that *ras* mutations may be early [5, 6, 15, 16] or late events [17, 18] depending on the cell type involved. It is not uncommon that the development of human oral squamous cell carcinomas is preceded by characteristic dysplastic leukoplakias [19]. These tissues, therefore, present an ideal opportunity to determine whether *ras* point mutations occur as an early or late event in human oral carcinogenesis. There are no data concerning the incidence of *ras* point mutation in human oral squamous carcinoma cell lines.

The purpose of this study was to analyse *c*-Ha-*ras*, *c*-Ki-*ras* and N-*ras* in three series of oral dysplasias known to have progressed to carcinomas, and in seven human oral squamous carcinoma cell lines which expressed a range of *in vitro* and *in vivo* phenotypes.

MATERIALS AND METHODS

Cell lines

Keratinocyte cultures were derived from oral squamous cell carcinomas which developed in 7 Caucasian patients, and were as previously described [20]. Cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS) 0.075% $NaHCO_3$, 0.6 μg/ml L-glutamine, 0.5 μg/ml hydrocortisone and 10 μg/ml cholera toxin at 37°C in 95% air/5% CO_2.

Fixed tissues

Archival material was obtained from the files of the Department of Oral Pathology, Eastman Dental Hospital, and comprised tissues from 3 patients who developed oral

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premalignant lesions which subsequently progressed to squamous cell carcinoma. 5 μm sections of paraffin-processed specimens were placed into 0.5 ml microfuge tubes, dewaxed in 400 μl xylene, centrifuged (12000 *g*, 10 min) and the tissue pellets washed in ethanol and dried under vacuum.

Preparation of DNA

DNA was prepared from cell lines by the method of Kreig *et al.* [21]. Briefly, cells were washed in phosphate buffered saline (PBS), lysed in 0.3 mol/l sodium acetate, 1 mmol/l EDTA, 1% sodium dodecyl sulphate (SDS), extracted with an equal volume of phenol/chloroform (twice, for 30 min) and the nucleic acids precipitated with ethanol. Following centrifugation and washing in 70% ethanol, DNA and RNA were resuspended in 10 mmol/l Tris-HCl, 1 mmol/l EDTA pH 8.0 (TE buffer) and the RNA digested with DNase-free RNase A (50 μg/ml) at 37°C for 30 min. Samples were extracted three times with phenol/chloroform, ethanol precipitated, washed in 70% ethanol, resuspended in TE buffer and quantified spectrophotometrically.

Oligonucleotides

Twenty nucleotide long oligomers for use as PCR primers were synthesised on a Du Pont Coder 300 DNA synthesiser by Dr L. Hall, Department of Biochemistry, University of Bristol. The nucleotide sequences are listed in Table 1.

Polymerase chain reaction

PCR was carried out in a Geneamp DNA Thermal Cycler (Perkin Elmer Cetus) as previously described [22]. Briefly, 1 μg of cell line DNA (1 μg/μl) was added to a 0.5 ml microfuge tube containing 98.5 μl of PCR reaction mix (50 mmol KCl, 10 mmol/l Tris–HCl pH 8.3, 1.5 mmol/l $MgCl_2$, 200 μmol/l dNTPs, 1 μmol/l of primers), overlaid with 0.5 volumes of light paraffin oil and denatured at 95°C for 10 min. Tubes were placed on ice, 2.5 U of *Taq* DNA polymerase added and returned to the thermal cycler for 30 amplification cycles of 95°C (1 min), 50°C (1 min) and 72°C (1 min). Alternatively, dried, dewaxed tissue sections were resuspended in 30 μl of distilled water, incubated at 100°C for 30 min, ice-cooled, reaction components added to final concentrations as listed above and PCR performed as for cell line DNA. PCR products were electrophoresed in 6% polyacrylamide, stained in gel buffer containing 0.5 μg/ml ethidium bromide and visualised by ultraviolet transillumination. Bands were excised, the DNA eluted by overnight incubation at 37°C in 0.5 mol/l NH_4Cl, 10 mmol/l EDTA, ethanol-precipitated and resuspended in 7 μl of distilled water.

Table 1. Oligonucleotide sequences of sense (S) and antisense (A) PCR primers used to amplify c-*Ha*-ras *(H),* c-*Ki*-ras *(K) and N*-ras *(N) exons 1 and 2 DNA sequences*

	Target sequence
H1S 5′-GCAGGAGACCCTGTAGGAGG-3′	168 bp
H1A 5′-TAGGCTCACCTCTATAGTGG-3′	
H2S 5′-CAGGATTCCTACCGGAAGCA-3′	157 bp
H2A 5′-ACTTGGTGTTGTTGATGGCA-3′	
K1S 5′-GTACTGGTGGAGTATTTGAT-3′	206 bp
K1A 5′-CTCTATTGTTGGATCATATT-3′	
K2S 5′-ATCCAGACTGTGTTTCTCCC-3′	215 bp
K2A 5′-TAAACCCACCTATAATGGTG-3′	
N1S 5′-CAGGTTCTTGCTGGTGTGAA-3′	144 bp
N1A 5′-CACTGGGCCTCACCTCTATG-3′	
N2S 5′-CCAGGATTCTTACAGAAAAC-3′	190 bp
N2A 5′-CTAGTACCTGTAGAGGTTAA-3′	

The expected fragment sizes are indicated in base pairs

DNA sequence analysis

Products of PCR reactions were sequenced using a commercially-available kit (Sequenase, United States Biochemicals). Briefly, 2 ng of either PCR oligonucleotide (to be used as sequencing primer) and 2 μl of 5 × sequencing buffer (200 mmol/l Tris–HCl pH 7.5, 100 mmol/l $MgCl_2$, 250 mmol/l NaCl) were added to 7 μl of DNA template, the reaction mixture denatured at 100°C for 5 min and chilled in a dry ice-ethanol bath for 5 min. To the thawed primer–template mix was added 1 μl of dithiothreitol, 2 μl of labelling mix, 1 μl of α-[^{35}S]-dATP (55.5 TBq/mmol) and 2 μl of a 1:8 dilution of Sequenase v2.0 enzyme. The extension step was allowed to progress for 3 min at ambient temperature and then terminated for 2 min at 37°C by addition of equal volumes of reaction mix to each of four termination mixes. Loading buffer was added, the samples heated to 80°C for 2 min and electrophoresed in 7.5% polyacrylamide. Gels were dried under vacuum and exposed to Kodak-X-AR5 film for several days.

Southern blot analysis of cellular DNA

Five microgram aliquots of keratinocyte DNA, prepared as described above, were digested overnight at 37°C with a 4-fold excess of the restriction endonuclease MspI. Samples and 50 pg of pUCEJ6.6 (23) plasmid containing an activated *c*-Ha-*ras* sequence were electrophoresed in 1% agarose, stained with ethidium bromide (0.5 μg/ml in running buffer) and photographed under ultraviolet transillumination. Gels were denatured in 0.25 mol/l HCl, rinsed in distilled water and transferred in 0.4 mol/l NaOH onto Hybond N^+ membrane (Amersham). Membranes were prehybridised in 5 × standard saline citrate (SSC) (1 × SSC is 0.15 mol/l NaCl, 15 mmol/l sodium citrate), 5 × Denhardt's solution (0.1% BSA, 0.1% Ficoll 400, 0.1% polyvinylpyrollidone), 0.5% SDS and 50 μg/ml denatured salmon sperm DNA overnight at 65°C. The DNA probe was prepared by oligolabelling [24] 25 ng of BamHI-excised *c*-Ha-*ras* DNA insert from pUCEJ6.6 to a specific activity in excess of 2×10^9 cpm/μg using a commercially available kit (Prime-It, Stratagene). Hybridisation was carried out overnight at 65°C, and filters washed twice in 2 × SSPE/0.1% SDS (65°C for 15 min), once in 1 × SSPE/0.1% SDS (15 min at 65°C) and a final high stringency wash in 0.1 × SSPE/0.1% SDS (10 min at 65°C). Autoradiography was carried out at −70°C with intensifying screens for up to 3 days.

RESULTS

ras *Sequences in premalignant and malignant oral lesions*

Biopsy specimens from patients with premalignant lesions of varying degrees of oral dysplasia but all of which later progressed to carcinoma, and others from established oral squamous cell carcinoma were examined for the presence of

Table 2. Details of fixed specimens of premalignant and malignant oral lesions from 3 different patients, which developed over periods from 3 to 14 years

Series	Biopsy date	Site	Histology	Sequence	Codon	Amino acid change
1(i)	1988	Lower lip	Solar keratosis, mild dysplasia	N	—	—
(ii)	1988	Lower lip	Mild dysplasia	N	—	—
(iii)	1990	Lower lip	SCC	N	—	—
2(i)	1982	FOM	Severe dysplasia	N	—	—
(ii)	1983	FOM	Severe dysplasia	N	—	—
(iii)	1983	FOM	Moderate/severe dysplasia	N	—	—
(iv)	1987	FOM	Severe dysplasia	N	—	—
(v)	1990	Tongue	SCC	N	—	—
(vi)	1990	FOM	SCC	GGC-GTC	Ha 12	Gly–Val
3(i)	1976	Lower lip	Severe dysplasia	N	—	—
(ii)	1981	Lower lip	SCC	N	—	—
(iii)	1990	Lower lip	SCC	N	—	—

Only one *ras* point mutation was detected, in the most recent of six biopsies from patient 2, at codon 12 of *c*-Ha-*ras* in one allele. This would result in substitution of valine-12 in place of the normal glycine-12. FOM = floor of mouth; SCC = squamous cell carcinoma; Ha12 = *c*-Ha-*ras* codon 12; N = normal sequence.

activating point mutations in exons 1 and 2 of *c*-Ha-*ras*, *c*-Ki-*ras* and N-*ras*. Table 2 lists the site and histological grading of these lesions.

The only *ras* mutation noted in 12 samples, each of which were analysed at two sites (exons 1 and 2) for three proto-oncogenes, was present in a moderately-differentiated oral squamous carcinoma which was the latest in a series of floor of mouth lesions in patient 2. There was a hemizygous mutation over codon 12 of *c*-Ha-*ras* and resulted in a GGA–GTA change (Fig. 1). This enables a mutant form of p21 to be encoded with a substitution of the normal glycine residue with valine at this position. The mutation was not detected in premalignant lesions from this patient, or in a co-existing carcinoma from the tongue which was histologically no different from the floor of mouth lesion. There was no evidence of *c*-Ki-*ras* or N-*ras* mutations in the tissue samples of patient 2.

No *ras* mutations were evident in the biopsy specimens of patients 1 and 3.

ras *Sequences in oral squamous carcinoma cell lines*

In order to examine the frequency of point mutational activation of *ras* genes late in oral epithelial carcinogenesis, we applied a similar protocol to DNA prepared from cultured keratinocytes derived from seven invasive oral squamous carcinomas. One out of seven oral carcinoma cell lines (14.3%) was found to contain point mutations both at codon 13 (data not shown) and codon 61 (Fig.2) of *c*-Ha-*ras*. In each case a guanine residue had been mutated to adenine. This had the effect of altering the coding potential at codon 13 from glycine

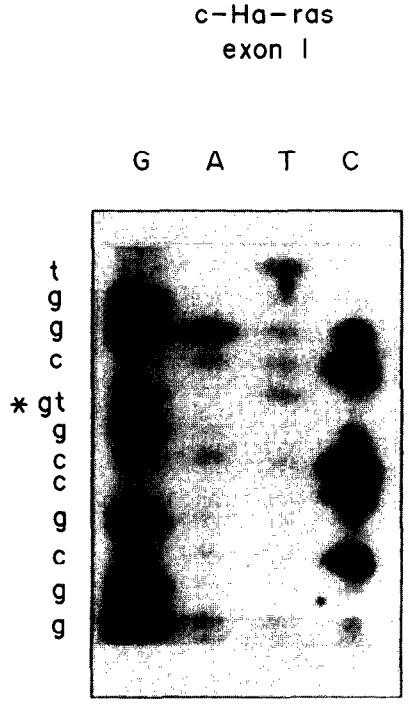

Fig. 1. Nucleotide sequence of *c*-Ha-*ras* exon 1 DNA from floor of mouth biopsy specimen (vi) from patient 2 (see Table 2). A heterozygous guanine to thymidine transversion was detected at codon 12.

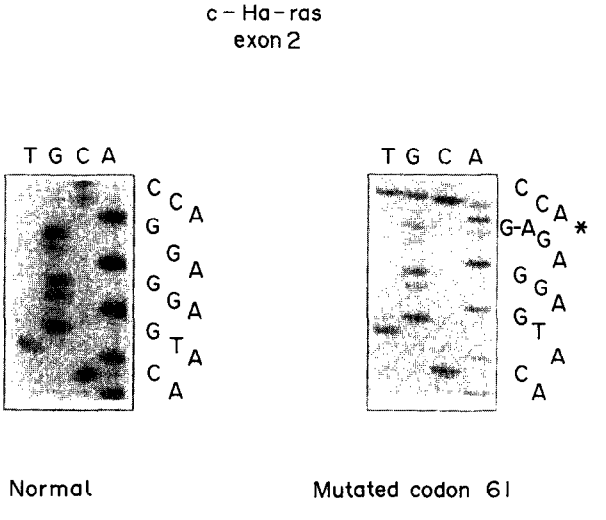

Fig. 2. Nucleotide sequence of *c*-Ha-*ras* exon 2 from oral squamous carcinoma cell line H357 (see Table 3), showing hemizygosity for a G-A mutation at codon 61. A G-A mutation was also observed at codon 13 (not shown). The coding potential is altered in exon 1 from glycine-13 to serine-13, but the mutation at codon 61 would not result in an amino acid substitution. The sequence shown was obtained using the antisense primer and the sequencing gel reversed to read the sense strand.

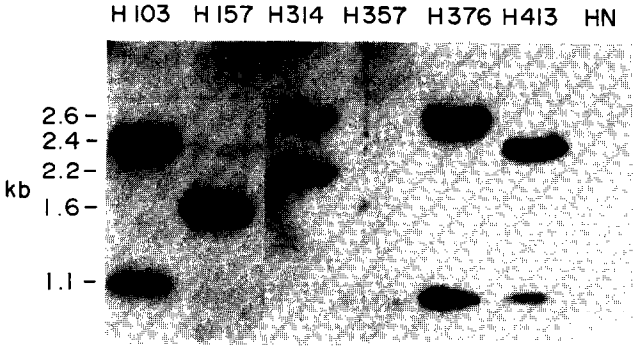

Fig. 3. Southern blot of MspI-digested genomic DNA from oral squamous carcinoma cell lines hybridised with the 6.6 kb insert from pUCEJ6.6 under conditions of high stringency. Only common *c*-Ha-*ras* alleles were detected, as indicated.

to serine. The base change at codon 61 was conservative. No mutations were detected in *c*-Ki-*ras* or N-*ras* over the region sequenced.

The above data imply a low incidence of point-mutational activation of *ras* genes in oral carcinoma.

c-*Ha*-ras *alleles in oral squamous carcinoma cell lines*

In order to determine whether *ras* gene activation was occurring perhaps as a result of the presence of rare alleles, we analysed the oral carcinoma cell line DNA by Southern blotting. Figure 3 shows the result of hybridisation under stringent conditions of MspI-digested genomic DNA from oral cancer cell lines with a ^{32}P-oligolabelled probe comprising the 6.6 kb BamHI insert of pUCEJ6.6.

Only the common *c*-Ha-*ras* alleles (2.6 kb, 2.4 kb, 2.2 kb, 1.6 kb, 1.1 kb) were detected in these carcinoma cell lines.

DISCUSSION

The somatic mutation theory of carcinogenesis was verified with the demonstration that *ras* oncogenes acquire transformation-inducing properties by single point mutations of their coding sequences [25, 26]. This was substantiated by the demonstration of a high frequency of *ras* mutations in a number of different human malignancies (reviewed in ref. 1). The present study examined Ha-, Ki- and N-*ras* mutations in tissue sections of premalignant and malignant oral epithelial lesions and in seven human oral carcinoma cell lines.

Table 3. Ras *mutations in oral squamous carcinoma cell lines. Two point mutations were detected in* c-*Ha*-ras, *both in a single cell line* (*H*357) *and both involving a hemizygous G–A transversion*

Cell line	Tumour histology	Sequence	Codon	Aminoacid change
H103	WD	N	—	—
H157	WD	N	—	—
H314	MD	N	—	—
H357	WD	GGT-AGT	Ha 13	Gly–Ser
		CAG-CAA	Ha 61	none
H376	WD	N	—	—
H400	MD	N	—	—
H413	MD	N	—	—

WD = well-differentiated; MD = moderately-differentiated; N = normal sequence; Ha13 = *c*-Ha-*ras* codon 13; Ha61 = *c*-Ha-*ras* codon 61.

The results of this study indicate that mutation of *ras* genes is uncommon in human oral premalignant and malignant tissues and in human oral carcinoma cell lines from Caucasian patients. The latter data also suggest that point mutation of *ras* genes did not occur as a consequence of growth *in vitro*. The findings support previous observations of a low frequency of *ras* mutation in oral malignancies in the western world [10–12, 26]. Interestingly, *c*-Ha-*ras* mutations are common in oral cancers in an Asian population [9]. Together the data strongly indicate that western and Asian populations differ in their exposure to the aetiological agents of oral cancer and that such exposure may influence the frequency of *ras* mutations. There is conflicting evidence as to whether cigarette smoking correlates with elevated expression of p21^{ras} [27, 28]. Interestingly, patient 2 in this study was a smoker, but information is not available for patients 1 and 3. These 2 cases, however, were lip lesions associated with actinic change. Nevertheless there is well-documented evidence that *ras* mutations are carcinogen-specific in several animal models [8, 13, 29, 30].

The stage at which *ras* mutations occur during malignant progression of epithelial cells is unknown. Data suggest that *ras* mutations may occur as an early [6, 15] or late [17, 18] event in carcinogenesis, depending on the tissue of origin. In the present study, *ras* mutations were detected only in tissues and cell lines of malignant origin, indicating the anomaly to be a late event in tumour progression.

Interestingly, the mutations detected in both tissues and cultured cells of this study were confined to *c*-Ha-*ras*. Furthermore, the only point mutations detected in the cultured cells were limited to one cell line (H357) and involved the same sequence alterations, G-A, at both codons 13 and 61. Whether other mechanisms of *ras* activation occur in head and neck cancer is not known. Limited information is available concerning the role of gene amplification and overexpression [11, 31–34], loss of heterozygosity [35] and alternative mutation sites [36] in head and neck cancer.

Other gene mutations may also be involved in the development of human oral malignancy. A close correlation between the tumour suppressor gene product p53 and cigarette smoking has recently been noted in oral cancer [37], and analysis of oral squamous cell carcinoma biopsy specimens [38] suggests that p53 mutation is a common feature of such tumours. We have recently extended this work and examined p53 mutation and expression in human oral carcinoma cell lines and have noted a high frequency of point mutations at specific regions of the gene (Yeudall *et al.*, manuscript in preparation)

In conclusion, the results of this study show that *ras* point mutations in malignant human oral epithelial cells are rare, at least in western examples of oral cancer. At best, *ras* mutations may contribute, but are not fundamental, to tumour development. If *ras* mutations do have a role in human oral epithelial carcinogenesis, it is more likely to be a late rather than an early event.

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