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Loss of allelic heterozygosity at the harvey *ras* locus in human oral carcinomas

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Summary. The Harvey *ras* locus was examined for restriction fragment polymorphism and loss of allelic heterozygosity in 62 oral cancer patients. Southern blot analysis on *Bam*HI digests of the tumour tissue DNA, revealed 23 patients with H-*ras*-1 heterozygosity. The probes used to study the polymorphism were the *Bam*HI 6.6-kb fragment encoding the complete H-*ras*-1 sequence plus the variable tandem repeat (VTR) region, and the 1-kb *Msp*I fragment encoding the VTR region. The allelic heterozygosity was better resolved by *Pvu*II and further confirmed by *Taq*I. In addition, *Taq*I digestion demonstrated a unique VTR rearrangement indicated by 2.1-kb, 0.9-kb and 0.6-kb fragments, implying additional *Taq*I sites, in three of the patients. Further analysis of matched tumor tissue and peripheral blood cell DNA from the same patient demonstrated tumor-associated loss of one of the allelic fragments in $^{7}/_{23}$ (30%) of the patients with H-*ras*-1 heterozygosity. However, the loss was not significantly correlated to clinicopathological parameters staging the disease. Thus, our data showing loss of H-*ras*-1 alleles and VTR rearrangement, with relatively high incidence ($^{9}/_{23}$; 39%) in the oral cancer patients at various stages of the disease, implies H-*ras*-1 involvement as an early event in the process of oral carcinogenesis.

Key words: H-*ras* – Allelic loss – Oral cancer

Introduction

Cancer of the oral cavity is one of the major types of cancer in India, comprising about 40% of the total cancer incidence (Sanghavi 1981). The oral cancers exhibit a unique pattern of high prevalence and an unequivocal relationship with the persistent tobacco-chewing habit (Gupta et al. 1987; Jussawalla and Deshpande 1971), and are preceded by a clinically distinctive premalignant stage such as "leukoplakia" (Daftary 1991). This is in contrast to their relatively low incidence – 2%–4% of oral malignancies – in the Western population (Binnie 1976; Field and Spandidos 1987), an association with tobacco usage in cigarettes or the habit of snuff dipping (Winn 1984), and de novo development of the malignancy without the consistent appearance of premalignant lesions (Binnie 1991).

In the oral cancers seen in the Indian subcontinent we have recently demonstrated involvement of the *myc* and *ras* family of oncogenes, specifically multiple oncogene amplification, in about 50% of patients (Saranath et al. 1989). In addition, we observed the L-*myc* S allele predominating in poor to moderately differentiated tumours, as well as in larger-sized tumours (Saranath et al. 1990). Furthermore, activation of the H-*ras*-1 oncogene, showing high-frequency mutations in codons 12 and 61 in these chewing-tobacco-related oral carcinomas, has been observed (Saranath et al. 1991). Interestingly, eight of the samples with H-*ras* mutations also showed loss of the wild-type H-*ras* gene.

The H-*ras*-1 locus includes a hypervariable region, designated as the variable tandem repetition region (VTR), which consists of a series of 28-base-pair random repeats 3′ to the gene (Capon et al. 1983). Polymorphism of the human H-*ras*-1 oncogene has been ascribed to changes in the number of repeat units (Capon et al. 1983; Krontiris et al. 1985), and can be defined by *Bam*HI, *Pvu*II and *Taq*I enzymes (Pierotti et al. 1986; Heighway et al. 1986). Recently tumour-specific allelic loss of the H-*ras*-1 gene has been demonstrated in several solid tumours including Wilm's tumours (Reeve et al. 1989), bladder carcinoma (Fearon et al. 1985), ovarian cancer (Lee et al. 1989) and breast carcinoma (Theillet et al. 1986). These studies implied a critical role either for the tumour-specific H-*ras*-1 allelic loss, or loss of adjacent normal growth-regulatory gene(s).

In the present study, we have investigated the status of the H-*ras*-1 locus in 62 patients with squamous cell carci-

Abbreviations: VTR, variable tandem repeat; PBC, peripheral blood cell; RFLP, restriction fragment length polymorphism; EGFR, epidermal growth factor receptor

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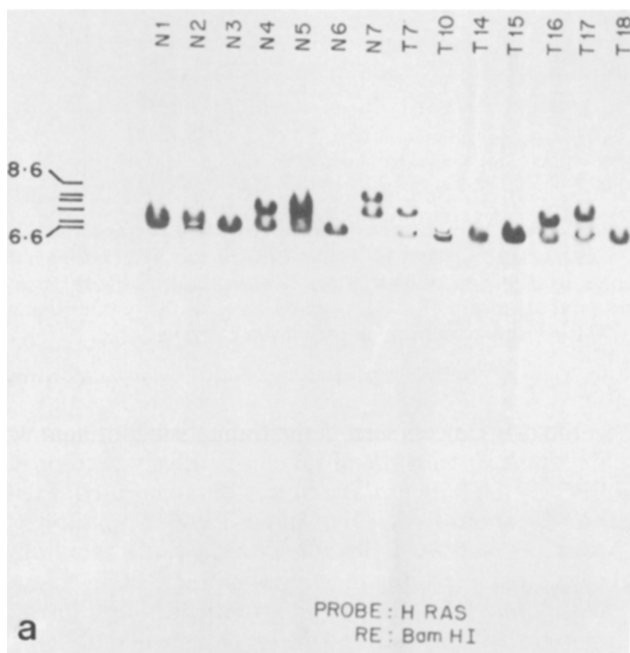
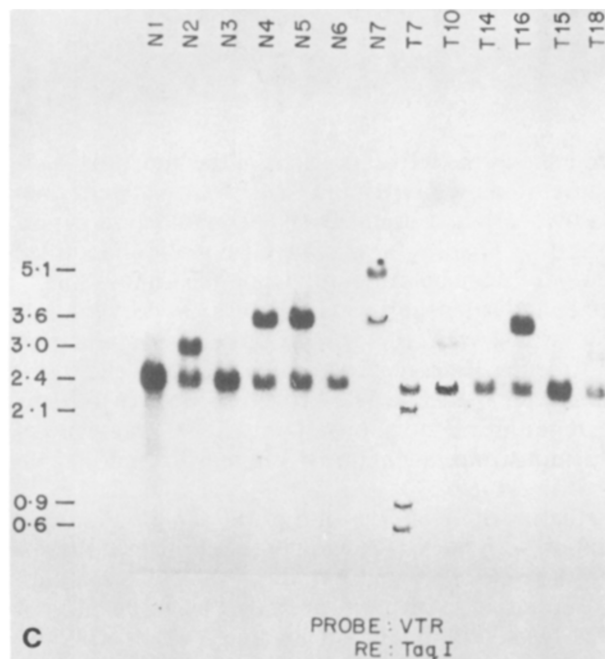
nomas of the oral cavity. Using restriction fragment length polymorphism (RFLP) analysis with *Bam*HI, *Pvu*II and *Taq*I enzymes, we demonstrate loss of the H-*ras*-1 allele in 30% of the patients showing heterozygosity at this locus, and VTR rearrangement in 3 of the patients.

Materials and methods

Patients. A group of 62 patients with squamous cell carcinoma of the oral cavity (48 male and 14 female), aged 28–70 years, were studied for H-*ras*-1 RFLP and loss in heterozygosity at the H-*ras*-1 locus. Material comprised 33 DNA samples from the buccal mucosa, 14 samples from the lower alveolus, 11 from the tongue, and 4 from the hard palate. The clinical diagnosis was confirmed by histological features of the biopsy material from the tumour. The staging was done according to the TNM classification (UICC 1988), and patients for surgical treatment with T_1–T_4, N_0–N_2, and M_0 tumours were investigated. Peripheral blood cells (PBC) were obtained from 28 of these patients, to be used as matching controls. In addition, PBC from healthy volunteers were also included in the study.

Preparation of DNA and Southern blot analysis. High-molecular-mass DNA was extracted from the tumour tissues and PBC by standard methods (Sambrook et al. 1989a). Samples of 20 µg DNA were digested overnight with *Bam*HI and *Pvu*II separately at 37° C, and with *Taq*I at 65° C. Aliquots of 10 µg digested preparations were electrophoresed and transferred to nylon membranes (Hybond-N, Amersham) according to the method of Southern (Southern 1975). The membrane was hybridized overnight with a radiolabelled human H-*ras*-1 probe in 6 × standard saline citrate (SSC; 1 × SSC = 0.15 *M* sodium chloride, 0.15 *M* sodium citrate), 50% formamide,





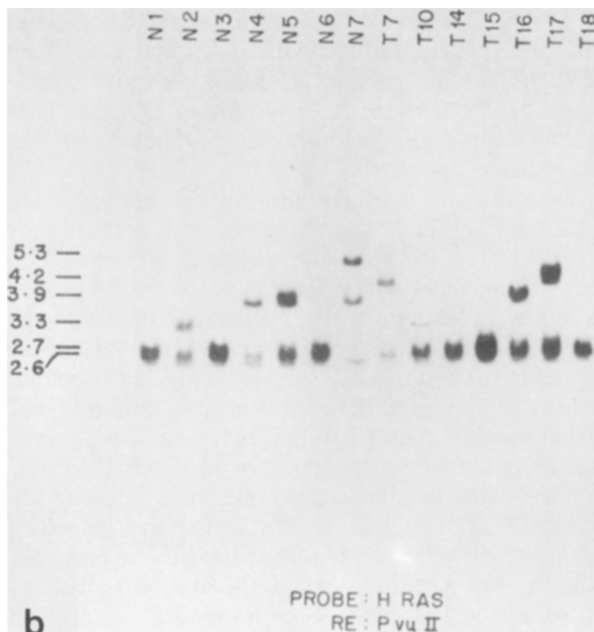


Fig. 1 a–c. Restriction fragment length polymorphism of the human H-*ras*-1 gene. Genomic DNAs from peripheral blood cells of healthy volunteers (N_1–N_7) and tumour DNAs of patients with oral cancer (T_7, T_{10}, T_{14}–T_{18}) were digested as follows. **a** With *Bam*HI: probed with the 6.6 kb *Bam*HI insert of the plasmid pUCEJ6.6 H-*ras*; polymorphic fragments ranging from 6.6 kb to 8.6 kb are indicated. **b** With *Pvu*II: probed with the probe as in **a**; 2.6-kb invariant fragment and variant fragments of 2.7 kb to 5.3 kb are indicated. **c** With *Taq*I: probed with the 1-kb *Msp*I variable-tandem repeat (VTR)-region-specific fragment of the H-*ras*-1 gene. VTR-encoding *Taq*I fragments range from 2.4 kb to 5.1 kb; the *Taq*I variant showing the 2.1-kb, 0.9-kb and 0.6-kb fragments is also indicated (T_{17} is not shown in this blot)

1% sodium dodecyl sulfate and sheared denatured salmon sperm DNA (100 μg/ml), at 42° C. After washing to a final stringency of 0.2 × SSC at 65° C, the filters were subjected to autoradiography at −70° C for 1–5 days.

Quantitative densitometric scanning was carried out on the heterozygous samples. Loss of heterozygosity was indicated when the residual fragment showed a minimum of 50% reduction in the signal intensity, as compared to the retained fragment.

Hybridization probe. Plasmid pUCEJ6.6 carrying the mutated human H-*ras*-1 gene (Shih and Weinberg 1982) was grown and purified by standard methods (Sambrook et al. 1989b). The two inserts used to study the polymorphism of the H-*ras*-1 gene were (a) the *Bam*HI 6.6 × 10^3-base (6.6-kb) fragment encoding the complete H-*ras*-1 sequence plus the VTR region, and (b) the 1-kb *Msp*I fragment encoding the VTR region. The two fragments were purified by preparative agarose electrophoresis, using a low-melting agarose procedure. Both probes were ^{32}P-labelled by nick translation at a specific activity of $>10^8$ cpm/μg.

Results

RFLP at the H-ras-1 locus

Figure 1 shows the RFLP patterns of the human H-*ras*-1 gene generated by *Bam*HI and *Pvu*II restriction enzymes in healthy normal volunteers (N_1–N_7) and oral cancer patients (T_7, T_{10}, T_{14}–T_{18}). *Bam*HI digestion identified restriction fragments ranging from 6.6 kb to 8.6 kb. Figure 1a indicates the normal samples (N_2–N_4, N_5, N_7) and tumor DNAs (T_7, T_{10}, T_{16}, T_{77}) displaying a heterozygosity at the H-*ras*-1 locus. The different allelic forms of H-*ras*-1 in the same DNAs were better resolved by *Pvu*II digestion, producing an invariable fragment of 2.6 kb, and variable fragments ranging from 2.7 kb to 5.3 kb (Fig. 1b).

Furthermore, *Taq*I digestion of the samples, and hybridization with the VTR region probe confirmed the homozygous or heterozygous pattern of the DNA samples. *Taq*I digestion resolved four variable fragments of 2.4 kb, 3.0 kb, 3.6 kb, and 5.1 kb (Fig. 1c). In addition, the *Taq*I restriction analysis indicated the presence of additional *Taq*I sites in the amplified VTR region of 3 patients. This distinguished a variant form of the H-*ras*-1 allele with *Taq*I fragments of 2.1 kb, 0.9 kb and 0.6 kb, substituting for a single 3.6 kb variable fragment as seen in patient T7 (Fig. 1c).

We determined the distribution of polymorphic fragments of H-*ras*-1 in 62 oral cancer patients. Heterozygosity of the gene was observed in $^{23}/_{62}$ (37%) of the samples analysed. These 23 samples were amenable to analysis for loss of heterozygosity at the H-*ras*-1 locus.

Loss of heterozygosity at the H-ras-1 locus

Twenty-three heterozygous samples were analysed for loss of heterozygosity by either analysis of matched PBC and tumour tissue DNA from the same patient, or the occurrence of a greater than 50% reduction in the signal intensity of one of the heterozygous alleles in the tumour tissue DNA. We observed complete or partial loss of heterozygosity in 7 of the patients. Figure 2 is a representative Southern blot of seven matched samples of PBC and oral cancer primary tumour tissue DNA. A complete loss of the 7.4-kb fragment in the tumour tissue of sample OCT21 (lane 6) with the allele constitutively present in the PBC of the patient (lane 5) was demonstrated. Furthermore, tumour tissue DNA of OCT19 (lane 2) showed a loss of a 7.2-kb *Bam*HI fragment. The faint signal observed is most likely due to contaminating PBC DNA.

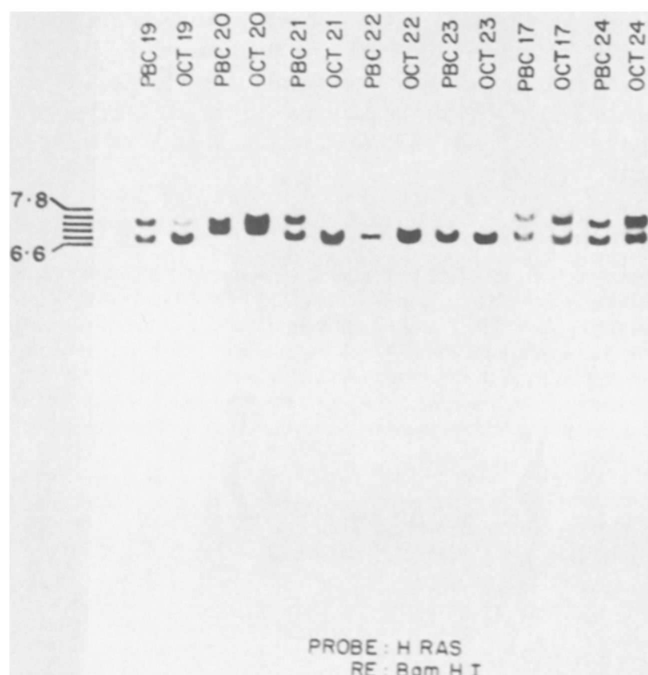


Fig. 2. Southern blot analysis demonstrating loss of one allelic H-*ras*-1 restriction fragment in human oral cancers. The peripheral blood cells (*PBC*) of patients 19 and 21 are heterozygous for H-*ras*, showing the presence of two fragments, whereas the tumour tissue (*OCT*) has lost one fragment in each instance

As summarised in Table 1, $^7/_{23}$ patients with H-*ras*-1 heterozygosity demonstrated the loss of one of the alleles. Furthermore, 1 of these patients, with moderately differentiated grade IV squamous cell carcinoma of the tongue, showed a *Taq*I variant allele, with additional *Taq*I sites, indicated by the presence of 2.1-kb, 0.9-kb and 0.6-kb fragments (Table 1, Fig. 1c). In addition, 2 more patients also showed the *Taq*I variant allele, although there was no loss of heterozygosity.

Earlier data for amplification/point mutation were available for 15 of the 23 heterozygous samples (Saranath et al. 1989, 1991). Of these, 5 demonstrated H-*ras* allelic loss, and 10 had lost no H-*ras* alleles. Of the 5 patients with allele loss, 2 demonstrated additional oncogene abnormalities (Table 1). Patient 21 indicated a point mutation in the H-*ras* codon 12.2, showing a glycine-to-valine substitution with loss of the wild-type, and patient 7 had a fourfold amplification of N-*myc* and a eightfold amplification of epidermal growth factor receptor (EGFR). The other 3 patients (10, 19 and 17) did not demonstrate *ras*/*myc*/EGFR oncogene aberrations. On the other hand, data on 10 heterozygous patients without H-*ras* allelic loss showed 5 of these with amplification/point mutations of *myc*/*ras*/EGFR oncogenes, and 5 without the abnormalities.

Table 1. Alterations of H-*ras* alleles in oral cancers

Patient no.	Site of tumour	Stage of differen-tiation	TNM staging and grade	H-*ras* alleles			Other oncogene aberrations[a]
				*Bam*HI fragments (kb)		*Taq*I	
				Constitutively present	Lost in OCT	variant fragments(kb)	
7	Tongue	Moderate	$T_3N_1M_0$ IV	7.8/6.8	6.8	2.1, 0.9, 0.6	Amplification of N-*myc* (×4) EGFR (×8)
10	Buccal mucosa	Well	$T_2N_0M_0$II	7.2/6.8	7.2	–	–
19	Lower alveolus	Poor	$T_4N_1M_0$IV	7.2/6.6	7.2	–	–
21	Buccal mucosa	Well	$T_4N_0M_0$IV	7.4/6.8	7.4	–	H-*ras* 12.2 point mutation, Gly-Val, loss of wild type
44	Tongue	Moderate	$T_4N_0M_0$IV	7.8/6.8	6.8	–	ND
57	Hard palate	Well	$T_3N_0M_0$ III	7.8/6.8	6.8	–	ND
62	Tongue	Moderate to poor	$T_2N_1M_0$IV	7.8/6.8	6.8	–	ND
17	Buccal mucosa	Moderate	$T_4N_0M_0$IV	7.8/6.8	–	2.1, 0.9, 0.6	–
37	Lower alveolus	Poor	$T_4N_2M_0$IV	7.8/6.8	–	2.1, 0.9, 0.6	ND

[a] Extended studies on reported data (Saranath et al. 1989, 1991) studying amplification of *myc*/*ras*/EGFR families and point mutations of *ras* oncogenes. –, No oncogene aberration; ND, not done; EGFR, epidermal growth factor receptor; OCT, oral cancer primary tumour tissue

Table 2. Correlation of allelic loss of H-*ras* with clinical parameters in oral cancers

Clinical parameters (total sample size) $n=62$		Heterozygous population $n=23$	Population with an allelic loss $n=7$	Population without an allelic loss $n=16$	χ^2
Tumour size					
2–4 cm	(16)	4	2	2	2.4, NS[a]
>4 cm	(46)	19	5	14	
TNM stage					
I–II	(6)	2	1	1	2.09, NS
III–IV	(56)	21	6	15	
Level of differentiation					
Well	(23)	7	3	4	2.3, NS
Poor-moderate	(39)	16	4	12	
Nodal involvement					
N0	(29)	10	4	6	0.18, NS
N+	(33)	13	3	10	

[a] NS, not significant

The loss in heterozygosity of the H-*ras*-1 allele was not associated with any of the clinical parameters of the malignancy, such as size of the tumour, level of differentiation, nodal involvement and the TNM staging (Table 2).

Discussion

The H-*ras*-1 protooncogene is highly polymorphic, mainly because of a hypervariability in the length of the VTR region. Besides being associated with polymorphism of the H-*ras*-1 gene, the VTR region has been implicated in a regulatory role, acting as an enhancer element of the H-*ras*-1 gene (Spandidos and Holmes 1987). To date, the specific incidence of loss of H-*ras* heterozygosity has not been investigated in oral cancers. Our data, analysing H-*ras*-1 RFLP in oral cancer patients, demonstrated $^{23}/_{62}$ samples with heterozygous alleles on *Bam*HI digestion. The heterozygosity was better resolved and confirmed by *Pvu*II and *Taq*I analysis. We also observed 3 patients with a variant *Taq*I pattern, represented by additional 2.1-kb, 0.9-kb and 0.6-kb fragments. Such variant VTR fragments could be generated by either mutational events creating the *Taq*I sites, or reiteration of the existing *Taq*I site present 18 bp from the 3′ end of the VTR region during its amplification/duplication, as suggested by Pierotti and coworkers (Pierotti et al. 1986).

In the oral cancer patients, we observed tumor-associated loss of H-*ras*-1 heterozygosity in $^{7}/_{23}$ (30%) samples. *ras* family oncogenes are known to be activated by amplification or point mutation of the gene. Earlier data from our laboratory have shown amplification of N-*ras* and Ki-*ras* oncogenes in squamous cell carcinoma of the oral cavity (Saranath et al. 1989). The H-*ras*-1 oncogene, though not amplified in our patients, demonstrated high-frequency point mutations selectively, with Ki-*ras* and N-

ras showing no point mutations in codons 12, 13 and 61 (Saranath et al. 1991). An equal proportion, i.e. 2/5 with H-*ras* allelic loss, and 5/10 without the loss, demonstrated other modes of oncogene activation such as amplification/point mutation of *myc*/*ras*/EGFR. The chromosome 11 losses we observed were probably important for some stage of tumorigenesis in the cases in which they occurred. They seemed to be present in most of the cells of the tumour specimens and may have a selective advantage by allowing these cells to outgrow all other cells. Loss of heterozygosity for H-*ras*-1 has been associated with several human tumours (Klein 1987; Fearon et al. 1985; Ali et al. 1987; Lee et al. 1989). In addition, on chromosome 11, in the vicinity of the H-*ras*-1 locus, a putative suppressor gene has been recently localised between the β-globin and parathyroid hormone loci (Ali et al. 1987). Thus, the loss of the H-*ras*-1 allele, observed in our patients, may also encompass the functional loss of a suppressor gene on chromosome 11, further influencing the process of oral carcinogenesis in these patients. This envisages an earlier event involving inactivation of one chromosome 11 suppressor locus, followed by loss of chromosome 11 containing the normal suppressor locus. Loss of the chromosome 11 suppressor locus and its role in tumorigenesis will remain speculative, until the advent of studies revealing the molecular effect of such a loss. Alternatively, loss of a normal H-*ras*-1 gene may give a selective functional advantage to the mutated H-*ras*-1 involved in cell transformation. However, no correlation was observed between the loss of heterozygosity and the clinical parameters analysed. The loss of the H-*ras*-1 allele observed in our studies may be an underestimate, as the oral cancer patients constitutively homozygous at the H-*ras*-1 locus are not amenable to the RFLP analysis. Furthermore, depending on the contaminating normal stromal and inflammatory cells and PBC, the loss of the H-*ras*-1 allele in the tumour tissue may be masked.

In conclusion, the present data demonstrate loss of heterozygosity at the H-*ras*-1 locus, and VTR rearrangement in a high proportion (39%) of oral cancers. Along with our earlier data on the activation of *ras* oncogenes by amplification of N-*ras* and Ki-*ras* (Saranath et al. 1989), and point mutation of H-*ras* (Saranath et al. 1991), a crucial role for *ras* oncogenes in the process of oral carcinogenesis seems imperative. As no correlation was observed between the loss of the H-*ras*-1 allele and the advanced stages of the disease, the loss perhaps represents an early event in oral carcinogenesis.

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