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Detection and Cloning of Potent Transforming Gene(s) from Chewing Tobacco-related Human Oral Carcinomas

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High molecular weight DNA isolated from 14 primary tumour tissues of human oral carcinoma patients was analysed for transforming activity by NIH3T3 co-transfection assay using pSV2neo gene as a selectable marker, followed by nude mouse tumorigenicity assay. Ten of the patient tumour tissues demonstrated molecular lesions in *myc*, *ras* or/and EGF-R genes, whereas 4 patients did not show tumour associated aberrations in these oncogenes. The G418-resistant transfected cells from 12 of 14 individual patients demonstrated transforming potential by colony formation in soft agar and tumour induction in nude mice within 25–80 days. DNAs from the transfected cells, consequent nude mice tumours and corresponding cell lines, contained human Alu sequences. Southern blot hybridisation with *ras*, *myc*, EGF-R oncogenes demonstrated the presence of human H-*ras* oncogene in one of the 12 sets of nude mice tumours. In contrast, DNA from the other 11 sets of nude mice tumours indicated absence of c-*myc*, N-*myc*, L-*myc*, H-*ras*, K-*ras*, N-*ras* and EGF-R genes on Southern analysis. Further, DNAs from five first cycle tumorigenic transformants were subjected to a second cycle of transfection, and induced tumours in nude mice with a shorter latency period of 21–50 days. The secondary transformants contained discrete human Alu sequences; however, the DNA did not hybridise with *myc*/*ras*/EGF-R probes. A genomic library was constructed from a second cycle nude mice tumour, using EMBL-3 as the vector. Four human Alu sequence positive clones were isolated on screening 2×10^5 plaques, and one of the recombinant clones subjected to fine restriction mapping using 16 restriction enzymes. The lack of association of the nude mice tumour DNA with *myc*/*ras*/EGF-R showing aberrations in the primary human tumour, implies activation of an alternative potent transforming gene(s) in the chewing tobacco-related oral carcinomas in India.
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INTRODUCTION

ORAL CANCER is one of the ten most common malignancies in the world with the prevalence varying from 1 to 2% of total malignancies in Japan, U.K., U.S.A., to 30–40% in India, Bangladesh, Pakistan and Sri Lanka [1, 2]. There is unequivocal evidence that tobacco either chewed, dipped or even smoked, constitutes a major source of intraoral carcinogens on a global scale, and almost 92% of oral cancers are avoidable [2, 3]. The tobacco-specific carcinogens such as tobacco-specific nitrosamines and polycyclic hydrocarbons, interact with cellular macromolecules including oncogenes and tumour suppressor genes, to result in the malignant phenotype leading to oral cancer [3–5].

Previous studies from our laboratory investigating primary oral tumour tissue of long-term (10–30 year) habitual tobacco chewers in India, demonstrated multiple tumour-associated oncogene aberrations in more than 90% of the patients. The oral tumour tissues showed activation of *myc*/*ras*/EGF-R genes via amplification, rearrangement, point mutations and loss of allelic heterozygosity [5–11]. With a view to better defining the predominant oncogene in oral carcinogenesis, our current studies focused on detection and isolation of a functionally dominant oncogene in the oral tumours, using a NIH3T3 co-transfection assay with pSV2neo as a selectable marker, followed by induction of tumours in nude mice. Several oncogenes including *met*, *ret*, *trk*, *cot*, *axl* and *mas* have been isolated from solid tumours using this technology [12]. Although molecular aberrations in *myc*/*ras*/EGF-R oncogenes were associated with the primary tumour tissues, the

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potent transforming gene(s) from the nude mice tumours did not show homology to these oncogenes in Southern hybridisation analysis, with the exception of one set of nude mice tumours. Our studies indicate activation of potent transforming gene(s), primarily other than *myc*/*ras*/EGF-R, in the tobacco-induced oral cancers. At this stage two strategies could be used for identification of the potent oncogenes, comprising either hybridisation with the known oncogene probes available, which are about 100 in numbers, or the other alternative was to make a genomic library of the second cycle nude mice tumour, using the human Alu probe to screen the library. The gene could then be identified by nucleotide sequencing of the gene. We constructed a genomic library from a nude mouse tumour induced by a second cycle transfectant, and four human Alu sequence positive clones were isolated. One of the clones was subjected to fine restriction mapping, using 16 restriction enzymes. The recombinant clone and EMBL-3 restriction maps were compared and the human sequences localised in a recombinant clone.

MATERIALS AND METHODS

Patient sample

Primary oral cancer tissues (OCT) from patients with squamous cell carcinoma (SCC) were used in the study and taken from resections of the primary tumours. The patients were diagnosed as having SCC of the oral cavity based on clinical examination and histopathological features of the biopsy specimen. The patients showed poor, moderate or well differentiated carcinoma, and T2-T4, N0-N3 and M0 stages according to TNM staging [13].

The tissues were collected in liquid nitrogen and stored at −70°C until extraction of DNA.

Transfection assay

The transfection tumorigenecity assay was performed as previously described [14]. Briefly, cellular DNA (40 μg) and pSV2neo DNA (2 μg), were co-precipitated with calcium phosphate in 100 mm culture dishes seeded 24 h earlier with 5×10^5 NIH3T3 cells, in 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% calf serum (complete medium). After incubation for 15–18 h, the precipitate was removed and replaced by fresh medium. After an additional 24 h, the cells in each dish were trypsinised and seeded into two 100 mm dishes containing fresh complete medium. Twenty four hours later, the medium was replaced by complete medium containing 0.5 mg/ml of the antibiotic G418 (selective medium). The cultures were fed with selective medium every third day. After 14 days of growth, the cells from all the plates from a particular set of transfected DNA were trypsinised, pooled and assayed for transformation *in vitro* by soft agar cloning assay and tumorigenecity *in vivo* by nude mouse tumorigenecity assay.

Soft agar cloning assay

G418 resistant transfected cells were trypsinised and seeded at a density of 1×10^3 cells per 60 mm plates in triplicate, in 2 ml of complete medium containing 0.3% agar (Difco), layered on top of a preformed 0.6% agar-complete medium basal layer. After a 14-day incubation at 37°C in a 5% CO_2 humidified incubator, colonies comprising a minimum of 10 cells were scored.

Nude mouse tumorigenicity assay

OCT DNA transfected G418 resistant cells were assayed for their ability to induce tumours in nude mice. 5×10^6 cells were injected subcutaneously into J6(nu–nu) nude mice maintained at the National Institute of Virology, Pune. With every series of nude mice inoculation, one set of mice was inoculated with NIH3T3 cells transfected with cloned mutated H-*ras* plasmid pEJ6.6H-*ras* was used as a positive control. As a negative control, one set of mice was inoculated with NIH3T3 cells transfected with either calf thymus DNA or normal PBC DNA. The mice were monitored for tumour formation, and the second cycle of transfection-tumorigenicity was initiated from five individual patient DNA transfected nude mice tumours. Cell lines were established from both the first and second cycle nude mice tumours.

Southern blot hybridisation

DNA isolated from various nude mice tumours and cell lines was digested with *Eco*RI or *Bam*HI, at 37°C overnight. Aliquots of 10 μg of the digested preparations were electrophoresed and transferred to Nylon membranes (Hybond-N, Amersham, U.K.) by the method of Southern [15]. The *myc*/*ras*/EGF-R/Blur-8/pSV2neo probes used are outlined in Table 1 [16–24]. The filters were prehybridised for 12–18 h, at 42°C in 6 × standard saline citrate (SSC: 1 × SSC = 0.15 mol/l sodium chloride, 0.15 M sodium citrate), 50% formamide, 1% sodium dodecyl sulphate (SDS), sheared denatured salmon sperm DNA (100 μg/ml), followed by hybridisation for 20 h at 42°C in the same buffer containing ^{32}P-labelled probe. Filters were washed to a final stringency of 0.2 × SSC at 65°C and exposed at −70°C for 1–4 days.

Northern blot hybridisation

Twenty micrograms of total cellular RNA extracted by the method of Chomczynski and Sacchi [25] from the nude mice tumour were electrophoresed on a 1.2% formaldehyde–

Table 1. Hybridisation probes

Clone	Plasmid	Enzymes used for digestion	Size of probe (kb)	Reference
Human c-*myc* pMC41HE	pBR322	*Cla*I/*Eco*RI	1.5	16
Human N-*myc* p9D	Sp64	*Eco*RI	1.3	17
Human L-*myc*	pJB327	*Sma*I/*Eco*RI	1.8	18
Human H-*ras* pUCEJ 6.6	pUC	*Bam*HI	6.6	19
Human K-*ras* p640	pBR322	*Eco*RI/*Hind*III	0.64	20
Human N-*ras* p52C	pUC	*Pvu*II	1.6	21
EGF-R cDNA	pBR322	*Cla*I	2.3	22
Human Alu *Blur*-8	pBR322	*Bam*HI	0.3	23
pSV2neo	pUC18	*Eco*RI	6.3	24

Table 2. Clinicopathological data, oncogene profile and tumorigenecity potential of human oral cancer

Samples	Tumour type	Histological diagnosis	TNM staging	Oncogene aberrations*	Alu probe	Soft agar colony formation	Nude mice tumorigenecity animals with tumours/injected Nos. (%)	Tumour appearance (days)
OCT-1	Buccal mucosa	Moderately differentiated	$T_4N_0M_0$	N-*ras* amplification *Taq*I variant allelle of H-*ras*	+	+	2/4 (50)	56
OCT-2†	Buccal mucosa	Well differentiated	$T_4N_0M_0$	N-*ras* amplification	+	+	4/4 (100)	25
OCT-3†	Tongue	Moderately differentiated	$T_4N_0M_0$	—	+	+	2/6 (33)	80
OCT-4†	Buccal mucosa	Well differentiated	$T_4N_0M_0$	N-*ras* amplification H-*ras* 12.2 mutation H-*ras* LOH	+	+	2/2 (100)	35
OCT-5	Tongue	Moderately differentiated	$T_3N_1M_0$	N-*myc*, EGF-R amplification; H-*ras* LOH	+	+	0/3 (—)	—
OCT-6	Buccal mucosa	Moderately differentiated	$T_4N_0M_0$	—	+	+	3/3 (100)	49
OCT-7†	Tongue	Moderately differentiated	$T_4N_2M_0$	N-*myc*, N-*ras*, EGF-R amplification H-*ras* 12.2 mutation	+	+	2/3 (66)	56
OCT-8	Lower alveolus	Poorly differentiated	$T_4N_2M_0$	N-*myc*, c-*myc*, K-*ras*, N-*ras*, EGF-R amplification	+	+	1/2 (50)	77
OCT-9	Buccal mucosa	Poorly differentiated	$T_4N_2M_0$	c-*myc* amplification	+	+	0/2 (—)	—
OCT-10†	Lower alveolus	Moderately differentiated	$T_4N_1M_0$	N-*myc*, K-*ras*, N-*ras* amplification H-*ras* 61.3 mutation	+	+	2/3 (66)	35
OCT-11	Lower alveolus	Well differentiated	$T_4N_2M_0$	H-*ras* 12.2 mutation	+	+	2/2 (100)	25
OCT-12	Tongue	Moderately differentiated	$T_4N_1M_0$	—	+	+	2/2 (100)	49
OCT-13	Buccal mucosa	Moderately differentiated	$T_4N_1M_0$	—	+	+	1/2 (50)	35
OCT-14	Tongue	Moderately differentiated	$T_2N_0M_0$	H-*ras* 61.2 mutation	+	+	2/3 (66)	35
CT-1/ NPBC-1	—	—	—	—	—	+	2/20 (10)	77
H-*ras*-1 pEJ6.6	—	—	—	—	—	+	11/11 (100)	14–21

*Oncogene aberrations in patient primary oral tumour. Amplification of c-*myc*, N-*myc*, L-*myc*, K-*ras*, N-*ras*, H-*ras*, and EGF-R; H-*ras*: point mutation, loss of heterozygosity and *Taq*I variant RFLP.
†DNA extracted from primary nude mice tumours induced by OCT DNA was used in a second cycle of transfection-tumorigenecity.

agarose gel, and transferred to Nylon membranes according to standard methods [26]. Membranes were prehybridised, hybridised and washed by standard methods as described [26].

Molecular cloning

A genomic library from a second cycle transfection-tumorigenicity nude mouse tumour DNA-NMT2.2, was constructed in the phage vector EMBL3, as described [27]. EMBL3 was obtained from Dr S.K. Mahajan, Bhabha Atomic Research Centre, Bombay. Briefly, DNA was partially digested with Sau3A to obtain fragments in the range of 15–20 kb and ligated to *Bam*HI digested EMBL3 vector arms. The ligated DNA was packaged *in vitro* and transduced in LE392 competent cells. The recombinant library of 2×10^5 plaques was screened with ^{32}P-labelled Blur-8 probe. Positive clones were isolated, amplified and DNA extracted. Clone-I DNA was characterised by mapping with 16 restriction enzymes namely: *Bam*HI; *Eco*RI; *Sal*I; *Hin*d III; *Sma*I; *Ava*I; *Bgl*II; *Cla*I; *Pvu*II; *Kpn*I; *Pst*I; *Sph*I; *Pvu*I; *Xho*I; *Not*I and *Fok*I, using optimal conditions as stated by the manufacturers (Amersham).

RESULTS

Tumorigenic activity of human oral tumour DNA

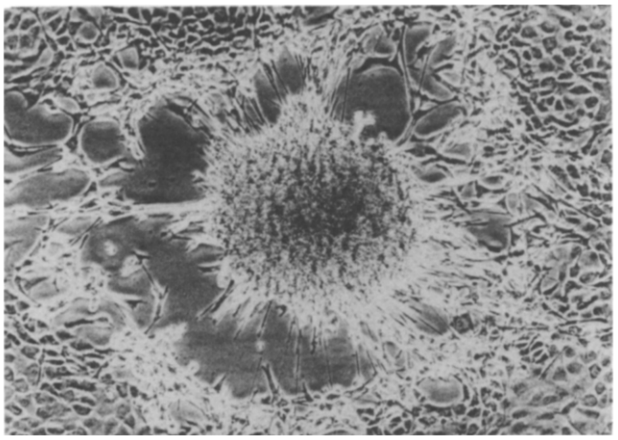
Co-transfection of NIH3T3 cells with oral cancer tissue (OCT) DNAs from 14 human oral cancer patients (clinical picture given in Table 2), normal peripheral blood cell (NPBC) DNA or calf thymus (CT) DNA, and the selectable marker pSV2neo DNA, yielded 200–400 G418 resistant colonies per 60 mm Petri dish. The colonies transfected with OCT or H-*ras* DNA, showed foci formation (Fig. 1), in contrast to CT or NPBC transfected cells which grew in monolayers. Southern analysis of DNAs prepared from all the primary OCT NIH3T3 transfectants using the Blur-8 probe, showed the presence of human Alu sequences as a smear ranging from 0.5 to 23 kb on autoradiography (data not shown).

Soft agar cloning of G418 resistant OCT DNA transfectants, showed 100–400 colonies per 60 mm plate on day 14, with an initial seed of 10^3 cells, thus indicating cloning efficiency of 10–40%. The colonies scored comprised a minimum of 10 cells (Fig. 2). On the other hand, CT DNA or NPBC DNA transfectants were present as single cells or small clumps of two to four cells and did not form colonies in soft agar. Whereas NIH3T3 cells transfected with pEJ H-*ras*6.6 plasmid formed soft agar colonies with high efficiency (50–60%) forming 500–600 colonies per Petri dish plate.

To detect the *in vivo* tumorigenic potential of oral cancer DNA, 5×10^6 G418 resistant OCT DNA transfectants were injected subcutaneously into nude mice. The transfectant DNAs from 12 of 14 (86%) individual patients induced progressively growing tumours in the nude mice by day 25 to 80 postinoculation in the first cycle tumorigenicity experiments (Table 2). The normal human PBC or CT DNA transfectants induced a low background of spontaneous tumours in two of 20 (10%) nude mice, whereas the mutated cloned H-*ras*, pEJ H-*ras*6.6, DNA transfectants, induced tumours in 11/11 (100%) nude mice within a period of 14–21 days (Table 2). Five of the primary OCT DNA transfectants which initiated nude mice tumours, were used in a second cycle of transfection-tumorigenicity. The second cycle transfectants induced tumours with a shorter latency period of 21–50 days. Cell lines were established from the induced nude mice tumours. Southern analysis of all the primary and secondary OCT DNA induced nude mouse tumours (NMT), and the corresponding established cell lines (NMT/CL), demonstrated the presence of several human Alu fragments (Fig. 3). Further, second cycle nude mouse tumours including NMT2.2 induced by OCT4 patient DNA (the patient data is given in Table 2), showed the presence of discrete *Eco*RI fragments. The NMT2.2 DNA demonstrated three discrete *Eco*RI fragments of 10 kb, 7.2 kb and 6.0 kb, also present in the first cycle of transfectant induced tumour (NMT2.1) (Fig. 3), from patient (OCT4) tumour DNA.

Relationship of the oral cancer transforming sequences with myc/ras/*EGF-R*

To determine the relationship between the tumorigenic sequences in the nude mice tumours and tumour associated oncogene aberrations observed in the patient, the first and second cycle nude mice tumours and the corresponding cell lines, were analysed for the presence of c-*myc*, N-*myc*, L-*myc*, H-*ras*, K-*ras*, N-*ras* and EGF-R genes by Southern analysis. The clinicopathological profile of the individual patients and the oncogene aberrations detected in the primary tumours are included in Table 2.



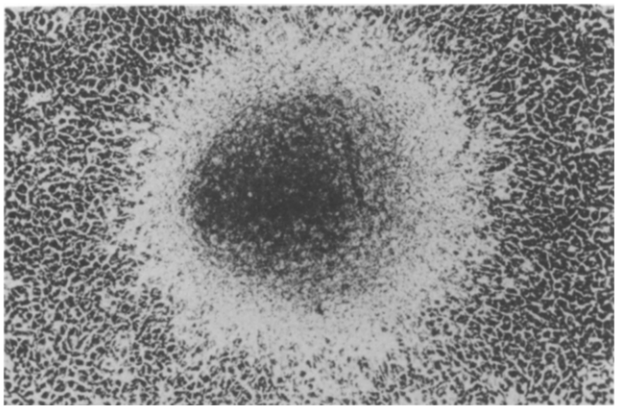


Fig. 1. Transformation of NIH-3T3 cells by plasmid pEJ6.6 H-*ras* or oral tumour DNA, cultured in G418 containing medium, in a SV2neo co-transfection assay. Phase contrast photographs showing foci formation.

Southern hybridisation analysis demonstrated the presence of a 7.8 kb *Bam*HI H-*ras* fragment (Fig. 4a), in one set of nude mice tumours (NMT4.1) induced by NIH3T3 transfectant of oral cancer origin from the series of 12 sets of patient tumour DNA induced nude mice tumours. Further, a 1.2 kb human H-*ras* specific transcript was also observed on Northern analysis of the particular nude mouse tumour (Fig. 4b). The other 11 sets of nude mice tumours and the corresponding cell lines did not hybridise with the oncogene probes used for screening, i.e. c-*myc*, N-*myc*, L-*myc*, H-*ras*, K-*ras*, N-*ras* or EGF-R in Southern analysis.

Genomic cloning of human oral tumour transforming sequences from second cycle nude mouse tumour

The second cycle nude mouse tumour NMT2.2 induced by OCT4 DNA, exhibited consistent presence of human Alu specific *Eco*RI fragments of 10 kb, 7.2 kb and 6.0 kb (Fig. 3), implying association of the transforming gene(s) in the nude mice tumour with human Alu repeat sequences. To isolate the OCT4 DNA derived transforming sequences, a genomic library from secondary nude mouse tumour NMT2.2 was constructed in EMBL-3 phage vector. A library of 2×10^5 recombinant phages was screened using Blur-8 probe, and four human Alu positive plaques were detected, isolated and amplified. Each of the clones contained an insert of about 14 kb. Clone-1 was further characterised by 16 restriction enzymes (Fig. 5a) and the restriction pattern compared with EMBL-3 vector (Table 3). *Sal*I digestion of clone-1 DNA characterised a unique *Sal*I site in the insert, generating 5.5 kb and 9.5 kb fragments (Fig. 5a). The recombinant Clone-1 DNA did not reveal restriction sites for *Pvu*I, *Xho*I and *Not*I on repeated digestion with the enzymes, and was digested to small fragments by *Fok*I enzyme. *Kpn*I enzyme digestion revealed the presence of a 27 kb fragment, which was due to partial digestion. The approximate fragment sizes of the various enzyme digests of both the recombinant clone-1 and the EMBL-3 vector are detailed in Table 3. As indicated, the EMBL-3 vector DNA was not cleaved by *Pvu*II, *Pvu*I, *Xho*I and *Not*I enzymes whereas *Fok*I enzyme digested the DNA to

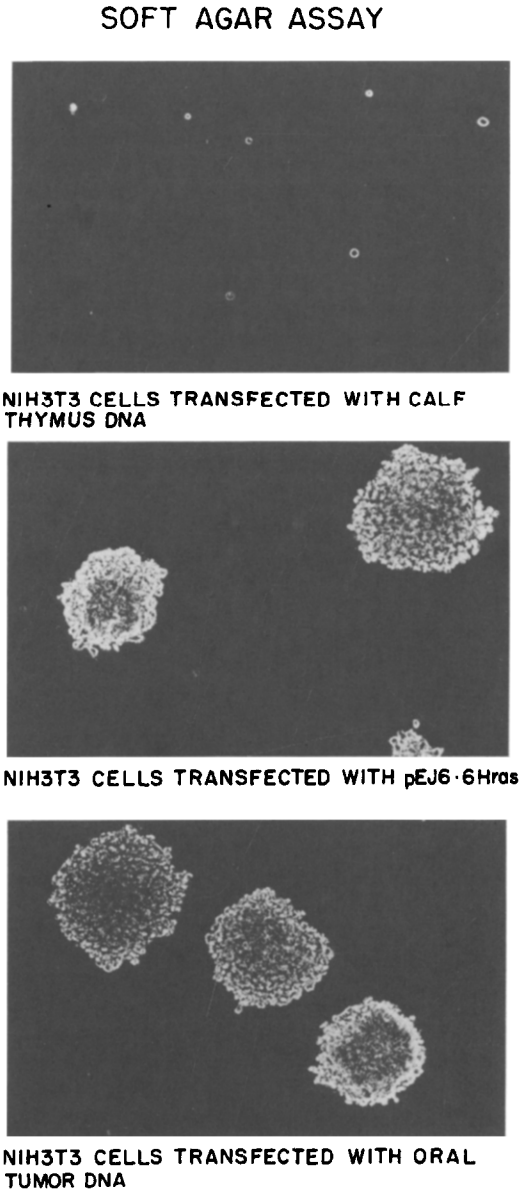


Fig. 2. Soft agar cloning assay showing presence of single cells of NIH-3T3 transfected with calf thymus (day 14); and large colonies of NIH-3T3 transfected with H-*ras* or OCT DNA (day 14). 10^3 cells were plated in DMEM medium with 10% FCS, in 0.3% agar, over a basal layer containing 0.6% agar.

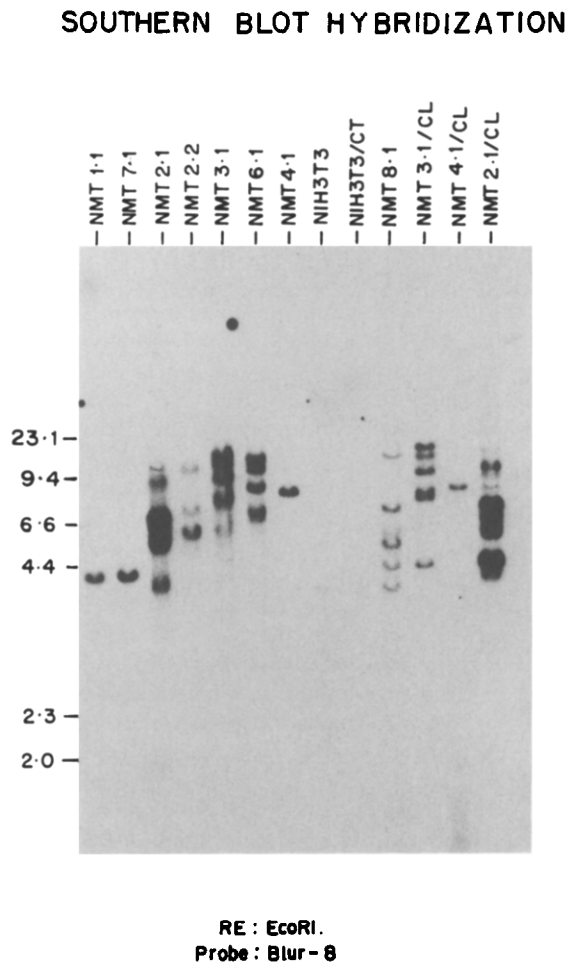


Fig. 3. Southern blot analysis of *Eco*RI digested DNA (10 µg) showing fragments containing human Alu sequences in first cycle nude mice tumours (NMT 1.1, 7.1, 2.1, 3.1, 6.1, 4.1, 8.1), and nude mice tumour cell lines (NMT3.1/CL, NMT4.1/CL, NMT2.1/CL); DNA (10 µg) from NIH3T3 cells or calf thymus. DNA transfected NIH3T3 cells do not show presence of human Alu sequences. The probe used was ^{32}P-labelled Blur-8.

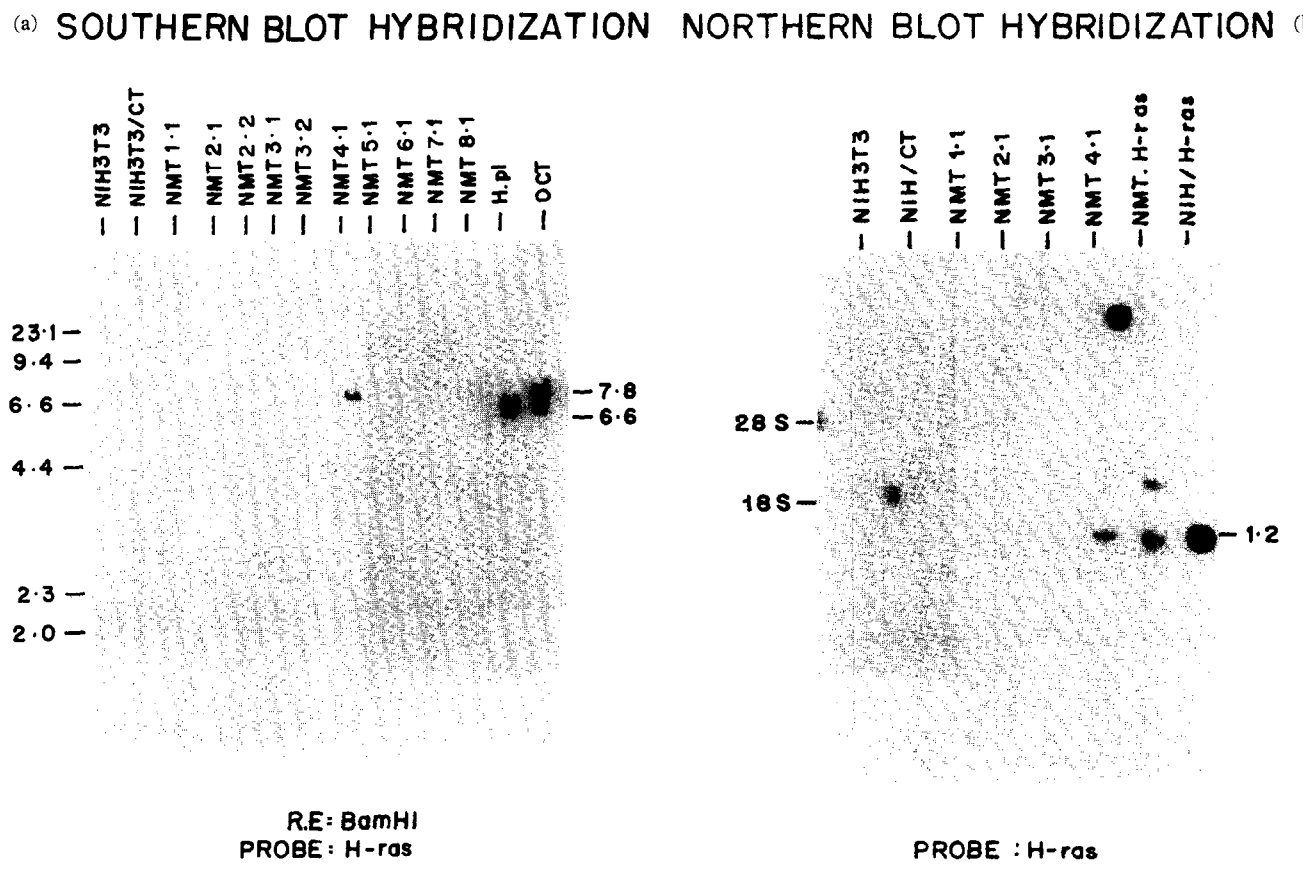


Fig. 4. (a) Southern blot analysis of *Bam*HI digested DNA (10 μg) of NIH3T3 cells, NIH3T3 transfected with calf thymus DNA (NIH3T3/CT), first cycle nude mice tumours (NMT1.1 to NMT8.1), second cycle nude mice tumours (NMT2.2 and NMT3.2), human placenta (H.pl) and oral cancer primary tumour (OCT). The probe was ^{32}P-labelled human H-*ras* plasmid pEJ6.6H-*ras*. (b) Northern blot analysis of total cellular RNA (20 μg) from NIH3T3 cells, NIH3T3 transfected with calf thymus (NIH/CT), H-*ras* (NIH/H-*ras*) and first cycle nude mice tumors (NMT1.1, NMT2.1, NMT3.1, NMT4.1, NMT H-*ras*). The probe was ^{32}P-labelled H-*ras* plasmid pEJ6.6 H-*ras*.

small fragments, and *Cla*I showed the presence of additional bands due to incomplete digestion.

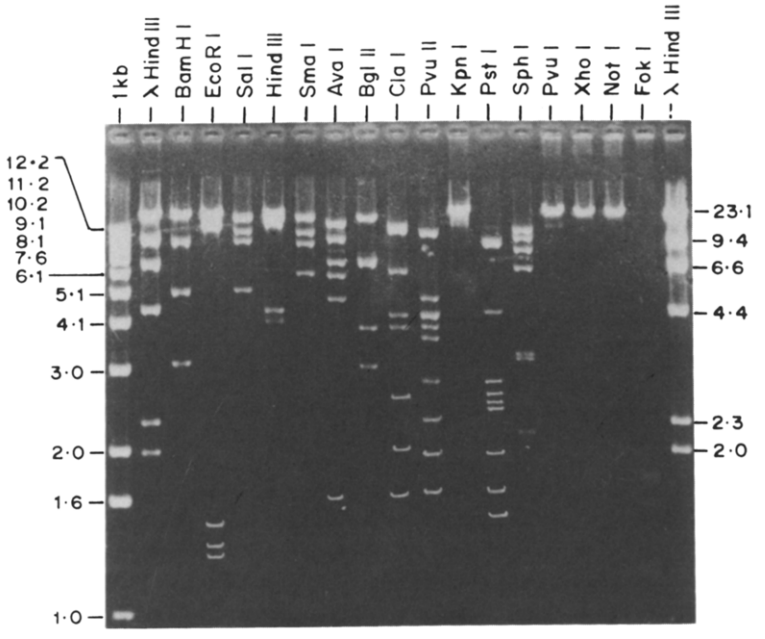
Southern analysis of the restriction fragments of the recombinant clone 1 using the Alu probe demonstrated the fragments containing Alu sequences (Fig. 5b). A restriction map of best fit according to the restriction enzyme digest fragment sizes, and Southern blot hybridisation with the Alu probe, indicated location of the Alu sequences towards the 3′ end of the insert (Fig. 6), with the Alu sequences flanked by *Kpn*I and *Bam*HI/*Pvu*II enzymes.

DISCUSSION

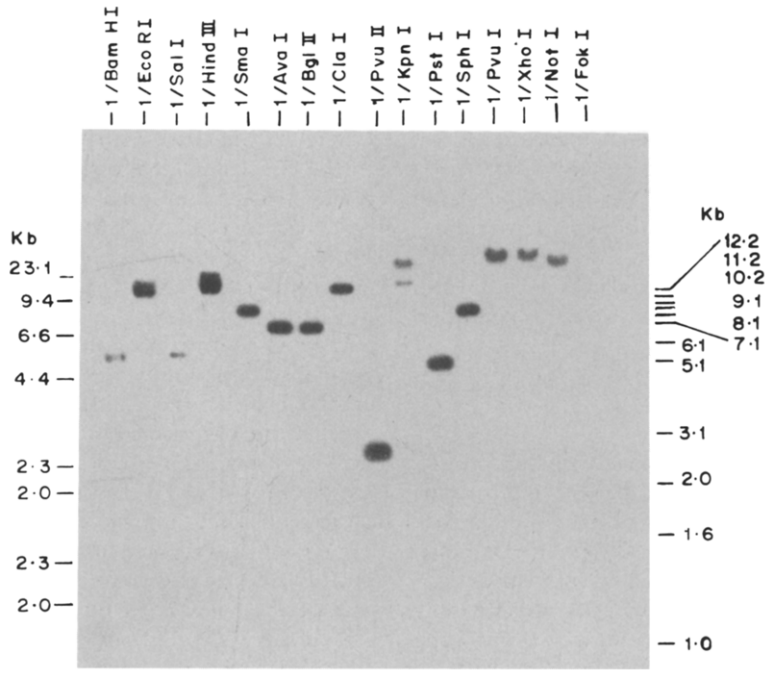
Earlier studies from our laboratory, investigating molecular aberrations in tobacco associated oral cancers in India, demonstrated that more than 90% of the oral tumours contained *myc*/*ras*/EGF-R oncogene lesions [5–11]. Dominant mutations leading to activation of cellular transforming genes have been induced by chemical carcinogens and efficiently transmitted by transfection [28]. Several activated oncogenes have been detected by foci formation on transfection of NIH3T3 fibroblasts with genomic DNA [12, 28]. However, this is an inefficient mode of DNA transfer with 80% of human tumour DNA ineffective in inducing transformed foci. Further, the assay has a bias towards *ras* oncogenes, being repeatedly detected by the focus assay. In the present study we have used the sensitive co-transfection assay in combination with the nude mouse tumorigenecity assay, and detected functionally activated oncogene(s) in primary oral tumour DNAs.

The oral tumour DNA transfected cells demonstrated colony formation in soft agar, indicating a malignant phenotype. Further evidence of the transforming ability of the OCT DNA transfected cells was provided by tumour formation in nude mice. Twelve of 14 (86%) individual patient oral tumour DNA transfected cells, demonstrated high efficiency transformation, as indicated by tumour induction within 25–80 days. Second cycle of transfection-tumorigenicity initiated from five of the Alu positive nude mouse tumours induced tumours with shorter latency periods of 21–50 days. This implied stable transfer and subsequent concentration of transforming sequences of OCT DNA origin. It was noteworthy that of the 12 patient DNAs capable of tumour induction, four of the primary tumour tissues represented tumours with no apparent *myc*/*ras*/EGF-R aberrations. Earlier studies from our laboratory had demonstrated tumour associated molecular aberrations in *myc*/*ras*/EGF-R oncogenes in greater than 90% of the long term tobacco habitues comprising our patient population. Our current studies used 10 of these patients and 4 patients with no apparent involvement of *myc*/*ras*/EGF-R [5, 11]. Hence, it was anticipated that one of the *myc*/*ras*/EGF-R oncogene would be identified as the predominant oncogene in the transfection-tumorigenicity assay. The nude mice tumours from one of the patient tumour (OCT-11) DNA with

(a) RESTRICTION ENZYME PATTERN OF CLONE-1



(b) SOUTHERN BLOT ANALYSIS OF RESTRICTION ENZYME DIGESTED CLONE-1 DNA



PROBE : Blur-8

Fig. 5. (a) Electrophoresis on 0.8% agarose gel of recombinant EMBL-3-Clone 1 DNA (2 μg), digested with different restriction enzymes. DNA molecular weight standards are marked on the left and right. The gel was viewed on a transilluminator after ethidium bromide staining. (b) Southern hybridisation analysis of the gel in (a), transferred onto Nylon membrane and probed with ^{32}P-labelled Blur-8 probe.

Table 3. Comparative restriction enzyme pattern of EMBL3 and clone-1

Enzyme	Fragment sizes EMBL-3	Fragment sizes Clone-1
*Bam*HI	20, 15, 9	25, 9.5, 5.5*, 3.5
*Eco*RI	20, 15, 9	26, 14*, 1.4, 1.35, 1.3
*Sal*I	20, 9, 9.5, 5.0, 0.5	20, 9.5, 9.0, 5.5*
*Hind*III	27, 5.8, 5.5, 4.4	20, 16*, 4.4, 4.0
*Sma*I	19.5, 13.5, 6.0, 4.5	19.5, 10.5, 8.0*, 6.0
*Ava*I	15, 10†, 8, 6.0, 4.5, 4.0, 2.6, 1.8, 1.6	15, 9.0, 7.0*, 6.0, 4.5, 1.6
*Bgl*II	26, 7.0, 5.5, 2.0, 1.2, 0.7, 0.3	23, 7.0*, 7.0, 3.8, 3.0
*Cla*I	13/8.0†, 6.5, 6.0, 4.2, 3.9, 3.0†, 2.5, 2.1, 2.0, 1.7, 0.8	12, 11*, 6.4, 4.3, 3.9, 2.5, 2.1, 1.7
*Pvu*II	Uncleaved†	10.5, 5.2, 4.5, 4.5, 4.0, 3.8, 2.7, 2.4*, 2.0, 1.7, 1.5
*Kpn*I	25.5, 17, 1.5	27†, 17, 14, 12*, 1.5
*Pst*I	10, 9.5, 4.2, 3.0, 2.8, 2.7, 2.6, 1.9, 1.7, 1.2, 1.1, 0.5	10, 9.5, 5.0*, 4.5, 2.8, 2.7, 2.65, 2.6, 2.0, 1.7, 1.5
*Sph*I	25, 10, 6.5, 2.3	11, 10, 8.0*, 6.3, 3.6, 3.5, 2.2
*Pvu*I	Uncleaved	Uncleaved
*Xho*I	Uncleaved	Uncleaved
*Not*I	Uncleaved	Uncleaved
*Fok*I	Digested to small fragments	Digested to small fragments

*Fragments positive for human Alu.
†Fragments arising due to incomplete digestion of DNA.

H-*ras* codon 12.2 mutation in the primary tumour tissue of the patient, demonstrated H-*ras* as a 7.8 kb *Bam*HI fragment on Southern analysis, and a 1.2 kb H-*ras* transcript was observed on northern analysis. Interestingly, the original human oral tumour had contained a 6.6 kb *Bam*HI H-*ras* fragment, as compared to the 7.8 kb fragment detected in the nude mouse tumour. This suggests a possible post-transfection H-*ras* rearrangement or the alternative of single nucleotide insertion/deletion resulting in loss of the *Bam*HI site in H-*ras* gene during transfection or post-transfection. Rearranged H-*ras* fragments have been detected in the NIH3T3 cells transformed by DNA from skin cancers [29]. Alternatively, the increased size of the H-*ras* fragment in OCT11 may represent integration with NIH3T3 DNA, with the probe detecting pEJ6.6 DNA and flanking cellular sequences.

Several transforming genes with no known homology to viral genes, including *hst* [30], *met* [31], *trk* [32], *raf* [33], *axl* [34], *cot* [35] have been detected and isolated from tumours using transfection-tumorigenicity assays, followed by cloning of the gene. Interestingly, two of these oncogenes, *met* and *raf*, were isolated from chemical carcinogen induced tumours, indicating activation of oncogenes due to the carcinogen. Recently, a putative dominant transforming gene, as yet unidentified, was detected in methyl nitroso-urea (MNU) induced rat colon tumours, using the transfection-tumorigenicity assay [36]. Although, MNU is known to induce *ras* mutations [37], the activated oncogene detected in the rat colon tumours was not related to such identified oncogenes as *ras*, *raf*, *neu*, *met* or *hst*, indicating involvement of an alternative dominant oncogene.

In our studies, with the exception of one of the 12 OCT DNA induced nude mouse tumours which demonstrated the presence of H-*ras*, the other 11 sets of tumours demonstrated the presence of Alu associated transforming sequences not related to either L-*myc*, N-*myc*, c-*myc*, H-*ras*, N-*ras*, K-*ras* or EGF-R. Of the 14 patient OCT DNAs tested in the transfection-tumorigenicity assay, five had point mutations in H-*ras*, besides alterations in *myc* and EGF-R. Five additional samples showed involvement of c-*myc*, N-*myc*, N-*ras*, K-*ras* and EGF-R. Interestingly, none of these genes could be detected as the transforming genes in the tumorigenicity assay, indicating the presence of an alternate potent oncogene. It is possible that this alternative gene could be related to other identified oncogenes or an alternative oncogene specifically associated with the chewing tobacco induced oral cancer. To identify the transforming gene in our patient group with oral cancer, two alternative approaches are possible. The first comprises Southern hybridisation analysis with all the identified oncogenes, which to date, number around 100. We have checked the transforming gene homology with the seven oncogenes, c-*myc*, N-*myc*, L-*myc*, H-*ras*, K-*ras*, N-*ras* and EGF-R, activated by amplification, point mutation, rearrangement or deletion in the primary tumours. The other approach to identify the transforming gene is to clone the gene, further sequence the gene and analyse DNA sequence homology with identified gene sequences. Hence, we constructed a genomic library from one of the second cycle nude mice tumour. We have obtained a 15 kb insert and have subjected it to fine restriction mapping. We are currently testing the recombinant clone restriction fragments in transfection/tumorigenecity assays.

Studies by Reynolds and co-workers have reported activated oncogenes in 11 of 13 (85%) lung tumour DNA from smokers, by the transfection assay [38]. The authors detected activated *ras* oncogenes in eight of the tumours; further c-*raf* and an as yet unidentified transforming gene were detected in two of the lung tumour DNAs. In our studies, tumour DNA from oral cancer patients with 10–20 years of tobacco chewing history, demonstrate comparable high incidence of transformation (12/14, 86%) in the transfection-tumorigenicity assay. It is likely that with the long term tobacco insult to the cells in the habitues, tobacco specific carcinogens may activate a specific gene, other than *myc*/*ras*/EGF-R, capable of efficient transformation in the complex multistep process of oral carcinogenesis.

Restriction mapping of the cloned DNA from the nude mouse tumour genomic library using EMBL-3 vector and human Alu sequences for screening, identified a 5.5 kb *Sal*I fragment which was Alu positive, and 9.5 kb *Sal*I fragment which was Alu negative. The Alu negative cloned DNA fragment (9.5 kb *Sal*I) may be used as a probe to detect homologous fragments in human oral tumours, putative premalignant lesions and normal tissue DNA, to reveal the role of the gene in oral carcinogenesis. Further identification of the gene from a cDNA library using the genomic cloned fragments as probes for screening the library, followed by sequencing, will allow precise identification of the predominant oncogene(s), and should provide a better insight into the role of the transforming gene in various stages of oral neoplasia. Besides, the status of the transforming gene in putative premalignant lesions such as leukoplakia, will indicate the probable use of the gene as a prognostic marker. Parallel studies to identify the smallest fragment of the cloned

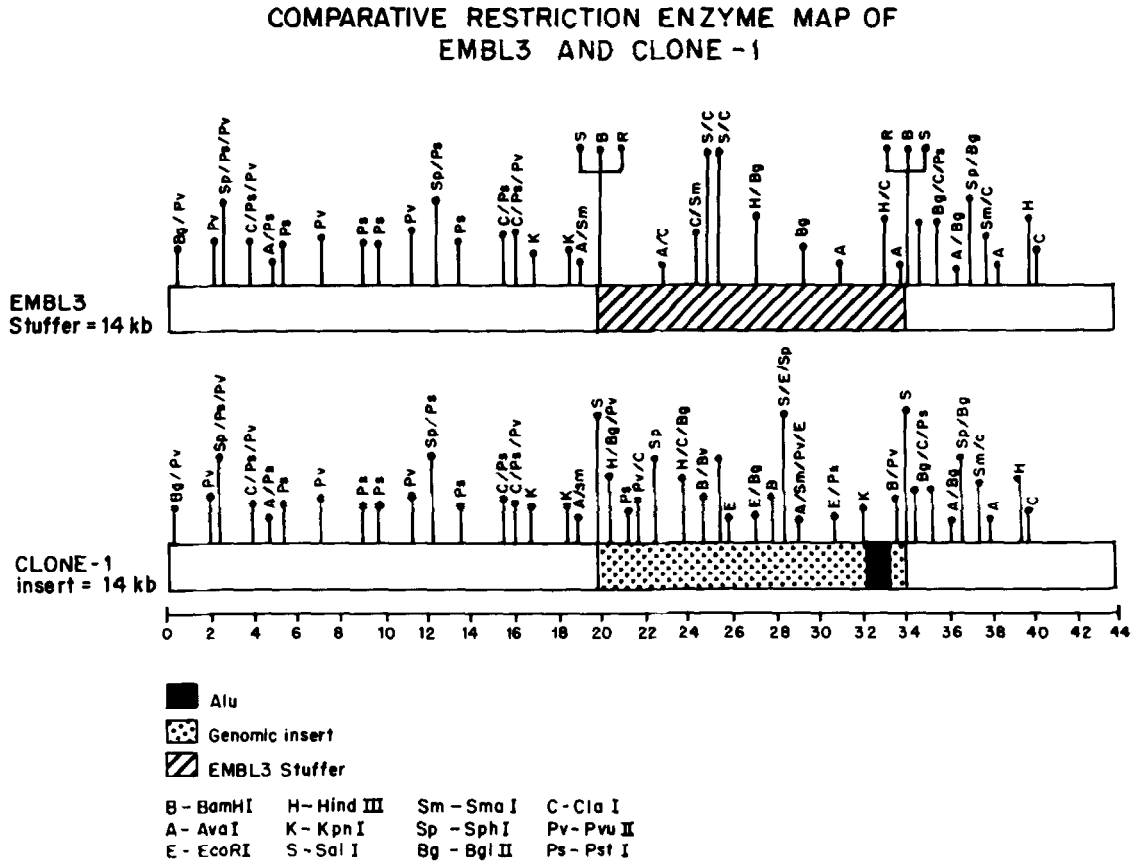


Fig. 6. Restriction enzyme map of recombinant EMBL-3-Clone 1, schematically represented, and a comparative map of the vector EMBL-3 obtained after RE digestion at our laboratory.

gene from the genomic library by transfection of the cloned gene and various restriction fragments in a NIH3T3 assay, followed by tumour induction in nude mice, are in progress.

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