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Oncogene Amplification in Squamous Cell Carcinoma of the Oral Cavity

Dhananjaya Saranath,[1] Rekha G. Panchal,[1] Rama Nair,[1] Ashok R. Mehta,[2] Vikram Sanghavi,[2] Janos Sumegi,[3] George Klein[3] and Madhav G. Deo[1,4]

[1]*Cell & Developmental Pathology Division, Cancer Research Institute and* [2]*Tata Memorial Hospital, Tata Memorial Centre, Bombay-400012, India and* [3]*Department of Tumor Biology, Karolinska Institute, S-10401, Stockholm, Sweden*

We have determined the prevalence of amplification of c-*myc*, N-*myc*, L-*myc*, H-*ras*, Ki-*ras*, and N-*ras* oncogenes in 23 cases of squamous cell carcinoma of the oral cavity, using Southern hybridization analysis of DNA extracted from the primary tumor tissues. Nick-translated oncogene probes and oncogene inserts labeled to high specific activities were used. We observed a 5- to 10-fold amplification of one or more of c-*myc*, N-*myc*, Ki-*ras* and N-*ras* oncogenes in 56% of the tumor tissue samples, with these oncogenes not being amplified in the peripheral blood cells of the same patients. L-*myc* and H-*ras* were not amplified in any of our samples. The oncogene amplifications seemed to be associated with advanced stages of squamous cell carcinomas, with the *ras* and *myc* family oncogenes being amplified in stages 3 and 4. Hybridization with N-*myc* detected an additional 2.3 kb *Eco*RI fragment, along with the normal 2.1 kb fragment. Our data also demonstrated amplification of multiple oncogenes in the same tumor tissue sample. About 60% of the samples with amplified oncogenes showed simultaneous amplification of 2 or more oncogenes. The results showing different oncogene amplifications in similar tumors, as well as multiple oncogene amplifications in the same tumor, suggest that these oncogenes may be alternatively or simultaneously activated in oral carcinogenesis.

Key words: Oncogene — Amplification — Oral cancer

Cancer of the oral cavity is one of the major cancer types in India, comprising about 40% of the total cancer incidence in India,[1] in contrast to 2–4% of the total of malignant tumors in Western society.[2] Epidemiological and experimental evidence indicates a causal relationship between chewing tobacco and oral cancer.[3,4] However, the molecular basis of cancer of the oral cavity is still an enigma.

In the last decade, oncogenes have been implied to play a critical role in various stages of human tumors. The proto-oncogenes in normal cells may be activated and contribute to neoplastic transformation through point mutations, translocation, deletions, amplification or other genetic mechanisms.[5] Gene amplification represents an important adaptive mechanism allowing selective increased expression of genes, whose products are needed by the cell.[6] Oncogene amplification is thought to be an important mechanism of aberrant oncogene expression, contributing to tumorigenesis.

Amplification of several oncogenes, including c-*myc*, c-*myb*, c-*ras*^{Ha}, c-*abl*, c-*erb B*, N-*ras* and N-*myc* has been documented, mainly in established tumor-derived cell lines.[7] There is also evidence showing amplification of oncogenes in primary and metastatic human tumors, linking this phenomenon to actual *in vivo* tumor development.[8–10] To date, the oncogene picture, including amplification of oncogenes, has not been investigated in oral cancers. With a view to studying the altered molecular events during oral carcinogenesis and elucidating the involvement and role of oncogene amplification in oral cancers, we have examined the oncogene profile in human oral cancer primary tumor tissues.

MATERIALS AND METHODS

Patients Twenty-three patients (16 males and 7 females), diagnosed as having primary squamous cell carcinoma (SCC) of the oral cavity, aged 35 to 60 years, were selected for the studies. The diagnosis was based on clinical examination and histologic features of the biopsy material. The majority of the patients under investigation had carcinoma of the buccal mucosa (14 patients), four patients showed carcinoma of the tongue and three patients showed carcinoma of alveolus, while two patients had carcinoma of the floor of the mouth (Table II). The staging was done according to the TNM classification (UICC, 1978), and patients with T_1 to T_4, N_0–N_3 and M_0 tumors, who were acceptable for surgery, were investigated. For each case, the portion of the tumor chosen as a sample was resected near the advancing edge of the tumor, avoiding the more necrotic center. As soon as the surgical specimens were available, the samples were minced, washed extensively in 0.1 *M* phosphate-buffered saline pH 7.2, homogenized and stored at −70°C, until used for DNA extraction. To check for the association of amplification of the oncogenes with the

[4] To whom correspondence should be addressed.

Table I. Cloned Genes Used as Hybridization Probes in the Analysis of *Eco*RI-digested Oral Cancer DNA

Genes	Enzymes for digestion	Size of probe (kb)	Size of cellular genome fragment detected[a] (kb)	References
Human c-*myc* pMc41HE	*ClaI*/*Eco*RI	1.5	12.7	14
Human Ki-*ras* p^{640}	*Eco*RI/*Hin*dIII	0.64	2.8	15
Human N-*myc* p9D	*Eco*RI	1.3	2.1, 2.3	16
N-*ras* p^{52c+}	*Pvu*II	1.6	7.2	17
Human H-*ras* pUC EJ6.6	*Bam*HI	6.6	30.0	18
L-*myc*	*SmaI*/*Eco*RI	1.8	10, 6.6	19

a) Cellular fragment size detected in the oral cancer *Eco*RI-digested cellular DNA.

tumor tissue, peripheral blood cells (PBC) were also obtained from the oral cancer patients on admission to the wards for surgery; PBC from healthy volunteers, normal human placenta and a multinodular goiter were also included as controls.

DNA biochemistry and hybridization analysis DNA was extracted from the tumor tissues and the cells by established methods.[11] After complete digestion with *Eco*RI (BRL), DNAs were run through agarose gels in TBE, and blotted onto Nylon membranes (Hybond-N, Amersham), according to the method of Southern.[12] DNAs were fixed by UV cross linking, and hybridized according to established procedures,[12] against both nick-translated oncogene probes labeled to specific activities of $1\text{–}5\times10^8$ cpm/μg[12] and gel-purified oncogene inserts labeled to high specific activities (1×10^9 cpm/μg) with [^{32}P]dCTP, using the method of Feinberg and Vogelstein.[13] After washing to a final stringency of $0.2\times$SSC at 65°C, the filters were exposed to Kodak X-Omat X-ray films with intensifying screens for a period of 3–5 days. The DNA probes used in the study are detailed in Table I.[14–19]

RESULTS

Oncogene amplifications In our preliminary experiments, we had observed no amplification of H-*ras* and L-*myc* oncogenes. Hence, the H-*ras* and L-*myc* oncogenes served as internal controls for the amount of DNA available for hybridization on the filters. After stripping the blot of radioactivity, the same blot was probed with a minimum of 4 oncogenes. The degree of hybridization was thus standardized and evaluated by densitometric scanning. Figures 1 to 6 are representative pictures of the same samples. As a threshold for amplification, we set a level of 5 copies of the gene per genome. Using this criterion, the c-*myc*, N-*myc*, L-*myc*, H-*ras*, Ki-*ras* and N-*ras* oncogenes were not amplified in normal human placenta, normal leucocytes or multinodular goiter. We also did not observe amplification of any of the six oncogenes in the PBC DNAs extracted from the oral cancer patients. In contrast, 13/23 (56.5%) of the oral cancer tumor tissues showed amplification of one or more of the oncogenes examined, with the other ten samples (43.5%), showing no amplification of the oncogenes examined (Table II); 5/23 (22%) tumor tissue samples contained a single amplified oncogene, and the other 8/23 (35%) samples showed amplification of two or three oncogenes in the same tumor tissue samples (Table II). The amplification of the oncogenes was not associated with either the size of the tumor as shown by the actual T value for each tumor, or the stage of differentiation of the tumor tissue. However, a comparison of amplification of the *ras* and *myc* family oncogenes with the TNM staging (Table III) demonstrated that the *ras* and *myc* oncogenes were amplified in stages 3 and 4 SCC. Further, about half (8/17) of the stage 4 cases had N-*myc* amplification. Amplification of the oncogenes was observed in the tumor tissues obtained from different sites in the oral cavity, with the exception of the floor of the mouth. The two tumors from the floor of the mouth were moderately differentiated and small in size (T_2), and these did not show amplification of any of the six oncogenes studied.

Amplification of *myc* family oncogenes c-*myc* was amplified 6-fold in 4/23 (17%) of the primary tumor tissues (Table II and Fig. 1), whereas 9/23 (39%) samples showed a 5- to 10-fold amplification of the N-*myc*

oncogene (Table II, Fig. 2a). The L-*myc* was not amplified in any of the tumor tissues (Table II, Fig. 3). Neither the 10 kb nor the 6.6 kb L-*myc* specific allele was associated disproportionately with the oral tumors. PBC from the oral cancer patients and normal donors, human placental DNA or multinodular goiter DNA did not show amplification of any of the *myc* family oncogenes.

An interesting observation was an additional 2.3 kb N-*myc* specific *Eco*RI fragment, besides the 2.1 kb band, in our samples. A single oral cancer tissue with N-*myc* not amplified exhibited distinct 2.3 and 2.1 kb fragments of equal intensity. Five samples showed only the 2.3 kb band, whereas the other 17 samples showed both fragments, with a stronger 2.3 kb signal. Amplification was associated with the 2.3 kb fragment. Non-amplified 2.3 kb N-*myc* specific fragment was observed in our control samples as well. Figure 2b shows the non-amplified 2.1 and 2.3 kb N-*myc* fragments in the PBC DNA from our oral cancer patients, and the 2.1 N-*myc* specific fragment from normal caucasian placental DNA (from Prof. George Klein's laboratory, Department of Tumor Biology, Karolinska Institute, Sweden).

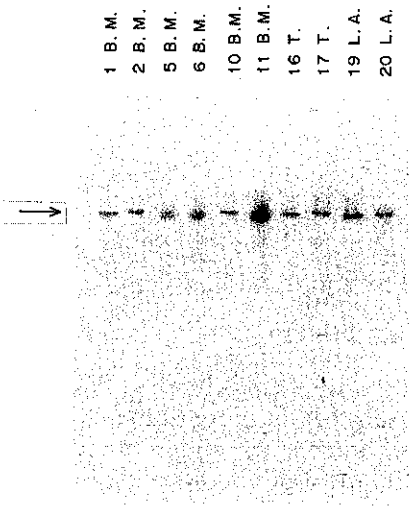

Fig. 1. Southern analysis of genomic DNAs from human oral cancer primary tumor tissues. Lanes 1–6, buccal mucosa; lanes 7 and 8, tongue; lanes 9 and 10, lower alveolus; digested with *Eco*RI (10 μg of DNA was loaded in each lane). The arrow indicates 12.7 kb c-*myc* specific fragment.

Amplification of the *ras* family oncogenes It was found that 7/23 (30%) of the tumor tissue samples showed a 5- to 10-fold amplification of the 7.2 kb *Eco*RI N-*ras* specific fragment (Table II, Fig. 4). Four (17%) oral cancer tissues exhibited a 5-fold amplification of Ki-*ras* oncogenes (Table II, Fig. 5), whereas H-*ras* was not amplified in any of the samples studied (Table II, Fig. 6).

Multiple oncogene amplification Of the samples showing amplification of oncogenes (13), more than half (8/13) showed simultaneous amplification of two or three of the oncogenes (Table II). Thus, 61.5% of the samples showing amplification of oncogenes exhibited multiple oncogene amplification with two or more oncogenes amplified

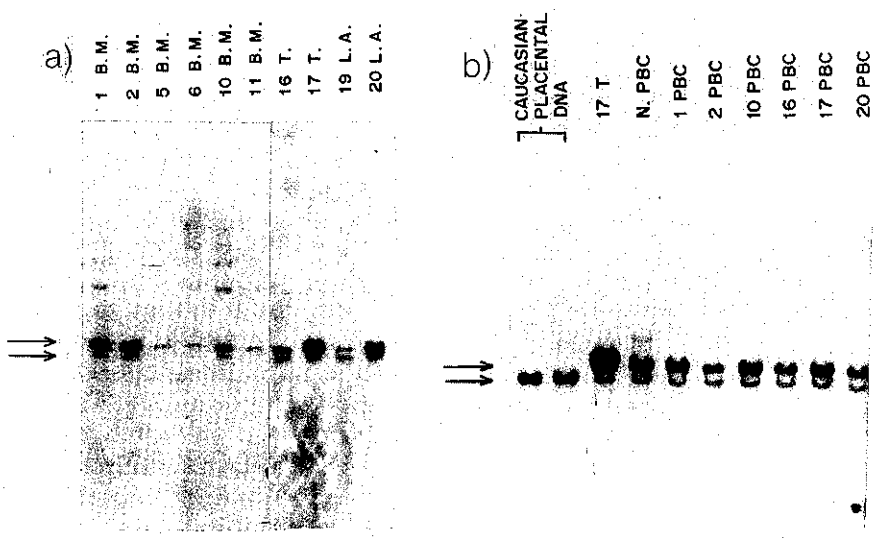

Fig. 2. a) Same as in Fig. 1. The lower arrow indicates 2.1 kb and the upper arrow 2.3 kb N-*myc* specific fragments. b) Lanes 1,2, Caucasian human placenta; lane 3, No. 17 oral tumor tissue; lane 4, normal PBC; lanes 5–10, PBC from oral cancer patients numbers 1, 2, 10, 16, 17 and 20 of Table II.

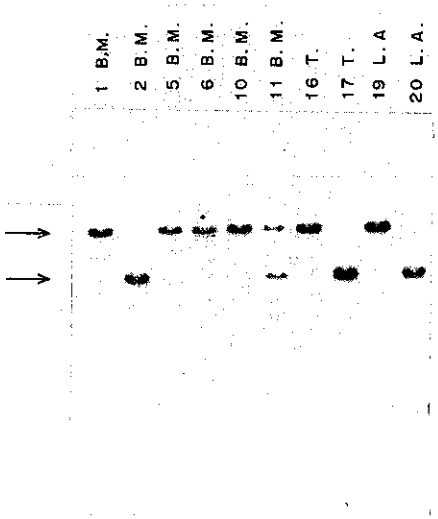

Fig. 3. Same as in Fig. 1. The lower arrow indicates 6.6 kb, and the upper arrow 10.0 kb L-*myc* specific fragments.

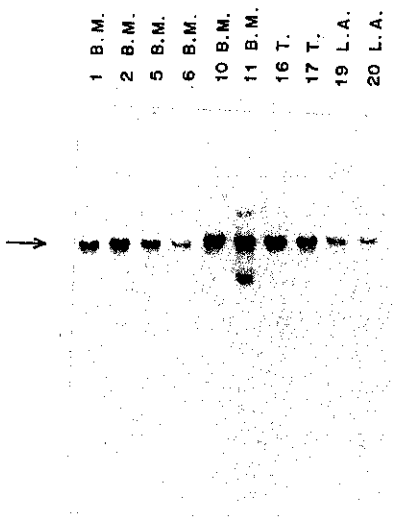

Fig. 4. Same as in Fig. 1. The arrow indicates 7.2 kb N-*ras* specific fragment.

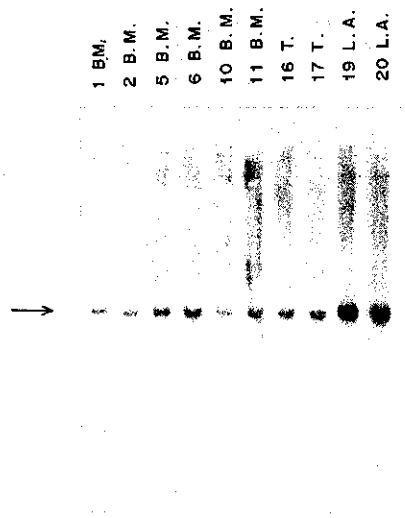

Fig. 5. Same as in Fig. 1. The arrow indicates 2.7 kb Ki-*ras* specific fragment.

in the same tumor tissue. We observed amplification of c-*myc* and N-*myc* oncogenes in three of our tumor tissue DNAs. Four tumor tissues showed concurrent amplification of N-*myc* and N-*ras*. N-*myc* and Ki-*ras* were also coamplified in two tumor tissues. Three samples showed a simultaneous amplification of three oncogenes, with one sample showing c-*myc*, N-*myc* and N-*ras* coamplified, one tumor tissue showing simultaneous amplification of N-*myc*, N-*ras* and Ki-*ras*, and one sample showing c-*myc*, N-*myc* and Ki-*ras* amplified.

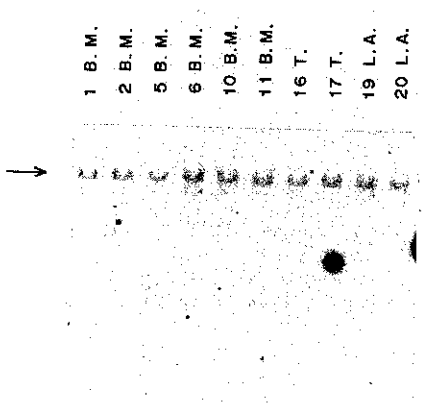

Fig. 6. Same as in Fig. 1. The arrow indicates 30 kb H-*ras* specific fragment.

DISCUSSION

Amplification of oncogenes has been shown to play a critical role in several human tumors. Oncogene amplification has been correlated with progression of malignancy, aggressive biological behavior of the tumor and poor response to therapy.[5, 7, 8–10] Thus, it has important implications in the diagnosis and treatment of cancer patients. With a view to investigating the involvement of oncogenes in oral cancers, we have examined the amplification of six oncogenes in primary tumor tissues from the oral cavity. The results clearly indicate a 5- to 10-fold amplification of c-*myc*, N-*myc*, N-*ras* and Ki-*ras* oncogenes in 20–40% of the samples examined, with 56% of the oral cancer tissues showing at least one of the oncogenes amplified. Cytogenetic evidence of gene amplification has been reported in less than 10% of the squamous cell carcinomas that have been karyotyped. The frequency of amplification in our samples was high, albeit the level of amplification was low, and might not be detectable as cytogenetic aberration on karyotyping by conventional banding techniques. L-*myc* and H-*ras* oncogenes were not amplified in the tumors. Karyotyping of short-term primary cultures from oral tumor tissues obtained from Tata Memorial Hospital did not exhibit polyploidy (A. Bhisey, personal communication). Besides, N-*ras* oncogene (amplified in 30% of our patients) and the single copy L-*myc* oncogene seen in the same samples are both present on the same chromosome 1. We observed multiple oncogene amplification of 2–3 oncogenes in 35% of the total samples studied. The amplification of the oncogenes in the oral cancers was not associated with

Table II. Amplification of Oncogenes in Squamous Cell Carcinoma of the Oral Cavity[a]

Sources of DNA & Histological diagnosis[b]	Sample[c] No.	TNM Classification	c-*myc* (12.7 kb)	N-*myc* (2.3 kb)	L-*myc* (10.0, 6.6 kb)	H-*ras* (30 kb)	Ki-*ras* (2.7 kb)	N-*ras* (7.2 kb)
Buccal mucosa								
Well differentiated	1 BM	$T_4N_1M_0$	1.0	6.9	1.0	1.0	1.0	3.0
	2 BM	$T_3N_1M_0$	1.0	6.9	1.0	1.0	1.0	5.5
	3 BM	$T_4N_0M_0$	1.7	2.7	1.0	0.9	1.2	1.3
Moderately differentiated	4 BM	$T_4N_2M_0$	6.0	9.3	1.0	0.9	0.9	2.4
	5 BM	$T_4N_2M_0$	1.5	1.0	0.9	0.9	2.2	3.0
	6 BM	$T_4N_2M_0$	1.8	1.0	0.9	1.0	2.6	1.4
	7 BM	$T_4N_1M_0$	1.1	1.3	1.0	1.0	2.0	1.0
	8 BM	$T_4N_1M_0$	5.7	5.9	0.9	1.1	3.7	6.6
	9 BM	$T_4N_1M_0$	1.0	6.8	1.0	1.0	5.1	5.9
	10 BM	$T_3N_0M_0$	1.0	2.7	1.0	1.0	0.7	6.6
	11 BM	$T_3N_0M_0$	6.0	1.0	0.8	1.0	2.0	6.8
	12 BM	$T_2N_0M_0$	1.0	1.2	1.0	1.0	1.1	1.0
Poorly differentiated	13 BM	$T_4N_3M_0$	1.0	2.1	1.0	1.1	0.9	1.0
	14 BM	$T_4N_0M_0$	2.0	7.9	1.0	1.0	1.1	1.2
Tongue								
Moderately differentiated	15 T	$T_2N_3M_0$	1.2	0.9	1.0	1.0	1.0	1.1
	16 T	$T_2N_2M_0$	1.3	3.0	1.0	1.0	2.2	6.6
	17 T	$T_2N_2M_0$	1.3	9.3	1.1	1.0	2.0	5.8
	18 T	$T_4N_1M_0$	5.9	5.0	0.9	1.0	5.5	1.2
Lower alveolus								
Well differentiated	19 LA	$T_4N_3M_0$	3.0	1.4	1.1	1.0	5.8	1.6
Moderately differentiated	20 LA	$T_4N_2M_0$	1.3	7.9	1.0	0.9	5.8	0.9
	21 LA	$T_4N_1M_0$	1.0	1.6	1.0	1.0	0.9	3.6
Floor of the mouth								
Moderately differentiated	22 FM	$T_2N_0M_0$	1.1	1.0	1.0	1.1	1.7	2.9
	23 FM	$T_2N_0M_0$	1.2	3.4	1.1	1.0	1.3	1.9

a) The autoradiograms of Southern blots using nick translated oncogene-plasmid probes or oligonucleotide-labeled oncogene inserts were scanned. H-*ras*/L-*myc* signals, and signals in control DNA were used as standards for a single copy of the oncogenes. The tumors showing 5-fold or more increase in signal intensity were considered amplified.
b) Histological examination was carried out in part of the specimen, the remaining tissue being used for DNA extraction.
c) BM, buccal mucosa; T, tongue; LA, lower alveolus; FM, floor of mouth.

Table III. Relationship between Clinical Staging and Oncogene Amplification

TNM stage	No. of patients	No. of patients with oncogene amplification			
		c-*myc*	N-*myc*	Ki-*ras*	N-*ras*
1	0	—	—	—	—
2	3	—	—	—	—
3	3	1	1	—	3
4	17	3	8	4	4

the size or degree of differentiation. However, we observed a correlation between the oncogene amplifications and advanced disease stage, with the *ras* and *myc* family oncogenes amplified in TNM stages 3 and 4 SCC. Amplification of N-*myc* gene has been associated with advanced stages of malignancies, as shown in neuroblastoma.[8,9] Our studies demonstrate that both c-*myc* and N-*myc* amplifications are associated with advanced stages of SCC in oral tumors, with 8/17 of the stage 4 tumors showing N-*myc* amplification, and 4/18 cases of advanced stages 3 and 4 of this malignancy having c-*myc* amplification. Spandidos and his colleagues[20] have demonstrated a significant difference between c-*myc* oncogene expression in stages 1 and 2 when compared to stage 3 and combined stages 3 and 4, indicating clinical relevance of oncogene expression in head and neck tumors. Amplified oncogenes, in most cases, have been

found to be abundantly expressed at the RNA level, generally in proportion to the amount of DNA amplification.[21] Our data indicate clinical relevance of oncogene amplification in advanced stages of SCC of the oral cavity.

Amplification of oncogenes in head and neck cancers, including tongue cancers has been suggested by Friedman and his colleagues, using the NIH-3T3 transfection assay.[22] They reported that all six of their tumor tissue DNAs tested from tongue, larynx and nasopharyngeal carcinoma yielded malignant transformation of NIH-3T3 cells. This high rate of transformation was suggested to be due to amplification of oncogenes in their samples of squamous cell carcinoma of the oral cavity, which correlates with the high frequency of amplification of oncogenes shown in our samples. Generally, a maximum of 30% transformation rate has been observed in NIH-3T3 transfection assays, using DNA from solid tumors.[23] Yokota *et al.*[10] have reported c-*myc* amplification in 2 of 7 cases of squamous cell carcinoma of the head and neck region. Escot *et al.*[24] observed a 2- to 15-fold amplification of c-*myc* oncogene in human primary breast carcinoma. These authors suggested a role for this oncogene in development of breast carcinoma, thus indicating the significance of a low level of amplification of oncogenes in solid tumors in humans.

A hamster cheek pouch model for oral cancers induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) has been examined for oncogenes by Wong and Biswas.[25] They observed a 5-fold amplification of H-*ras* and c-*erb* in the carcinoma, akin to the 5- to 10-fold amplification of one or more oncogenes in our tissues. Wong and Biswas considered their model to resemble closely the development of human oral cancers. However, the oncogenes involved were H-*ras* and *erb B*, neither of which were amplified in the human oral cancer samples we examined (*erb-B* — data not shown). The differences could be due to the mechanism of development of these cancers in humans and hamsters being different, as in the hamsters the cancer was DMBA-induced, and in the humans the cancer was associated with tobacco chewing. Alternatively, the amplifications of *erb-B* and H-*ras* could be rare events in oral carcinogenesis, hence not seen in our samples.

An interesting aspect of our study was the amplification of multiple oncogenes in the same tumor tissue samples. Interaction of two classes of oncogenes in malignancies has been documented.[26, 27] Multiple proto-oncogene alterations, involving a 5-fold amplification of c-*myc*, a partial deletion of H-*ras* and a 4- fold amplification of Ki-*ras*, in a cystadenocarcinoma of the ovary have been recently reported.[10] On screening of human hepatomas for oncogene expression, elevated expression of multiple oncogenes *erb-B*, *erb A+B*, H-*ras*, *fos* and *fms* has been reported.[28] As reviewed by Field and Spandidos,[2] more than one c-*onc* gene has been found to be transcriptionally active in renal, lung, colon, colorectal, breast, cecal, small bowel and ovarian carcinomas. In squamous cell carcinoma originating from tongue, buccal mucosa and floor of the mouth, Spandidos *et al.*[29] have demonstrated elevated expression of three oncogenes H-*ras*, Ki-*ras* and c-*myc*, using RNA spot hybridization analysis. Thus, multiple increased transcriptions of oncogenes in oral cancers have already been reported. We observed co-amplification of c-*myc* and N-*myc* in 13% and N-*myc* and N-*ras* in 17% of our samples. Coamplification of c-*myc* and N-*myc* has not been reported earlier. In small cell lung carcinoma cell lines, Nau *et al.*[30] observed either c-*myc* or N-*myc* amplified. The simultaneous amplification of c-*myc* and N-*myc* oncogene seems to be a unique feature of oral cancer tumor tissues. We also observed an additional 2.3 kb *Eco*RI fragment specific to the N-*myc* probe, in our samples. This could indicate restriction endonuclease fragment length polymorphism (RFLP) of the N-*myc*. The possibility of somatic rearrangement with amplification of the N-*myc* region that is consistent in a number of different squamous cell tumors also exists. An N-*myc*-specific 5.5 kb *Eco*RI additional fragment has been reported in a small cell lung cancer cell line.[30] RFLPs of the human N-*myc* oncogene have recently been reported by Kurosawa and his colleagues.[31] The presence of a prominent 2.3 kb band in the peripheral blood cells, though not amplified, indicates a preferential distribution of this fragment in the Indian population. The high incidence of oral cancers in the Indian population poses the question of whether there is any involvement of this allele in predisposition to oral malignancy. We are presently evaluating the unusual N-*myc* *Eco*RI band after digestion with other enzymes and its preponderance in the Indian population, as well as any association with predisposition to oral tumors.

The finding of different oncogene amplifications in tumors of similar type, and multiple oncogene amplifications in the same tumor, suggests that these oncogenes may be alternatively activated in the same pathogenetic step. Multiple oncogene amplification perhaps implies a cooperative action of the oncogenes in certain of these tumors. This form of genetic heterogeneity could be further explored in relation to variability in other parameters such as drug sensitivities. Determining the oncogenes regularly or occasionally amplified in oral cancers and also the involvement of oncogenes in oral cancer-associated lesions such as leukoplakia, should prove useful in understanding the role of these specific amplified oncogenes in the tumorigenesis of oral cancers.

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