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Molecular Lesions in Human Oral Cancer: The Indian Scene

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Carcinogenesis is a multi-step process including aberrant expression of two interacting classes of genes—oncogenes and tumour suppressor genes. With recent technological advances, it is feasible to identify the various molecular lesions underlying the different stages of neoplasia. Squamous cell carcinomas of the head and neck, although representing 2–4% of the malignancies in the West, comprise a large fraction (40%) of total cancers in India, posing a major health problem. Further, epidemiological and experimental evidence unequivocally confirms a causal association between tobacco chewing habit, highly prevalent in India, and oral cancers. Thus, the oral cancers offer an excellent *in vivo* system for the study of the environmental tobacco-carcinogen induced molecular alterations in the malignancy, and associated premalignant lesions such as leukoplakia. With a view to elucidating the molecular lesions involving oncogenes in oral carcinogenesis, we have investigated *myc/ras*/EGF-R activation by amplification, point mutation, gene rearrangement and allelic losses. Further, a functionally activated potent transforming gene was detected in a NIH3T3 transfection/tumorigenicity assay, unrelated to *myc/ras*/EGF-R. Studies on the involvement of p53 gene in oral cancer, indicates p53 allelic loss as an event observed in leukoplakia and tumour tissues. Advanced oral cancer stages demonstrate cumulative molecular aberrations, with greater than 95% samples showing oncogene involvement, thus indicating a multi-step process of oral carcinogenesis. The review presents a comparative picture of the oral malignancies seen in Western countries and India, significance of molecular lesions and future perspectives of oncogenes and tumour suppressor gene involvement in oral cancer.
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INTRODUCTION

ORAL CANCER is one of the 10 most common cancers in the world. Globally the age-adjusted incidence rates in males for oral cancer (ICD-9, 140–145) show a wide variation ranging from 2.2 per 10^5 (Japan) to 22.5 per 10^5 (Brazil), with females generally showing much lower rates than males [1]. In India, oral cancer is highly prevalent, comprising 35–40% of all malignancies, due to the habit of tobacco chewing in betel quid commonly observed in the population [2, 3]. About 56 000 new cases have been estimated to occur each year, leading to 100 000 individuals suffering from oral cancer in the Indian population, in any given year. The exceptionally high incidence of oral cancer far exceeds that of countries such as the U.K., U.S.A. or Australia, where oral cancer constitutes 2–4% of all malignancies [4]. Besides the oral cancer picture in India is distinct from the malignancy seen in the Western population in several aspects. The incidence rates for oral cancer in females is equivalent to males, in India. Further, cancer of the tongue and buccal mucosa constitute the bulk of oral cancers in India, with cancer of the lip being relatively infrequent. Cancer of the tongue is the most aggressive lesion of the oral cavity and although deceptively small, infiltrates deeply with high propensity for lymph node metastasis. In contrast, the Western registries show cancers of the floor of the mouth as the most frequent, with cancer of gum and tongue being rare [1]. In the Indian population with the habit of tobacco chewing, a latent period of 5–15 years from start of the habit to diagnosis of the malignant lesion, is common; and almost every tobacco related oral malignancy is preceded by a clinically distinctive premalignant stage such as leukoplakia, at the particular site of cancer development [3].

Clinical, epidemiological and laboratory studies confirm an aetiological relationship between prolonged tobacco chewing and oral cancer in India [5, 6]. *N*-nitroso compounds constitute the most abundant carcinogens present in tobacco, with tobacco-specific nitrosamines (TSNA), representing an important class of genotoxic carcinogens [7]. Carcinogenicity bioassays show that *N*-nitrosonornicotine (NNN) and 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) are the most potent carcinogens amongst the TSNA [8]. The TSNA are metabolically activated to yield electrophiles which react with cellular components, including nucleophilic centres of DNA, leading to miscoding [9], followed by point mutation and other molecular aberrations, particularly involving protooncogenes and tumour suppressor genes. These two interacting classes of genes are highly conserved in nature and are involved in various regulatory pathways controlling normal cellular growth and differentiation [10]. The protooncogenes and tumour suppressor genes can be deregulated by various mechanisms such as amplification, point mutation, gene rearrangement and deletions leading to subsequent preneoplastic and neoplastic alterations in the cell [11, 12]. The oral tumours from the habitual tobacco chewers in India, provide

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a unique opportunity to investigate the role of proto-oncogenes and tumour suppressor genes, in an environmental carcinogen-associated human tumour type.

The aim of this article is to present a review of the studies on molecular lesions involving oncogenes and tumour suppressor genes, in human oral cancers from the Indian subpopulation. The technology commonly used for the analysis of molecular alterations in human oral malignancies include investigations at the genomic level using Southern analysis, polymerase chain reaction (PCR) technology, flow cytometry and cytogenetic analysis; quantitative/qualitative aberrations of the RNA transcripts by northern/dot blot analysis; and quantitative/qualitative alterations of the oncoproteins by western blotting analysis/immunohistochemical staining/enzyme linked immunosorbent assays. The primary sites in the oral cavity showing carcinoma include buccal mucosa, tongue, lower alveolus, soft palate, lip and floor of the mouth.

ACTIVATION OF ONCOGENES IN ORAL CANCER

Oncogene amplification

Oncogenes most commonly associated with solid tumours, belonging to the *myc* and *ras* family, have been analysed in head and neck cancers. In addition, *erb*B-1, implicated in several squamous cell carcinomas has also been studied in oral cancers, as 95% are histologically diagnosed as squamous cell carcinomas (SCC) [13–15].

Studies in our laboratory demonstrated amplification of c-*myc* oncogene in 36% patients, the sample size being 102 SCC. N-*myc* was amplified in 26% of the patients [13, 15], and L-*myc* was not amplified in the tumour tissues. Coamplification of c-*myc* and N-*myc* was observed in 30% tumour tissue samples, thus indicating existence of two different clones with c-*myc* or N-*myc* amplified. Alternatively, the two *myc* oncogenes could be amplified in the same cell. Co-expression of c-*myc* and N-*myc* has been recently demonstrated in a neuroblastoma cell line by Gazitt [16], as well as in undifferentiated pre-B cells in mice [17]. The coamplification implies a role for both the oncogenes in early developmental stages, and/or a particular differentiation window in a cell. A significant association of c-*myc* and N-*myc* amplification with nodal metastasis of the tumour was noted in the study [15]. Correlation with other clinicopathological parameters including grade of differentiation, tumour size, TNM staging, or recurrence of the tumour, was not observed.

Further, the *ras* oncogenes K-*ras* and N-*ras*, were amplified 3–8 fold in 33% patient tumour tissue DNAs analysed, with no correlation to clinical parameters. The epidermal growth factor receptor gene (EGF-R), a homologue of the *erb*B-1 gene was amplified in 29% (19/66) of the oral tumour samples screened [14]. A consistent feature of the oral tumours in the Indian population screened, was the coamplification of *myc*/*ras*/EGF-R oncogenes in 44% of the samples with oncogene involvement. The multiple oncogene amplification indicates a complex, multi-hit, process of oral carcinogenesis, and implies simultaneous or sequential activation of oncogenes in this malignancy.

Similar studies from head and neck cancers in the U.S.A., U.K. and Australia, generally demonstrate lower incidences of oncogene amplification. Table 1 gives comparative data on oncogene and tumour suppressor gene involvement in oral cancers [13–15, 18–38]. A recent review is cited for detailed information [39]. The presence of oncogene amplification in a study on Australian patients by Leonard *et al.* [24], demonstrated amplification of oncogenes in 9–18% of the 66 patients, screened with a panel of nine different oncogenes. Amplification of a minimum of one oncogene was observed in 12 samples (18%), with c-*myc* amplified in 9%, EGF-R in 10%, and *bcl*-1/*int*-2 in 7% of the tumour DNA samples. H-*ras*, TGF-α, c-*mos*, c-*erb*B-2 and c-*erb*A-2 were not amplified. Coamplification of the oncogenes was commonly observed in their patient population. Amplification of EGF-R in head and neck cancer has been reported by Hunts *et al.* [18] in 1/10 (10%), Yamamoto and coworkers [19] in 1 of 6 (16%), Yokota *et al.* [20] in 1 of 8 (12%), and Ishitoya *et al.* [22] in 4 of 21 (19%) patients. The other oncogenes reported as amplified in the oral tumour tissues include c-*myc*, *hst*-1/*int*-2 and *bcl*-1 [20, 25, 26, 40].

The amplified oncogenes generally show increased transcripts or overexpressed oncoproteins. Multiple transcriptional activation of H-*ras*, K-*ras* and c-*myc* has been reported [35]. Further, Field *et al.* [41] demonstrated overexpression of c-*myc* oncoprotein and observed significant correlation with advanced stages III/IV as well as poor prognosis of the patient. However, correlation with the patient age, sex, TNM staging, site of the tumour, histopathological grading, lymph node metastasis or encapsular rupture of the tumour was not noted. Azuma and coworkers [36] have reported increased *ras* expression, with correlation to poor prognosis.

Point mutations in ras *oncogenes*

Mutations leading to activation of *ras* oncogenes, have been identified in oral cancers [30]. To analyse activation of *ras* oncogenes by point mutations in oral tumour DNA, *in vitro* amplification by Taq polymerase, followed by detection of the mutation using allele specific oligonucleotide (ALO) hybridisation or direct sequencing of the PCR amplified products, is the method of choice. The codons 12/13 and 61 of H-*ras*, K-*ras* and N-*ras*, mutated in several human malignancies has been investigated in oral cancers from Indian group of patients [30]. In these studies, point mutations detected in 20/57 (35%) of the samples screened, were restricted to the H-*ras* oncogene. K-*ras* and N-*ras* activated by amplification in several oral cancer patients, were not mutated at codons 12/13 or 61. The H-*ras* mutations were primarily at codon 12.2 resulting in a glycine to valine substitution, and codon 61.2 causing a glutamine to arginine substitution [30]. Interestingly, several tumour samples (8/20) with the H-*ras* point mutations also showed loss of wild type H-*ras* allele, indicated by absence of signals for wild type codons 12 or 61 on dot blot analysis. Besides, three apparently novel mutations at codon 12.2: a G–A transition resulting in glycine to serine; at codon 13.2: a G–A transition resulting in glycine to aspartate; and at the same codon 13.2 a G–T transversion resulting in glutamine to histidine, substitutions were reported in the tumour tissues. Also, two tumour tissues demonstrated concurrent mutations in H-*ras* codons 12 and 61. The specific H-*ras* mutations in the Indian oral malignancies associated with tobacco chewing, may represent an important example of an environmental carcinogen induced step in a multistep pathway leading to malignant transformation.

On the other hand, analysis of oral cancer patients from the U.K., has demonstrated that point mutations in *ras* oncogenes were infrequent, with no *ras* mutations at codons 12, 13 or 61 in 17 patient tumour DNA examined [31]. Rumsby *et al.* [29] have reported 1 of 15 tumour DNA samples of U.K.

Table 1. Oncogene/tumour suppressor gene involvement in SCC of the oral cavity

Aberration	Oncogene/ tumour suppressor gene	Reference	Samples activated/ samples analysed	Percentage with aberration
Oncogene amplification (Southern analysis)	EGF-R	18	1/10	10
		19	1/6	16.6
		20	1/8	12.5
		21	0/17	0
		22	4/21	19
		23	7/66	10.6
		24	3/46	6.5
		14	19/66	29
	int-2	25	2/5	40
		26	7/20	33
		27	11/21	52
		28	10/21	47.6
		23	5/66	7.6
	bcl-1	26	8/23	34.8
		23	5/66	7.6
	c-*myc*	20	2/7	28.6
		26	0/17	0
		13, 15	4/23; 21/102	17; 21
		23	6/66	9
	N-*myc*	13, 15	9/23; 27/102	39; 26
	N-*ras*	13, 15	7/23; 28/102	30; 28
Point mutations (PCR)	H-*ras*	29	1/15	6.6
		30	20/57	35
		31	0/17	0
		32	0/42	0
RNA over-expression (dot blot analysis/northern analysis)	H-*ras*	33	14/20	70
		34	2/12	16.6
	k-*ras*	33	14/20	70
	c-*myc*			
Oncoprotein expression (immuno-histochemical staining, ELISA)	EGF-R	24	24/42	57
	c-*myc*	35	21/44	48
	ras	36	71/121	59
LOH: RFLP	H-*ras*	34	10/46	22
		37	7/23	30
Tumour suppressor gene (LOH: PCR and RFLP)	p53	Saranath *et al.* (unpublished data)	3/21	14
Over-expression: (immuno-histochemical staining)	p53	38	32/48	67.0

residents, with *ras* mutations. Similar low frequency mutations have been reported by Sheng and coworkers [34].

RESTRICTION FRAGMENT LENGTH POLYMORPHISM STUDIES

Rearrangement of oncogenes may be detected as, cytogenetic translocations in karyotypic analysis studies; restriction fragment length polymorphisms (RFLP) using Southern analysis or by using PCR technology. In the Indian subjects with oral cancers, RFLP investigations for the L-*myc* and H-*ras* oncogenes has been examined [42].

The L-*myc* RFLP has been examined for specific association of the alleles, which are represented as *Eco*RI 10 kb L-allele or 6.6 kb S-allele, with increased incidence of oral cancer, metastatic potential or prognosis of the disease [42]. The studies demonstrated equi-distribution of the two alleles in healthy individuals and oral cancer patients. Thus implying no predisposition to oral cancer by presence of either allele. However, the S-allele was correlated to poorly differentiated tumours and larger sized tumours. It is noteworthy that N-*ras* and *jun* oncogenes located on the same chromosome 1 as L-*myc* gene, have been incriminated in cancer progression [43]. Rhesus gene locus on the short arm of chromosome 1, has recently been demonstrated to be of independent prognostic value in oral SCC patients [44]. Besides, the chromosome area may also include a tumour suppressor gene [45, 46].

The H-*ras* RFLP studies in the Indian oral cancer patient group, revealed tumour associated loss of heterozygosity (LOH) in 30% of the patients [37]. Further, a rearrangement in the regulatory element—variable tandem repeat (VTR) of H-*ras* was observed in 10% of the samples [37]. The rearrangement suggests altered function of VTR, representing one of the varied molecular lesions in the tobacco induced oral cancers. In accordance with the data on H-*ras* point mutations, it seems feasible that the H-*ras* allelic loss may provide a selective advantage to the activated H-*ras* gene. Further, the loss of the H-*ras* allele may encompass a tumour suppressor gene, located on chromosome 11, in the vicinity of H-*ras* gene at 11p15 [47]. From recent studies, it appears that more than one tumour suppressor gene may be present on chromosome

11 including loci at 11p15 and 11p13 [48], with one of the 11p13 locus characterised as encoding a zinc finger protein [49]. The loss of chromosome 11 alleles in several other tumours including Wilms, breast and hepatoblastoma, indicates an important role for tumour suppressor mechanisms in such tumours.

DNA PLOIDY STATUS IN ORAL CANCER

The ploidy status and the proliferative activity of tumours, has been investigated in the Indian group of oral cancer patients, by flow cytometric measurements [50]. The authors observed that a majority (58%) of the tumours were diploid with a unimodal distribution. The percentage of cells in S phase ranged from 2.9% to 22%, with a mean value of 17.7%. A correlation between DNA-index and S-phase fraction was not observed. However, a correlation between tumour stage and ploidy was observed, with poorly differentiated grade III tumours primarily non-diploid, and tumours with lymph node metastasis showing greater percentage of cells in S-phase.

DETECTION OF ACTIVATED/FUNCTIONAL ONCOGENES

Reviewing the data on molecular aberrations in oral cancers in India, it was obvious that several oncogenes were activated in the tumour tissues, with greater than 90% (96/102) of the patients analysed, showing oncogene involvement. Hence, to detect and isolate a functionally active dominant oncogene from the oral tumour tissues, NIH3T3 transfection/tumorigenicity assay has been used in our current studies (unpublished data). Representative primary oral tumour DNAs (10 patients) with oncogene aberrations, as well as four primary tumours with no detectable molecular lesions, were investigated for the presence of a dominant oncogene(s). To summarise, the data demonstrated high transforming potential of oral tumour DNA on transfection into NIH3T3 cells, using SV2neo gene as a selectable marker. The cells were highly refractile, morphologically transformed showing foci formation and colony formation in soft agar. High incidence of tumour induction was observed, with 86% (12/14) oral tumour DNA transfected cells inducing tumours in nude mice within 5–10 weeks. Further, the tumours confirmed presence of human specific Alu sequences on Southern analysis using a Blur-8 probe. The primary and secondary tumours induced in nude mice did not indicate the presence of *myc*/*ras*/EGF-R oncogenes involved in the oral tumours from the patient population, with the exception of one of the nude mice tumour showing presence of *ras* oncogene. Incidentally, the patient group studied, included 4 patients with oral tumours not showing detectable oncogene aberrations. Thus, the results implied the involvement of alternative oncogenes, other than *myc*/*ras*/EGF-R, in the nude mice tumours, and perhaps presence of a potent novel oncogene, specifically associated with the chewing tobacco induced oral tumours. The oncogene may be activated by tobacco specific carcinogens, and is a potent transforming gene with high transformation/tumorigenicity potential. We are currently in the process of cloning the gene from secondary-transfectant induced nude mice tumours to isolate and identify the gene.

p53 GENE IN ORAL CANCER

Recent studies from our laboratory, indicates a role for the p53 tumour suppressor gene in our oral cancer patients (unpublished data). We examined the status of p53 Exon 4, amplified *in vitro* by polymerase chain reaction in tumour tissue DNA and corresponding PBC DNA, to examine LOH in the p53 gene. The PCR amplified Exon 4 product is a 259 bp fragment [51]. On *Bst*U1 digestion of the product, heterozygosity is discernible as three fragments, as 259 bp without *Bst*U1 site, and allele with *Bst*U1 site showing 160 bp and 99 bp fragments [51]. In our study, several heterozygous samples (3/21), showed tumour tissue associated LOH, with corresponding PBC DNA indicating the constitutional presence of the alleles.

Similar studies on leukoplakia which is considered as a premalignant lesion in people who chew tobacco demonstrated one of the seven heterozygous leukoplakia DNA, with a distinct LOH, and the corresponding PBC DNA showing constitutive presence of the two alleles. Currently, we are investigating point mutations of p53 Exon 5 through 9, in the malignant and premalignant tissues of the oral cavity in the Indian patients. However, it is apparent from our studies that tumour suppressor gene p53, is involved in a certain proportion of oral cancers as well as the precancerous leukoplakias. Perhaps, leukoplakias with p53 aberrations may define the high risk group of patients, progressing towards frank malignancy.

Recently, increased expression of p53 gene, possibly the mutated gene product, has been reported in head and neck cancers in the West, by two groups using a range of antibodies [52, 53]. Field *et al.* [36] demonstrated correlation between heavy smoking and elevated p53 expression, suggesting that alterations in this gene may be one of the early events in the development of these cancers, smoking being one of the factors in oral cancers from the West. Thus, it is possible that p53 alterations in oral cancer may represent an early event, with increased cancer risk in tobacco smokers or tobacco chewers, further predisposing the normal cells to malignant phenotype.

CONCLUSIONS AND FUTURE PERSPECTIVES

Thus, studies on molecular lesions in an Indian oral cancer group emphasises that oncogenes/tumour suppressor genes constitute common molecular targets to be hit by tobacco specific carcinogens. The possible stages at which the alterations are most likely to occur is represented schematically (Fig. 1), with the percentage molecular alterations computed in the Indian oral cancer patients. The oral cancers observed are invariably associated with tobacco habits and preceded by distinct premalignant lesions, such as leukoplakia, with a 5–15 year time lag period from the initiation of tobacco habits to the malignancy. The studies indicate an involvement of p53 and perhaps H-*ras* in the initial stages of oral cancer development, possibly the leukoplakia. These alterations may include inactivation of tumour suppressor genes including LOH of p53, LOH in H-*ras* with possible point mutations in H-*ras*. Successive sequential clonal expansion of cells containing these alterations may result in progression towards stages 1 and 2. These may involve further heterogenous genetic events including the activation of a potent transforming gene and amplification of *myc*/*ras*/EGF-R oncogenes. Cells with these cumulative lesions would have a proliferative advantage and likely to have a propensity to metastasise locally into regional lymph nodes. Thus, the progression from leukoplakia to frank malignancy may involve a series of genetic events and the final outcome would be a result of accumulated genetic alterations involving oncogenes and tumour suppressor genes. The

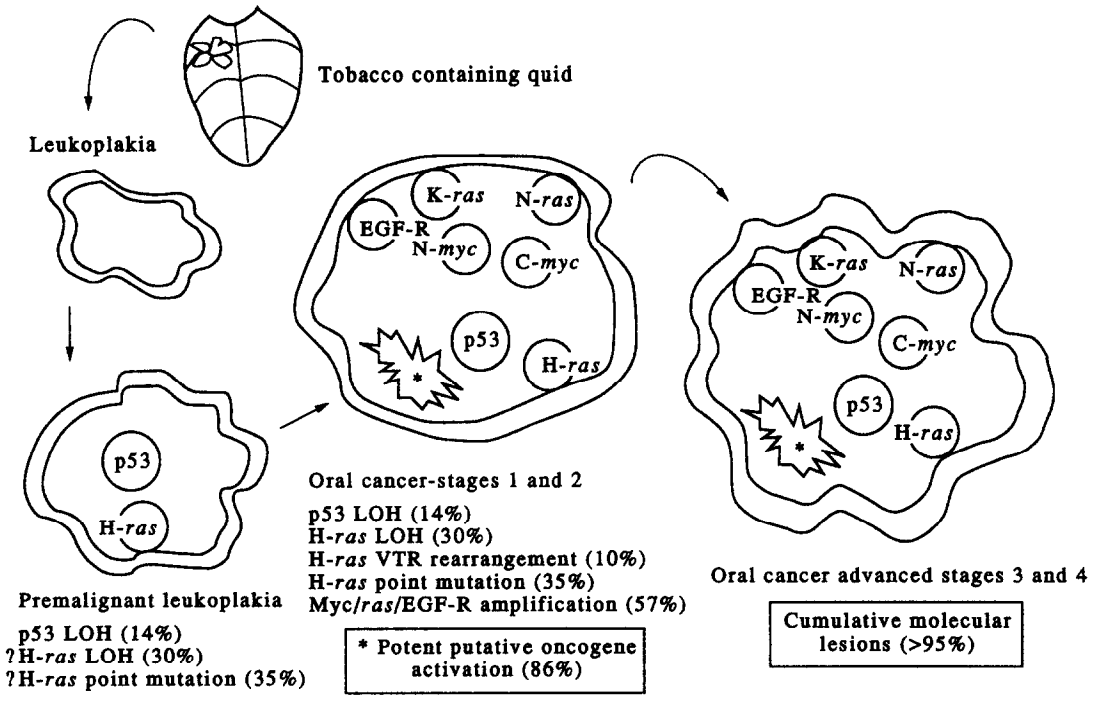

Fig. 1.

review of Field [39] on oncogenes and tumour suppressor genes in head and neck cancers, postulates on mechanisms operating in the development of these malignancies. The authors indicate H-*ras* and p53 involvement as early events, with c-*myc* activation and a cell adhesion molecule E-cadherin regulation, as a late event in the progression of the cancers. Further, the author suggests *erb*B-1 to be involved during the development of this malignancy.

We are currently in the process of cloning, isolating and identifying the potent transforming gene from the oral tumours. The status of the putative oncogene with high transforming potential in the premalignant and malignant tumour tissues will be imperative in understanding the molecular basis of oral carcinogenesis. Besides, such studies may provide clues to early diagnosis/prognosis of the malignancy and may indicate use of individualistic specific treatment modalities per se, or supplement conventional therapeutic approaches.

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