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Amplification and Overexpression of Epidermal Growth Factor Receptor Gene in Human Oropharyngeal Cancer

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The presence of epidermal growth factor receptor (EGF-R) gene was determined in 84 patients with squamous cell carcinoma (SCC) of the oropharyngeal region—a highly prevalent, chewing-tobacco associated malignancy in India, using Southern hybridisation analysis of DNA extracted from primary tumor tissues. We observed a 3- to 8-fold amplification of EGF-R gene in 19/66 (29%) of the SCCs of the oral cavity, and about 30-fold EGF-R amplification in 2/18 (11%) hypopharyngeal cancers. Dot blot analysis of total RNA from several tumour tissues, revealed overexpression of the EGF-R gene in the examined patients, with the EGF-R gene amplified. 4 patients with single copy EGF-R gene, did not exhibit overexpression of the gene. Within our sample set, no correlation was evident between EGF-R gene amplification and clinico-pathological parameters of the malignancy. The amplification and overexpression of the EGF-R gene observed in the primary tumour tissues of 25% (21/84) of the human oropharyngeal cancers, indicate possible involvement of the gene in the pathogenesis of these cancers.
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INTRODUCTION

In India, a minimum of 40% of all cancers recorded annually at Cancer Centres including Tata Memorial Centre, Bombay, are cancers of the oropharyngeal cavity, with this malignancy being one of the leading health problems in the country [1]. On a global basis, oropharyngeal cancer is also gaining importance, with 300×10^3 and 106×10^3 new cases per year of the cancer estimated to occur in multi developing and developed countries, respectively [2]. The high incidence of oropharyngeal cancers in India, has been attributed to the common habit of chewing tobacco [3]. A latent period of 5–15 years is common, and tobacco related oral cancer in the Indian community is preceded by precancerous lesions including leukoplakia [4]. Tobacco specific carcinogens such as tobacco-specific *N*-nitrosamines and polycyclic hydrocarbons have been identified, capable of causing neoplastic changes, in the oropharyngeal cancers [5]. Recently, the tumour suppressor gene p53 has been implicated in human malignancies such as lung cancer and head and neck cancer, with strong association between p53 mutations and smoking habit of the patients [6, 7]. However, the critical link between the tobacco specific carcinogens and the cellular macromolecules, in the chewing tobacco associated oral cancers, is an enigma.

Recent studies from our laboratory have demonstrated the involvement of *myc*/*ras* oncogenes in cancers of the oral cavity via amplification, point mutations and loss of the H-*ras* allele [8–11]. The oropharyngeal cancers observed in India, are primarily squamous cell carcinomas. The epidermal growth factor receptor (EGF-R) and the ligands are involved in the control of normal and neoplastic epithelium, with autocrine stimulation of growth involving this receptor identified in several types of neoplasias. Besides, the EGF-R gene is the homologue of avian erythroblastosis virus oncogene *erb*B, and is often deregulated through amplification and overexpression in squamous cell carcinomas [12, 13]. Hence, we were interested in determining the status of the EGF-R gene in oropharyngeal cancer, with respect to amplification and overexpression of the gene in the primary tumour tissues.

PATIENTS AND METHODS

Patient Information

84 untreated patients, 69 males and 15 females, aged between 28 and 65 years, diagnosed as having squamous cell carcinoma (SCC) of the oropharyngeal cavity, were examined for EGF-R status. The diagnosis was based on clinical examination and histological features of the biopsy material. The various sites included buccal mucosa—30 cases, lower alveolus—19 cases, tongue—15 cases, floor of the mouth—2 cases, and hypopharynx—18 cases, with either poor, moderate or well differentiated carcinoma, and TNM [14] stages T_2 to T_4, N_0 to N_3 and M_0. The hypopharyngeal patients comprised of 15 cases of pyriform sinuses and 3 cases were cancers of the aryepiglottic fold.

The tumour tissues were taken from resections of primary tumours near the advancing edges, care being taken to avoid the necrotic centre. The tissues were collected in liquid nitrogen and stored at −70°C until extraction of DNA or RNA. Peripheral blood cells (PBC) were also collected from the patients on admission to hospital.

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PBC from healthy volunteers were used in the study, as normal control for quantitation of single copy genes. Vimentin was used as an internal single copy control gene.

DNA extraction

High molecular weight DNA was isolated from the tissues and PBC, according to the standard method [15]. The cells were lysed with sodium dodecyl sulphate (0.5%), deproteinised with proteinase K (100 μg/ml, 4–5 h, 50°C) and phenol chloroform extraction, ethanol precipitation, ribonuclease treatment (100 μg/ml, 60 min, 37°C), and dialysis. The DNA was precipitated with chilled ethanol in the presence of high concentration of monovalent cations. The resultant pellet was dissolved in TE buffer (10 mmol/l Tris pH 7.6, 1 mmol/l EDTA). DNA was quantified using optical density at 260 nm, and high molecular weight of the DNA ascertained by electrophoresis in 0.7% agarose gel.

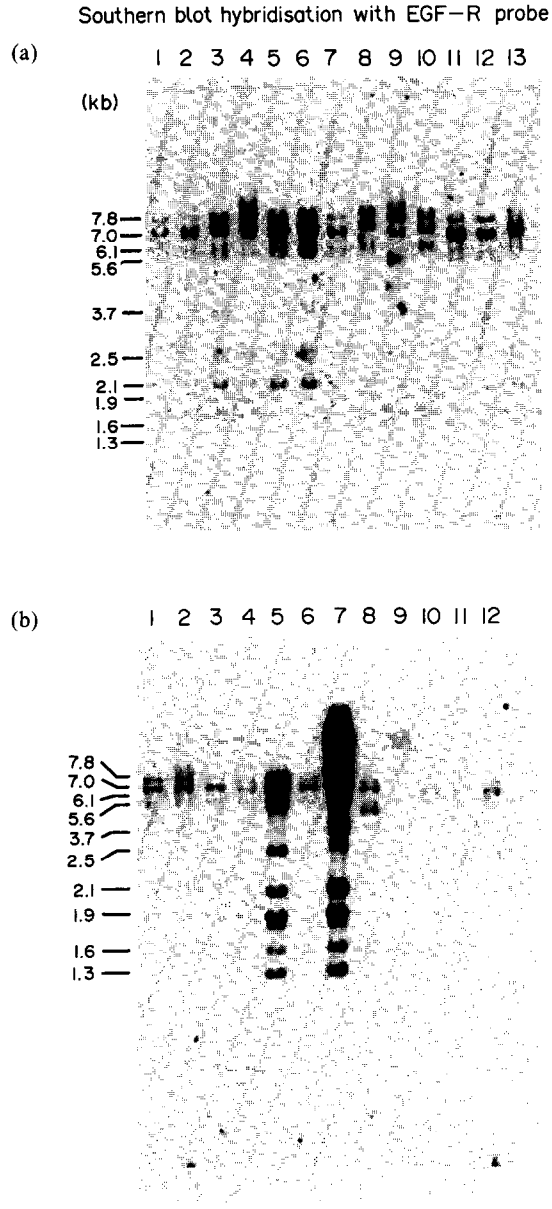

Fig. 1. Southern blot analysis of primary tumour tissue DNA from cancer patients, digested with *Eco*RI (10 μg DNA was loaded in each lane). Numbers on the left hand side indicate the molecular weight of EGF-R specific fragments. (a) Samples from oral cancer patients (lanes 2–13). Lane 1 is normal healthy volunteer PBC DNA; lanes 3, 4, 5, 6, 11, 12, 13 represent patient Nos. 3, 4, 11, 17, 5, 16, 18, respectively in Table 1; the other lanes represent patients with EGF-R gene non-amplified. (b) Samples from hypopharyngeal cancer patients (lanes 2–12). Lane 1 is normal PBC DNA; Lanes 5, 6 represent patient Nos. 21, 20 respectively, in Table 1; the other lanes represent patients with EGF-R gene non-amplified.

Hybridisation probes

The EGF-R cDNA probe was prepared from the pE7 clone, as a 2.3 kb ClaI fragment in pBR322 plasmid [16]. The 2.3 kb EGF-R fragment was purified by preparative agarose electrophoresis, using low melting agarose. The probe was labelled by ^{32}P dCTP to a specific activity of 10^8 cpm/μg, according to the method of Feinberg and Vogelstein [17]. Vimentin probe used as the house keeping gene, was isolated as a 1.8 kb *Eco*RI fragment, cloned in pGEM-3 [18].

Southern blot analysis

Genomic DNAs were digested to completion with the restriction enzyme *Eco*RI, under standard conditions. 10 μg of the digest was subjected to electrophoresis in 0.7% agarose gels using TBE (89 mmol/l Tris-borate, 89 mmol/l boric acid and 2 mmol/l EDTA pH 8.0). DNA fragments were blotted onto a Nylon membrane (Hybond-N, Amersham), according to standard procedure as described by Southern [19].

Hybridisation, washing and autoradiography were carried out as previously described [8]. λ *Hin*dIII digested DNA was used as size marker.

RNA extraction and dot blot analysis

Frozen oral tumour tissues and PBC, were homogenised in 4 mol/l guanidium isothiocyanate mixture. Total RNA was isolated by the single step method of RNA isolation combining guanidinium thiocyanate and phenol chloroform extraction as described previously [20].

Dot blot hybridisation for a semi-quantitative expression of the EGF-R gene was carried out as described. Briefly, 2-fold serial dilutions of RNA were prepared in 50 μl diethylpyrocarbonate water. To this 30 μl, 20 × SSC (1 × SSC = 0.15 mol/l sodium chloride, 0.15 mol/l sodium citrate pH 7.0) and 20 μl formamide was added. The RNA was denatured at 55°C for 15 min and spotted onto nylon membrane filter with a BioRad dot blot apparatus. Replicate filters were prepared and the RNA fixed by ultraviolet illumination. The filters were prehybridised, hybridised with vimentin, and/or EGF-R insert and washed as previously described [21]. The filters were exposed to Fuji X-ray films at −70°C using intensifying screens, for a period of 3–5 days.

RESULTS

Southern blot analysis

Primary tumour tissues from 84 oropharyngeal cancer patients were analysed for amplification and/or expression of the EGF-R gene. Genomic DNA digested with *Eco*RI and probed with EGF-R (pE7), generally exhibited a prominent 7.0 kb fragment and several consistent fragments ranging in size from 7.8 kb to 1.3 kb (Fig. 1a, b). A higher molecular weight 8.2 kb fragment was observed in several samples.

Amplification of EGF-R gene was estimated on densitometric scanning of the autoradiographs. We define amplification as a minimum of 3-fold increase in band intensity relative

Table 1. Amplification/expression of EGF-R in oropharyngeal cancers

Patient no.	Site	Differentiation	TNM classification	Stage	Amplification (DNA)	Expression (RNA)
1	Buccal mucosa	Well	$T_2N_1M_0$	III	3.0×	ND
2	Buccal mucosa	Well	$T_4N_1M_0$	IV	3.0×	4×
3	Buccal mucosa	Moderate	$T_4N_2M_0$	IV	3.0×	ND
4	Buccal mucosa	Moderate	$T_4N_2M_0$	IV	3.2×	4×
5	Buccal mucosa	Moderate	$T_4N_2M_0$	IV	5.0×	ND
6	Buccal mucosa	Moderate	$T_2N_0M_0$	II	3.0×	ND
7	Buccal mucosa	Poor	$T_4N_1M_0$	IV	8.0×	16×
8	Lower alveolus	Well	$T_4N_0M_0$	IV	4.0×	ND
9	Lower alveolus	Well	$T_4N_2M_0$	IV	3.2×	ND
10	Lower alveolus	Moderate	$T_3N_0M_0$	III	3.0×	ND
11	Lower alveolus	Moderate	$T_4N_2M_0$	IV	6.0×	8×
12	Lower alveolus	Moderate	$T_4N_2M_0$	IV	3.3×	ND
13	Lower alveolus	Poor	$T_4N_2M_0$	IV	3.0×	4×
14	Lower alveolus	Poor	$T_4N_1M_0$	IV	6.0×	ND
15	Tongue	Well	$T_3N_1M_0$	III	4.0×	4×
16	Tongue	Moderate	$T_2N_1M_0$	III	3.0×	ND
17	Tongue	Moderate	$T_3N_1M_0$	III	8.0×	ND
18	Tongue	Moderate	$T_4N_2M_0$	IV	4.0×	ND
19	Tongue	Poor	$T_2N_0M_0$	II	3.0×	ND
20	Hypopharyngeal carcinoma	Moderate	$T_2N_2M_0$	IV	32.0×	32×
21	Hypopharyngeal carcinoma	Poor	$T_3N_2M_0$	IV	12.0×	16×

ND = Not done due to insufficient sample.

to normal controls. Initial standardisation of the signal intensity was demonstrated using single copy Vimentin gene as an internal control, and presence of a control single copy EGF-R gene in the normal PBC DNA from healthy volunteers. We detected a 3- to 8-fold amplification of EGF-R gene in 19/66 (29%) of the oral SCCs. 2 of the hypopharyngeal cancer patients revealed much higher level of amplification, around 30-fold, of the EGF-R gene (Fig. 1b). Table 1 summarises the clinico-pathological information in the patients showing amplification of the EGF-R gene, EGF-R amplification and expression in these patients.

The oropharyngeal tumours were all classified histopathologically. A correlation between amplification of the EGF-R gene and clinico-pathological features of the oropharyngeal tumours, such as grade of differentiation, TNM stage, nodal involvement, and recurrence of the tumour during a 1–3 year follow up period, was not evident in our set of patients as shown in Table 2. A similar analysis with tumours restricted to the oral cavity, also did not show correlation between EGF-R amplification and the clinico-pathological features of the oral SCCs, the χ^2 values being 0.851 for grade of differentiation, 0.186 for TNM stages, 2.766 for nodal involvement and 0.345 for recurrence of oral tumour during a 1–3 year period.

RNA dot blot analysis

To determine the level of EGF-R mRNA, dot blot analysis of total RNA from 12 tumour specimens and six normal controls (normal PBC) was performed using the EGF-R (pE7) probe. Of the 12 samples examined for EGF-R expression,

Table 2. Correlation of EGF-R amplification with various clinico-pathological features of oropharyngeal cancer patients

Clinical pattern (n = sample size)		EGF-R non-amplified (A^-)	EGF-R amplified (A^+)	χ^2	P*
Grade of differentiation					
Well	(18)	14	4	0.00000374	NS
Poor–moderate	(66)	49	17		
TNM stages					
I and II	(7)	5	2	0.469	NS
III and IV	(77)	58	19		
Nodal involvement					
N^-	(30)	26	4	2.48	NS
N^+	(54)	37	17		
Recurrence†					
R^-	(70)	52	18	0.0000457	NS
R^+	(14)	11	3		

*P value calculated using the χ^2 test with 1 d.f. (Yates correction applied).
†Follow-up data for a period of 1–3 years was available on the patients.
NS = Non-significant.

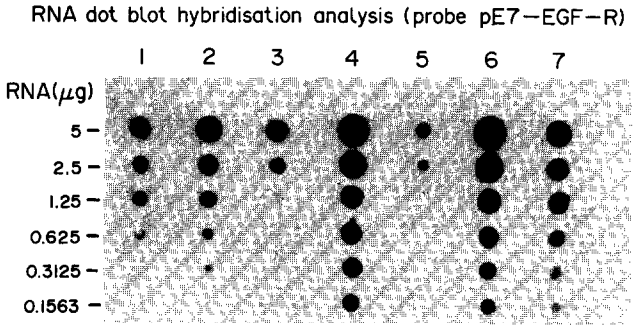

Fig. 2. Representative RNA dot bot analysis, showing expression of EGF-R gene in oropharyngeal cancer samples, on probing with ^{32}P-labelled EGF-R(pE7) probe. Lanes representing samples and the EGF-R expression are as follows: lane 1: oral cancer tissue (4×); Lane 2: Oral cancer tissue (8×); lane 3: oral cancer tissue (2×); lane 4: hypopharyngeal cancer patient tumour tissue (32×); lane 5: normal PBC (1×); lane 6: A431 cells (30×); lane 7: hypopharyngeal cancer patient tumour tissue (16×).

eight contained EGF-R gene amplified and 4 patients demonstrated presence of single copy EGF-R gene. All the eight tumour tissues with the EGF-R gene amplified, exhibited increased EGF-R specific RNA on dot blot hybridisation analysis. Figure 2 is a representative RNA dot blot. Tumours were evaluated as being overexpressed if the amount of EGF-R mRNA in the tumour, was at least 4-fold higher than those in the normal PBC (Fig. 2, lane 5). RNA from the A431 cell line with the gene amplified greater than 30-fold and showing comparable increased EGF-R expression, was used as a positive control in the RNA dot blot (Fig. 2, lane 6). The highest EGF-R expression being 32-fold greater than the single copy signal intensity of normal PBC sample, was noted in a hypopharyngeal cancer patient (Fig. 2, lane 4). Figure 2, lanes 1, 2 and 7 were quantitated as 4×, 8× and 16× EGF-R RNA, representing patients 2, 11, and 7 in Table 1, as compared with the single copy gene expressed in PBC from a normal healthy volunteer in lane 5. The lane 3 quantitated 2× RNA expression, which was not considered as overtly increased in the dot blot hybridisation. PBC from the patients did not show increased RNA expression (data not shown).

DISCUSSION

EGF-R/c-*erb*B-1 gene amplification is considered to play a significant role in the pathogenesis of malignant transformation, particularly in squamous cell carcinomas [12, 13, 22]. However, in SCCs of the oropharyngeal region, there is paucity of information on EGF-R gene involvement via gene amplification/overexpression. Earlier investigations on molecular lesions in oropharyngeal cancers, have primarily been grouped as cancers of the head and neck region, with few samples representing the oropharyngeal region. Despite oropharyngeal cancers being one of the 10 leading cancers in the world, molecular lesions associated with the malignancy are not extensively investigated. Thus, it becomes mandatory to investigate the basic molecular mechanism of oropharyngeal carcinogenesis. Another important aspect to be understood in head and neck cancers, is the clinical observation that these patients in comparable stages may respond differently to similar treatment. Routine clinico-pathological/TNM staging do not suffice in effectively highlighting the varied treatment responses. Towards defining more accurate markers outlining the biological behavior of the maglignant cell and consequently the malignancy, molecular studies of genes such as EGF-R and other oncogenes, may be a step in the right direction. In this report, we have demonstrated that the EGF-R gene is amplified and overexpressed in several oropharyngeal cancer patients, with amplification as one of the underlying molecular mechanisms of overexpression of the EGF-R gene in the patients. Of the 84 patients examined for EGF-R gene involvement, 21/84 (25%) oropharyngeal tumours comprising 19/66 (29%) cancers of the oral cavity and 2/18 (11%) hypopharyngeal cancer patients, demonstrated EGF-R gene amplification with several of these patients showing increased expression as well. The size of the EGF-R *Eco*RI genomic fragments, indicated that overexpression of EGF-R was not due to rearrangement or translocation of the gene to another chromosome, in these patients. A correlation between EGF-R gene amplification and clinical parameters of the malignancy was not observed in our patient population.

Amplification and overexpression of EGF-R has been reported in human cancers including glioblastomas [23] bladder cancers [24], gastric carcinoma [25] and oesophageal tumours [22], with several studies indicating utility of EGF-R as a prognostic marker. In head and neck cancers, EGF-R amplification studies demonstrate a 3- to 10-fold amplification of EGF-R gene in 0–19% of the patients screened [26–28]. Leonard *et al.* [26] reported a 3- to 10-fold amplification of the EGF-R gene in 10% (7/66) patients. Ishitoya and co-workers [27] demonstrated EGF-R amplification in head and neck cancers, with no correlation between EGF-R amplification/expression and clinical stages of the tumours. Whereas, on the other hand, Eisbruch and co-workers [28], demonstrated EGF-R amplification and rearrangement in head and neck cancers as uncommon events, with none of their 17 samples showing EGF-R gene amplification. In comparison, our data show 25% of oropharyngeal cancers with a 3- to 10-fold EGF-R amplification, and several of the tumours showing overexpression of the gene. Rikimaru *et al.* [29], have reported EGF-R amplification, increased level of EGF-R synthesis and hEGF binding in 4 cases of tumour lesions and corresponding cell lines in SCC of oral cavity. The authors concluded that increased capacity for hEGF binding plays a more important role than gene amplification in the development of SCC of head and neck. Gene amplification, increased synthesis or decreased degradation of EGF-R molecules may be responsible for increased hEGF binding. Our data emphasises that in a certain proportion of oral cancers, EGF-R gene amplification may be the mechanism underlying increased expression of EGF-R and consequent increased hEGF binding.

Consistent clinical and molecular differences have been reported in oral cancer as observed in the Indian population and the patients from the USA and the UK [30, 31]. A comparatively high incidence of 40% of total malignancies, clinically distinctive premalignant stages, high multiple oncogene amplification and high frequency of H-*ras* point mutations are hallmarks of the Indian oral cancers [1, 3, 31]. In contrast, in the USA and the UK in a 1–4% of total cancer incidence, there are infrequently observed precursor lesions, a low frequency of oncogene amplification and H-*ras* mutations are reported in oral cancers [31]. The striking differences could be due to several factors such as the tobacco-chewing habits, strain/species of tobacco used, mode of usage, the curing process and perhaps genetic predisposition. The EGF-R amplification and overexpression in our patient population

emphasises an additional molecular alteration in the oropharyngeal cancers, in the multi-step process of the carcinogenesis. Further, modulation of such a receptor by physiological agents could be of clinical interest in the malignancy.

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