EXPRESSION OF EPIDERMAL GROWTH FACTOR RECEPTOR AND p53 ONCOPROTEIN IN PREMALIGNANT AND MALIGNANT ORAL LESIONS

ANANDA KUTTAN NITHYANAND
(BDS, The University of Kerala, Trivandrum, Kerala, India, 1989)

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NAME: Ananda Kuttan Nithyanand

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EXAMINING COMMITTEE:

Chair: Dr. G.F. Tibbits

Dr. N.M.G. Bhakthan
Senior Supervisor

Dr. M.P. Rosin

Dr. L. Zhang
Faculty of Dentistry
Department of Oral Biology
U.B.C.

Dr. R.M. Shah
External Examiner
Faculty of Dentistry
Department of Oral Biology
U.B.C.

Date Approved: September 15th '94

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Expression of epidermal growth factor receptor and p53 oncoprotein in premalignant and malignant oral lesions.

Author: _____
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Ananda Kuttan NA
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ABSTRACT

Tissue markers such as epidermal growth factor receptor (EGFR) and oncoprotein p53 actively participate in oral carcinogenesis, in initiation and/or promotion of the process. The potent mitogen epidermal growth factor (EGF) mediates its growth responses through its receptor (EGFR), a transmembrane glycoprotein. The expression of mutant p53 has been shown to be present in many human solid tumours of squamous origin including oral squamous cell carcinomas. The wild-type p53 acts as a tumour suppressor recessive gene, the inactivation of which could lead to malignant transformation. Very few studies have been cited in the literature that deal with the over-expression of EGFR and the expression of mutant oncoprotein p53 in oral dysplasia and squamous cell carcinomas. These studies, however, are not consistent with each other in their results. It is hypothesized that the expression of growth factor receptors may increase with the appearance of carcinoma through various degrees of dysplasia when compared to normal stratified squamous oral epithelium. It is possible that the cells need more receptors as the disturbance in growth progresses to dysplasia of a higher grade or into malignancy from a state of benign hyperplasia. The wild-type p53 protein appears to block the cell cycle at the G1/S boundary of the cell cycle and to produce G1 arrest which provides sufficient time for DNA repair. On the other hand, its mutant variety forms complexes with the wild-type, inactivates the wild-type, and thus prevents the wild-type from carrying out its normal function. This may, in turn, lead to abnormalities in the cell cycle repair system and eventually to malignant transformation. Mutations in p53 gene may provide the cells with a growth advantage over the normal cells leading to malignant transformation.

This study investigated the expression of EGFR in different degrees of dysplasia and squamous cell carcinomas (64 Canadian samples) using monoclonal antibodies with the 'ABC' staining methods. There were no significant differences in EGFR staining either in intensity or in the epithelial layers stained among the normal oral epithelium, hyperplastic and dysplastic lesions. In addition, no significant difference was noted between keratinized and non-keratinized specimens and among lesions from different sites. Oral SCCs demonstrated significantly stronger staining than the normal oral mucosa, hyperplastic and dysplastic lesions ($p=0.0011$). At this time, the conflicting data on the EGFR expression in oral dysplastic lesions indicate that this receptor is not a good marker for oral dysplasia. Because most of the available data (including the present study) show that the majority of oral SCC over-express EGFR, this receptor may be useful in the diagnosis and treatment of some oral cancers. Monoclonal antibodies directed against EGFR may help with the

diagnosis, prognosis of premalignant and malignant lesions, and palliative treatment of malignant lesions in the head and neck region.

The expression of oncoprotein p53 in squamous cell carcinoma (23 Indian samples) of the oral cavity using monoclonal antibodies with the 'ABC' staining methods. 14/23 Cases were positive for anti-p53 antibody staining and most of the positive specimens showed diffuse pattern of staining, where the cells were seen as small islands. Statistical analysis shows that there is a significant correlation between the expression of p53 and the tobacco chewing habits. Frequent chewers (>5 pan/day) had Fisher exact value of 0.0011. As the mutant oncoprotein p53 seems to play a role in malignant transformation, more cells are likely to show a strong nuclear staining reaction when compared to normal or hyperplastic oral epithelia. The significance of p53 oncoprotein in head and neck tumourigenesis is yet to be understood. Further studies such as Direct Nucleotide Sequencing, Polymerase Chain Reaction (PCR) and Single Strand Conformation Polymorphism (SSCP), addressing the possible involvement of this oncoprotein are warranted to get an insight into the molecular details of the mechanism.

This work is dedicated to my parents Shrinivas and Sharada, and to my nephew Shajay Bhooshan.

"TAMASO MA JYOTIRGAMAYA."

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Chapter 1. INTRODUCTION

Cancer is the second most common cause of mortality in the world today. More than half of the new cases of cancer recorded in the world occur in developing countries, where 70-80% of the world population lives (Parkins, Läära and Muir, 1988). Oral squamous cell cancers (SCC) comprise a significant fraction (5%) of all the malignancies among the world population (ACS, 1988) and more than 40% of all malignancies in South Asia (Sanghvi, 1981; Daftary et al., 1991). Although it forms a small proportion of all malignancies in Europe and North America, the incidence is rising, especially among young adults (La Vecchia et al., 1992; Johnson and Warnakulasuriya, 1993). Males over 50 years of age are more affected than their female counterparts of the same age, at a ratio of 3:1. In Western countries, the most common intraoral site for SCC is the lateral border of tongue followed by floor of the mouth, gingiva and buccal mucosa. On the other hand, in South-East Asian countries, there is site predilection for buccal mucosa because of tobacco chewing habits. This is followed by lateral border of tongue, floor of the mouth and gingiva (Sankaranarayanan, 1990). Tobacco chewing and smoking, alcohol, nutritional deficiencies, and dietary habits have been implicated in the etiology of oral cancers (Jayant and Deo, 1986; Shklar, 1986; Blot et al., 1988; De Stefani et al., 1990).

The process of multistep carcinogenesis may be divided into the basic stages of initiation, promotion and progression (Spandidos, 1986). The initiation of cancer probably involves either activation of oncogenes such as c-erb-B_1 or mutations in tumour suppressor genes such as p53. The changes in the genes may be caused by chemical carcinogens, radiation or viruses, and are irreversible. The latter stages of promotion and progression involve further genetic alterations, culminating in full-blown cases of squamous cell carcinomas. It is likely that a combination of genetic lesions contributes to oral cancers, as there is no conclusive evidence to indicate which oncogenes are responsible for different stages of carcinogenesis in the aforesaid diseases (Field and Spandidos, 1987; Scully, 1993). The common mechanism for cancer initiation appears to be through DNA damage which leads to uncontrolled proliferation. Furthermore, cancer cells do not have the same restraints of proliferation and differentiation that affect normal cells. Some cases of disturbances in growth such as hyperplasia or dysplasia may be preceded by an increase in the expression of both the epidermal growth factor receptor (EGFR), and other substances related to cellular proliferation such as proliferating nuclear antigen, PCNA-Cyclin (Tsuji et al., 1992), and p53 (Rogel et al., 1985; Nishioka et al., 1993).

1.0 Normal Histology

Like all mucous membranes, the oral mucosa is composed of a combination of epithelial tissue and connective tissue. The epithelial portion of the oral mucosa is stratified squamous type, that is, the epithelial cells are mostly flat and are several layers deep. The basal layer of epithelial cells, which rests upon the connective tissues, is composed of cuboidal rather than flat cells. As new epithelial cells are produced by mitosis in the basal layer, some of the basal cells and the cells superficial to them are forced outward, and eventually they reach the surface. In some parts of the mouth where the oral mucosa is subject to considerable wear and tear, such as hard palate and the gingiva, the surface epithelial cells are not sloughed off. Instead, they lose their nuclei and their cell boundaries and form a noncellular, tough, protective layer on the surface of the stratified squamous epithelial cells. This tough layer is called the *keratin layer*, and the epithelium on which such a layer occurs is called *keratinized epithelium* (Bhaskar, 1986; Fig. 1). When there is prolonged low-grade irritation to the oral mucosa, the non-keratinized epithelium may become keratinized. The keratin layer wears with use, but it is continuously replaced by the aging cells beneath it.

The connective tissue of the oral mucosa is composed chiefly of fibrous connective tissue in which are blood vessels and nerves. It is separated from the overlying stratified squamous epithelium by a thin basement membrane. In most regions the junction between the connective tissue and the epithelium is an irregular boundary with projections of connective tissue extending like fingers up into the epithelium, but not reaching the surface. In some areas of the oral mucosa, these projections are characteristically much longer and more numerous than in other areas. This irregularity of contact surfaces between the two tissues serves to increase the area from which the epithelium can receive nourishment from the underlying connective tissue.

1.1 Premalignant and Malignant Oral Lesions

The World Health Organization (1980) has defined a precancerous lesion as "morphologically altered tissue that is more likely to turn malignant than its apparently normal counterpart." In the oral cavity, lesions such as leukoplakia, erythroplakia, and erythro-leukoplakia are considered to be premalignant. Leukoplakia is a white, non-

scrapable lesion of the oral mucosa, which cannot be characterized clinically or pathologically as any other disease (Pindborg, Daftary and Mehta, 1977). Erythroplakia is a clinical, descriptive term for lesions of the oral mucosa that constitute bright-red, velvety plaque, and which cannot be characterized clinically or pathologically as being due to any other condition (WHO, 1980).

1.11 Classification and High Risk Sites for Oral Premalignant Lesions

WHO (1980) has suggested that the incidence of the premalignant lesions usually shows a predilection for certain sites: the most common site is the commissures followed by the buccal mucosa, tongue, vermillion border, floor of the mouth and soft palate. Alveolar ridge, hard palate and gingiva form rare sites for leukoplakic lesions. Premalignant lesions associated with smoking usually appear on lips and commissure whereas the lesions associated with chewing habits tend to be limited to areas like the buccal mucosa and hard palate. Unlike a Western population, floor of the mouth leukoplakia is not that common among Asians (Banoczy, 1984). Erythroplakia and sublingual keratosis show the highest degree of risk of turning malignant (50% and >40% respectively). The degree of risk associated with oral precancerous lesions are: Leukoplakia 4-18, Erythro-leukoplakia 32, Oral submucous fibrosis 3-6, and Lichen planus 1-10 (values expressed in percent).

Pindborg et al. (1963) have classified leukoplakic lesions based on their clinical appearance; 1) A homogeneous type, where the surface presents with smooth or wrinkled consistency with cracks and fissures, and 2) A speckled or nodular type with white patches and erythematous bases or erythroplakic areas mixed with white excrescences, giving the whole lesion a 'speckled' appearance. Risk of premalignant lesions depends on: 1. Degree of dysplasia, and 2. Location of the lesion. Dysplasia is the name given to disordered proliferation of tissue which particularly affects epithelium where there is normally a well-defined pattern of maturation. Dysplasia is characterized by loss of the normal uniformity of individual cells and a tendency for disorganization of the tissue as a whole. It can be a prelude to malignant change (WHO, 1980).

The changes in the epithelium during the development of premalignant lesions can be classified into different degrees of dysplasia (mild, moderate and severe), a significant percent of which bears a certain risk of malignant transformation. The histologic features of dysplasia are: (1) Loss of polarity of the basal cells; (2) Basilar hyperplasia; (3) Altered nuclear cytoplasmic ratio (N/C ratio); (4) Drop-shaped rete pegs; (5) Irregular epithelial stratification; (6) Abnormal mitoses; (7) Mitotic figures in the superficial layers of the epithelium; (8) Cellular pleomorphism; (9) Nuclear hyperchromatism; (10) Enlarged nucleoli; (11) Loss or reduction of intercellular adhesion; and (12) Individual cell keratinization in the spinous layer of cells (Katz, Shear and Altini, 1985; Pindborg, Homstrup and Reibel, 1985; Regezi and Sciubba, 1993). Dysplasia can be graded as mild when two of the above-listed features are present, moderate when two-to-four features are present and severe when more than 5 of the aforesaid features are present. Carcinoma-in-situ (CIS) lesions show top-to-bottom dysplastic changes in the epithelium with no signs of invasion into the underlying connective tissue (Banoczy and Csiba, 1976). The likelihood of carcinoma developing from dysplastic lesions has been calculated to be 11%, 33% and 56% for cases of dysplasia in the ascending order of its degree respectively, suggesting that the risk of malignant transformation is directly related to the degree of dysplasia (Shibuya et al., 1986). It is noteworthy that not all dysplastic lesions turn into squamous cell carcinoma and some of them may even show regressive changes (Lind, 1987).

1.2 Oral Squamous Cell Carcinoma (OSCC)

The clinical appearance of small, early oral squamous cell carcinoma may vary from a white, thickened, or verrucous lesion to a velvety plaque or a chronic painless ulcer. More than 90% of oral SCCs are found to be moderately or well differentiated tumours and metastasis generally occurs in about 80% of the lesions to submandibular or cervical lymph nodes (Murthi et al., 1986). Patients with primary oral carcinoma have a higher risk (4-13%) of developing a second primary tumour of the upper aerodigestive tract, and the prognosis depends on the nature of metastasis (Mohit-Tabatabai et al., 1986; Kissane, 1990).

1.21 Etiology of Oral Cancer

A variety of local chronic irritations, acting alone or in combination with each other, will produce leukoplakic lesions in many susceptible individuals. The factors which have been suggested to be of etiologic importance in the case of premalignant oral lesions are tobacco (both smoking and chewing types), alcohol, spices (from diet), and genetic susceptibility (Shklar, 1986; Blot et al., 1988). Other factors like papilloma viruses, ionizing radiation, ultraviolet rays, and such chronic infections as candidosis may also play a synergistic role in the process of carcinogenesis. The lesion commences like a protective barrier to prevent further injuries to the underlying tissues and carries a greater risk of malignant transformation, especially in regions such as floor of the mouth where the lining epithelium is that of the non-keratinized type (William, 1990). The relative risk for oral cancer increases with duration and quantity of a carcinogen exposure and with the combination of several carcinogens (Langdon and Partridge, 1992).

1.22 Chewing and smoking habits and genotoxic effects

The most common source of chewing tobacco in several countries in the Asian region is in the betel quid or *pan*, which is a preparation of betel leaf (Piper betel), areca nut (the fruit of areca palm tree), tobacco, lime and catechu (a resinous extract from the Acacia tree). Sometimes various condiments such as cardamom or cloves are also added in the quids. This is commonly used in northern parts of India (Sanghvi, 1981) and also in Sri Lanka (Seneviratna and Uragoda, 1973). In Kerala, a southwestern province of India, the betel quid does not contain any catechu or spices (Sankaranarayanan et al., 1990). The chewing of betel quid with tobacco has been established as the principal etiological factor for the high incidence of oral cancer in India and some other South-East Asian countries (IARC, 1985; Warnakulasuriya, 1987; Sankaranarayanan, 1990; Daftary et al., 1991). Tobacco has also been implicated as one of the major etiological factors in the development of other cancers including lung (Iggo et al., 1990; Bartsch et al., 1992) and bladder (Hollstein et al., 1991; Sidransky et al., 1991). Potent carcinogenic agents such as tobacco- and areca-nut-specific nitrosamines have been separated from tobacco and areca-nut, the major ingredient of the betel quid (Stich et al., 1983; Nair et al., 1985; Stich and Anders, 1989). Many studies have revealed that the spontaneous generation of reactive oxygen species (ROS) occur during different stages of carcinogenesis (Stich and Stich, 1982; Stich et al., 1984) and that such free radical scavengers as β-carotene may

induce remission of some preneoplastic lesions (Stich et al., 1988; Stich and Anders, 1989; Nair et al., 1990). There is an increasing body of experimental evidence that such ingredients of the chewing mixture as slaked lime plays an important role in the production of reactive oxygen species (ROS) (Nair et al., 1987; Stich and Tsang, 1989). Free radicals such as superoxide anion, hydroxyl anion and hydroperoxide anions are highly reactive and may bring in changes such as oxidative damage to cellular organelles and DNA, in turn, leading to genetic damage. This has been experimentally proven by micronuclei test-the presence of micronuclei in exfoliated cells, especially that of the oral cavity (Nair et al., 1991; Jaju et al., 1992; Nair et al., 1992; Rosin, 1992; Hoffmann et al., 1994; Patel et al., 1994). Many investigators have shown that there is increased occurrence of micronuclei (Stich, Stich and Parida, 1982; Stich and Rosin, 1984; Dave et al., 1991) and/or a higher frequency of sister chromatid exchanges in peripheral lymphocytes in chronic tobacco/betel quid chewers (Ghosh and Ghosh, 1984; Ghosh, Sharma and Ghosh, 1988; Kayal et al., 1993). Tobacco-specific nitrosamines (TSNA), known to be potent carcinogens in experimental animals, may also be causative agents for oral cancers in humans; they may act alone or in combination with other ingredients such as gum catechu and special flavoring agents (e.g., *pan masala* used in northern parts of India) to bring about the genotoxic effects (Nair et al., 1985; Nair et al., 1987; Jaju et al., 1992). Studies have shown that lime together with tobacco and areca nut elevates the pH (becomes highly alkaline) in the oral cavity in betel quid chewers. Lime may increase the cell turnover by 'killing' the cells and also the likelihood of heritable mutations being transmitted to daughter cells before complete DNA repair (Cohen and Ellwein, 1990; Thomas and McLennan, 1992).

1.3 Proto-oncogenes, Oncogenes and Suppressor genes

Several genetic alterations that perturb normal cellular growth and developmental control mechanisms can lead to abnormal proliferation and differentiation (Brown, 1992). These include point mutations, deletions, translocations, amplifications and gene rearrangements, and occur primarily in two classes of genes, namely oncogenes and tumour suppressor genes. While mutation or amplification of certain genes can lead to abnormal cellular proliferation, loss or mutation of tumour suppressor genes (which normally suppress abnormal proliferation), can activate neoplastic transformation (Hunter, 1991). Proto-oncogenes (cellular oncogenes: c-onc) are present in normal cells, controlling cell proliferation and differentiation. Oncogenes are closely related to proto-

oncogenes, but they have mutated to produce abnormal protein products or to allow gene over-expression leading to a loss of the normal constraints on their activity (Bishop, 1991). The genes which act by regulating the oncogenes or by producing substances that antagonize the actions of oncoproteins, are termed tumor suppressor genes (emerogenes, anti-oncogenes, or recessive cancer susceptibility genes) (Scully, 1992). Mutations in tumor suppressor gene p53 is considered to be one of the salient features of a large spectrum of human malignancies (Hollstein et al., 1991). Amplification or over-expression of EGFR, *int-2*, and *c-myc* appear to be the most documented findings both in neuroblastoma cell lines (Libermann et al., 1985) and tumour tissue from oral squamous cell carcinomas (Leonard et al., 1991). Multiple oncogenes may be involved in oral carcinoma, especially *c-myc* with *H-ras* (Field and Spandidos, 1987) or *c-erb-B-1* (Yamada et al., 1989; Saranath et al., 1992).

1.31 Molecular Lesions in Oral Cancer

A mutation is a change in the nucleotide sequence of the DNA molecule which may occur within a gene or in the intergenic regions. If a mutation occurs in an intergenic region it will probably be silent and have no detrimental effect on the cell. But when the mutation occurs in a gene, it may alter the gene product and generate observable changes in the organism which is referred to as a change in phenotype. Mutation can happen at the level of DNA sequences (point mutation, insertion, deletion and inversion) or at the level of gene (Brown, 1992). The function of a gene can be blocked at the protein level by altered gene(s) which encodes a mutant product capable of inhibiting the wild-type gene product in a cell. Such a mutation would be 'dominant negative' because its phenotype is manifested in the presence of the wild-type gene, but it inactivates the wild-type activity. Herskowitz (1987) referred to this kind of mutations as "*antimorphs*, 'antagonistic mutant genes' having an effect virtually contrary to that of the gene from which they were derived." Basically, the process involves the inhibition of function of a wild-type gene product by an overproduced inhibitory variant of the same product. If a protein has multiple functional sites (e.g., multimeric nature), a derivative capable of interacting with wild-type polypeptide but otherwise defective will be inhibitory, especially if it forms multimeric forms which are non-functional. Hence, dominant negative mutant proteins will retain an intact functional subset of the domains of the parent wild-type protein, but have the complement of this subset either missing or altered so as to be non-functional (Rovinski et al., 1988; Lane and Benchimol, 1990). The

dominant negative function is achieved by the formation of inactive complexes (hetero-oligomers) between the mutant and wild-type p53 proteins (Lane, 1992), and this may, in turn, affect the sequence specific DNA-binding activity of p53 (Hinds et al., 1990; Bargonetti et al., 1991; Momand et al., 1992).

Mutation in one of the two alleles of the gene that act in a dominant manner over the normal phenotype (wild-type) of allele leads to a gain of function, or uncontrolled proliferation. Mutation of one of the allele followed by loss of, or a reduction to homozygosity in the second allele will lead to a loss of regulatory effect on cell proliferation (Levine, Momand and Finlay, 1991). p53 oncoprotein appears to block the progression of cells through the late presynthetic phase of replication: mutant forms may fail to maintain this control or may even stimulate cell proliferation (Field 1992; Nishioka et al., 1993). The most frequently documented genetic change in human oral cancer is in the short arm of chromosome 17 (17p), in the region of the suppressor gene p53. Mutations or deletions in onco-suppressor genes may give rise to defective suppressor proteins thereby leading to the loss of tumour suppressor function (Bishop, 1991; Scully, 1993). Such genetic markers as p53 may play an important role in the development of oral squamous cell cancers, and give some insight into the molecular mechanisms that are responsible for neoplastic transformation (Field et al., 1991; Zhang et al., 1993). Mitogenic agents such as epidermal growth factor produce pleiotropic response involving the activation of various biochemical processes that are responsible for cellular growth and development. The interaction of the growth factor and its effects on oral mucosa will be described in subsequent sections.

1.4 Mitogen-Activated Protein Kinases (MAP kinases)

The growth signals obtained at the cell surface are transduced to the nucleus through a cascade of tightly regulated phosphorylation events mediated by kinases such as protein kinase-C (PKC), casein kinase and mitogen activated kinases (MAPK). Recent studies have shown that the ligands that bind to epidermal growth receptor may also act through the receptor-specific threonine kinases (Northwood et al., 1991). There is an increasing body of evidence which suggests that these kinases play an active role in the cellular proliferation and/or differentiation (Blenis, 1990). Since many membrane-associated growth factor receptors have been shown to exhibit ligand-dependent protein-

tyrosine kinase activity, it is thought that tyrosine phosphorylation is an early event in the propagation of signal transduction cascade. Furthermore, oncogenes that are improperly regulated have been shown to induce cellular transformation via aberrant phosphorylation of tyrosine residues (Kohno, 1985; Ben-David et al., 1991). An important kinase among these enzymes, originally named microtubule-associated protein kinase-2 (MAP-2) because it phosphorylates microtubule-associated protein, is now referred to as mitogen-activated protein kinases (MAP kinase) (Anderson et al., 1990; Gómez and Cohen, 1991).

The MAP kinase (MAPK) requires both tyrosine and threonine phosphorylation for activity and recent studies has revealed that MAP kinase is not a single enzyme, but a family of kinases that have been to referred to as Extracellular signal Regulated Kinases (ERKs) (Sturgill et al., 1988; Boulton et al., 1990; Seger et al., 1991; Pelech and Sanghera, 1992; Pelech, 1993). Depending upon the *in vitro* substrates that have been used to characterize these kinases, they are again subdivided into myelin basic protein kinases or MBP (Ahn et al., 1991), ribosomal S6 protein kinase-kinases (RSKs) (Anderson et al., 1991; Davis, 1993), and EGF-receptor threonine kinases or ERK (Ahn et al., 1990, 1991; Northwood et al., 1991; Takishima et al., 1991). The role of MAPK in signal transduction pathways may be summarized as follows: Signals initiated by receptor tyrosine kinases (RTKs), by protein kinase-C or by signal transducing G-proteins rapidly converge and activate the cytoplasmic MAPK via phosphorylation of tyrosine and threonine (Fig. 2). The activated MAPK then activate the RSKs by serine/threonine phosphorylation, which helps the RSKs to phosphorylate their targets such as ribosomal S6 protein (Blenis, 1991; Davis, 1993; Hsuan, 1993). One of the MAPKs, epidermal growth factor receptor-tyrosine kinase (ERT kinase) is proposed to down-regulate the activity of EGFR soon after ligand binding while RSK has the potential to turn off a variety of signal transducers that are modulated by tyrosine- or serine/threonine phosphorylation via other kinases that may, in turn, activate RSKs (Pelech and Sanghera, 1992; Hsuan, 1993). Many extracellular stimuli that influence cell proliferation and differentiation, including growth factors, cytokines and hormones can stimulate MAP kinases and the activation of MAPKs require phosphorylation of tyrosine and threonine residues (Nishida and Gotoh, 1993). Studies concerning the mechanism of activation of MAPKs are in progress now (Nebreda, 1994).

1.5 Tumour Markers

Neoplastic transformation is a multistage process which involves a number of aberrant genetic events that are progressively associated with the acquisition of a state of malignancy. Many oral cancers are known to arise from premalignant lesions. The substances (e.g., oncoproteins, enzymes, growth factor receptors), that are used to study the changes in preneoplastic as well as neoplastic tissues, are referred to as tumour markers (Malkin, 1987). A number of growth factors such as epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) and -beta (TGF-β) show altered expression, or disregulated production during the process of carcinogenesis (Goustin et al., 1986). An increased production of growth factors may indicate the presence of abnormal growth or some disturbances in cellular proliferation (Wong et al., 1989; Eibling, Wagner and Johnson, 1990; Wong et al., 1990). Altered proliferation or differentiation may also be induced by deregulation of tumour suppressor genes such as p53 (Hinds et al., 1990; Xiong et al., 1993; Donehower, 1994).

1.6 Epidermal Growth Factor Receptor and Its Functions

Receptors may be defined as cellular components that monitor the status of the extracellular environment. They interact with regulatory molecules from outside the cell, and thereby transmit a biochemical signal into the cell (Scully, 1992). They are involved in many essential cellular functions including proliferation and differentiation. The expression of a structurally altered receptor or the over-expression of a normal receptor itself, may contribute to uncontrolled cellular proliferation and differentiation (Downward et al., 1984; Seemeyer and Cavenee, 1989). For cell growth and division to occur, a large variety of metabolic processes mediated through the growth factor-activated kinases must be carefully coordinated in the cell. Growth factors and hormones possess the ability to trigger a complex 'pleiotropic growth response' in their target cells. It has been shown that the epidermal growth factor receptor is involved in both normal and abnormal proliferations of epithelial tissues (Carpenter and Cohen, 1990). Mitogen-activated protein kinases may function as convergence points in pathways of cellular growth and differentiation (Michelle, 1989; Pelech, 1993). The determination of the functional role of the receptor-associated kinases in modulating receptor function or the process of mitogenic signal transduction, may give some new insights into the molecular basis of tumourigenesis in the head and neck region (Williams et al., 1993).

Activation of the EGF receptor occurs on binding to the ligand and this, in turn, initiates a cascade of cellular events by triggering the intrinsic tyrosine kinase activity (Carpenter, 1984). This is immediately followed by a rise in cytosolic calcium level and receptor internalization which results in degradation of the receptor (Downward et al., 1984; Carpenter, 1987). Studies have shown that the tyrosine kinase activity is necessary for all subsequent receptor actions, including internalization (Chen et al., 1987). Activation of the tyrosine kinase at the plasma membrane is sufficient for cell division and as a result the membrane-bound forms of the ligands such as pro-EGF or pro-TGF-α should be capable of eliciting cell growth signals to the neighboring receptor-bearing cells (Wells et al., 1990).

An increased density of EGF receptors is found in many epithelial tumour cells including those of the oral cavity (Burgess, 1989; Salomon et al., 1990; Shrestha et al., 1992). Binding of EGF, TGF-α, and other ligands to the EGF receptor (EGFR), activates tyrosine-protein kinase activity of the receptor resulting in phosphorylation of the receptor and other substrates in the immediate surroundings of the receptor (Ushiro and Cohen, 1980; Carlin et al., 1983; James and Bradshow, 1984; Sporn and Roberts, 1988). Activation of the kinase system generally induces a transient internalization of the EGF receptor (Beguinot et al., 1985; Michelle, 1989), and this could be one of the mechanisms that regulate EGF-induced proliferation in susceptible tissues (Damjanov, Mildner and Knowles, 1986; Schlessinger, 1988). Internalization and degradation of the receptor appear to abrogate its long-term actions and in the absence of this attenuation mechanism, even low quantities of the ligand would result in uncontrolled proliferation (Schlessinger, 1988; Wells et al., 1990; Carpenter and Cohen, 1990; Fantl et al., 1993). It is suggested that the over-expression of the receptor is due to gene amplification (c-erb-B-1; chromosome 7) as evident from the studies conducted on A-431 carcinoma cells or animal models (Wong and Biswas, 1986; Shin et al., 1990), and oral SCCs *in vivo* (Cowley et al., 1984; Yamamoto et al., 1986; Yamada et al., 1989; Saranath et al., 1989). The amplified oncogenes generally show increased transcripts or over-expressed oncoproteins along with co-amplification of other oncogenes (Lee, 1992). The co-expression of other oncogenes such as c-myc and H-ras also may account for the malignant transformation (synergistic action). The primary reason for over-expression of EGFR may be the over-production of the protein itself or over-expression of the messenger RNAs (Kawamoto et al., 1991; Kearsley et al., 1991; Yamada, Takagi and Shioda, 1992).

1.61 Epidermal Growth Factor Receptor and Oral Mucosa

Many investigators have studied the expression of epidermal growth factor receptor (EGFR) in normal and dysplastic and neoplastic lesions of oral mucosa SCCs. Over-expression of EGFR was observed in a certain percentage of the studied cases (Tables 1 and 2). In a series of studies conducted by Kearsley et al. (1991), 24/42 (57%) cases of oral carcinomas showed over-expression of the receptor protein. Although some studies failed to show any presence of EGFR amplification in head and neck squamous cell carcinomas (Eisburch et al., 1987), amplification of EGFR has been reported in oral cancer cases ranging from 4-29% (Ishitoya et al., 1989; Saranath et al., 1992). Amplification of the oncogene may result in above normal levels of transcripts for the oncoprotein or over-expression of the protein itself (Saranath et al., 1993). Over-expression of EGFR is likely to show a direct relationship to the degree of differentiation and aggressiveness of tumours of squamous origin (Ishitoya et al., 1989; Kearsley et al., 1991; Christensen et al., 1992; Kunikata et al., 1992; Christensen et al., 1993; Grandis and Tweardy, 1993). Tables 1 and 2 summarize the results of studies on over-expression and amplification of EGFR gene conducted by various investigators with the help of advanced techniques such as labeled biotinylated antibodies, and Southern and Northern Blots, respectively.

As shown in Tables 3 and 4, in normal epithelium, EGF receptors are detectable predominantly on basal cells with a plasma membrane distribution progressively diminishing in suprabasal layers (Partridge et al., 1988; Shirasuna et al., 1991). In areas such as lateral border of tongue, all layers of the epithelium tend to express the receptor (Whitcomb, Eversole and Lindermann, 1993). Immunoreactive EGF receptors also have been localized to the first 2 or 3 layers of basal and suprabasal epidermal keratinocytes (Nanney et al., 1984) and in conditions such as psoriasis where the tissue turnover rate is proposed to be relatively high (Nanney et al.,1986). A membrane-bound positive staining reaction of EGF receptors has also been reported in epidermal premalignant and malignant lesions (Groves, Allen and McDonald, 1992; Raikhlin, Petrov and Serre, 1992). In oral keratoses or where acanthosis is extensive, the spinous cell layers show moderate intensity of staining, but with the appearance of squamous cell carcinomas the staining extends to all layers of the epithelium (Shirasuna et al., 1991; Christensen et al., 1992).

There are clear differences in the distribution of the EGFR in neoplastic epithelia when compared to adjacent 'normal' mucosa. Areas in the adjacent normal epithelium where there is microinvasion, the tumor cells may show a strong staining (*the honeycomb pattern*) to anti-EGFr staining. Many tumour islands show a strong staining to anti-EGFr antibody but some tumours may show a weak staining pattern (Kearsley et al., 1991). Poorly differentiated SCCs tend to show a weaker staining than well differentiated cases and keratin pearls may remain negative for anti-EGFr staining (Partridge et al., 1988; Yamada, Takagi and Shiyoda, 1992). Dysplastic epithelium expresses above normal levels of mRNA transcripts for the receptor protein leading to the conclusion that there is over-expression of the receptor (Todd et al., 1991; Grandis and Tweardy, 1993; Prime et al., 1994). The distribution of EGFR can be classified into two types according to the immunohistochemical pattern of staining: 1) Cell membrane positive type (CMB), where all cell membranes of the epithelial cells in a given field show a positive reaction to the antibody (Kearsley et al., 1991; Yamada, Takagi and Shioda, 1992); 2) Focal cell membrane positive (FCMB), where the positive cells are scattered in nature in a given field (Kunikata et al., 1992; Yamada, Takagi and Shioda, 1992). In normal squamous cells of the oral mucosa, the staining for EGF receptor is confined to cell membranes of the basal and suprabasal layers (Whitcomb, Eversole and Lindermann, 1993), while squamous cell carcinomas will show focal cell membrane positive staining, whole plasma staining, and sometimes no staining or a combination of these patterns (Kunikata et al., 1992). The 'normal' cells adjacent to squamous cell carcinoma tend to show a plasma membrane-bound staining. Hyperplastic and dysplastic lesions will be positive in the basal and suprabasal layers with a progressive decrease in the intensity of staining as it moves towards the superficial layers (Shirasuna et al., 1991).

Over-expression of epidermal growth factor receptor and c-erb-B_1 amplification has been reported in squamous cell carcinomas of bladder (Neal et al., 1985), breast (Nicholson et al., 1988; Ozawa et al., 1988; Lewis et al., 1990), cervix (Gullick et al., 1986), colo-rectum (Koike et al., 1993; Yamaguchi et al., 1993), liver (Fukusato et al., 1990), lung (Berger et al., 1987; Takahashi et al., 1991), and stomach (Lee et al., 1991; Jang et al., 1993). It is possible that, during the process of carcinogenesis, there exists an autocrine mechanism of regulation for the secretion of the growth factor and its receptor (Partridge et al., 1989; Todd et al., 1989, 1991).

1.7 Onco-suppressor gene p53

p53, a proto-oncogene product, is a cellular protein expressed in low levels in non-transformed cells. On the other hand, tumour-derived cells and transformed cell lines show a many-fold increase in the expression of p53 (Koeffler et al., 1986). Unlike the proteins of non-transformed cells, the mutant protein is likely to form complexes with transforming proteins of viruses such as SV40 leading to the acquisition of a stable conformation than the wild-type protein. The results of several studies have supported the view that increased levels of p53 effect changes in the phenotype of normal cell and that over-expression of the protein leads to the immortalization of cell lines (Rogel et al., 1985; Lane and Benchimol, 1990). It is suggested that over-expression of p53 is a common event in the multistep carcinogenesis in colo-rectal carcinomas (Baker et al., 1989; Hollstein et al., 1991) and oral squamous cell carcinomas (Warnakulasuriya and Johnson, 1992; Zhang et al., 1993).

1.71 Functions of p53

Finlay et al. (1988) have demonstrated that the wild-type sequence of the proto-oncogene does not code for a transforming protein, but loss of function or mutation in the gene possibly transactivates the protein culminating in malignant transformation. The p53 protein appears to block the progression of cells through the late G_1 phase of replication: mutant forms fail to perform this function and may even stimulate cell proliferation (Fearon and Vogelstein, 1990; Montenarh, 1992). A clear relationship has been established between p53 expression and heavy smoking and alcohol consumption and the protein appears to play a central role in oral carcinogenesis, possibly being mutated by carcinogens in tobacco and alcohol (Gusterson et al., 1991). Studies using 'high-tech' devices has revealed that p53 has a possible role in the control of mRNA synthesis and/or maturation (Lee, 1992a; Soussi et al., 1994). Analysis of non-transformed cells, non-SV40-transformed cells, and growth-arrested and -stimulated mouse cells has revealed that the p53 plays an important role in the cell cycle and the subcellular distribution of the protein varies throughout the cell cycle (Gusterson et al., 1991; Kastan et al., 1991; Nishioka et al., 1993). p53 forms high molecular weight complexes with E6 proteins of the human papilloma virus types 16 and 18 (Werness et al., 1990; Park et al., 1992; Kim et al., 1993), heat shock proteins (Finlay et al., 1988) and protein kinases (Kraiss et al., 1991). Since p53 is a nuclear protein which seems to be

involved in the regulation of cell cycle, especially DNA replication and transcription (Eliyahu et al., 1984; Eliyahu et al., 1989; Shaulsky et al., 1990; Nishioka et al., 1993), the mutant variety of the protein shows a relatively high affinity for binding to single-stranded DNA than to double-stranded DNA (Rovinski and Benchimol, 1988; Kern et al., 1991). Although this is true, the wild-type protein ($p53^{wt}$) is more efficient in binding SV40 DNA fragment which consists of the sequences known to regulate the activity of SV40 promoters. Hence, it is possible that the protein plays a dual role by involving in DNA replication and in the regulation of gene expression (Bargonetti et al., 1991).

The p53 tumour suppressor gene is a frequent target for structural alterations in a large number of human malignancies (Hollstein et al., 1990, 1991; Levine, Momand and Finlay, 1991). The levels of wild-type p53 increase in response to DNA-damaging agents such as ionizing radiation, leading to G1 arrest. Because of high levels, p53 may transcriptionally induce the expression of the growth arrest and DNA damage protein, GADD45, resulting in the inhibition of progression from G_1 into S phase (Kastan et al., 1992). Over-expression of wild-type p53 in transformed cells can arrest cell proliferation, reverse a tumourigenic type, and sometimes induce apoptosis or differentiation (Kern et al., 1992; Donehower, 1994). The wild-type p53 has been shown to be capable of attaching to specific DNA sequences thereby acting as a transcriptional modulator. Furthermore, over-expression of wild-type p53 represses transcription from a variety of promoters (Santhanam, Ray and Seghal, 1991) probably through basal transcription factors (Seto et al., 1991; Oliner et al., 1993; Ragimov et al., 1993). Studies have revealed the existence of a p53-binding protein, which can associate with both wild and mutant types of the protein (Hinds et al., 1990). Momand et al. (1992) have identified this protein as the product of murine double minute (*mdm2*), a putative proto-oncogene, over-expression of which interferes with sequence-specific transcriptional activation by wild-type p53. Over-expression of wild-type p53 is capable of sequence-specific binding to a region within the *mdm2* gene and of transactivating directly this gene (Juven et al., 1993). High levels of MDM2 protein, similar to the DNA tumour virus oncoproteins may inactivate the tumour suppressor activity of p53 by complexing to it (Lane, 1992). It is also possible that the increased levels of wild-type p53 may transactivate EGF receptor promoter sequences and that there exists a common regulatory mechanism to control cell proliferation mediated through both p53 and EGF receptor (Deb et al., 1994).

Mutations that activate transformation may be the ones that result in a loss of function of the wild-type p53. Finlay et al. (1989) suggested that over-expression of p53 mutants enhances the transformation process by the formation of nonfunctional multimeric complexes which can inactivate wild-type of the protein thereby giving a growth advantage over the other cells. Since the p53 acts as a tumour suppressor gene, it possibly gets involved in the negative control of growth and differentiation via its protein product, $p53^{wt}$ (Montenarh, 1992). This shows that the interaction of mutant forms of the oncoprotein with that of the wild-type would switch off the activity of the latter or considerably decrease its endogenous tumour suppressor activity (dominant negative); thus, making the cell to be deficient in the function of that gene product (Fig. 3). There is also evidence that at least some mutant p53 proteins may also play a key role in allowing cells to enter the S-phase which fails to correct DNA damage (Wolf, Harris and Rotter, 1984; Mercer, Avignolo and Baserga, 1984; Shohat et al., 1987). In other words, over-expression of the mutant p53 and its interaction with that of the wild-type protein makes the cell more susceptible to uncontrolled proliferation (Hicks et al., 1991; Levine et al., 1991). It seems that p53 mutation is an important step for cell transformation in vivo (Soini et al., 1992).

1.72 p53 Expression in squamous tumours other than OSCC

Although the expression of proto-oncogene product p53 is very low in normal non-transformed cells, most of the neoplastically transformed cell lines show a many-fold increase in the levels of the oncoprotein (Lane and Crawford, 1979; Rogel et al., 1985; Koeffler et al., 1986; Rovinski and Benchimol, 1988; Finlay et al., 1989). Mutations of p53 gene have been reported in most of malignant tumours: breast (Bartek et al., 1990), liver (Bressac et al., 1990); lung (Chiba et al., 1990; Iggo et al., 1990; Westra et al., 1993); colo-rectum (Fearon and Vogelstein, 1990; Rodrigues et al., 1990); and bladder (Sidransky et al., 1991; Habuchi et al., 1993; Soini et al., 1993; Zhang et al., 1994).

1.73 p53 and Oral Lesions

Heavy smoking and drinking (Langdon and Partridge, 1992; Ogden et al., 1992) or tobacco smoking coupled with tobacco/betel quid chewing (Warnakulasuriya and Johnson, 1992; Ranasinghe et al., 1993) are the major parameters which correlate with

the over-expression of oncoprotein p53 in patients with oral squamous cell carcinoma. The high percentage of p53 positivity, in heavy smokers, may be due to accumulation of chemical carcinogens in the tissues over time. Field et al. (1991) suggested that the excess carcinogen may damage the integrity of the gene leading to its over-expression and that there might be a loss of suppressor activity with a gain in the dominant transforming activity. It has been established that G→T transversions are the most frequent p53 mutations in non-small cell lung cancer (Iggo et al., 1990) and in esophageal squamous cell carcinomas (Hollstein et al., 1990). It is worth noting that benzo(a)pyrene, a major carcinogen in tobacco smoke, is known to induce G→T transversions (Suzuki et al., 1992). This strongly suggests that tobacco use, a known risk factor in oral squamous cell cancer, is linked with the mutations that occur in the gene on prolonged exposure to tobacco smoke (Somers et al., 1992).

A number of publications have shown that there is over-expression of p53 oncoprotein in oral SCCs. As shown in Table 5, the percentage of oral SCCs positive for p53 varies from 35% to 93.5%, with most of the reports showing a positive reaction in more than 50% of the cases. The average percentage of p53 staining in oral SCCs is 60.5. However, a recent paper by Ranasinghe et al. (1993) showed a very low percentage (11%) of oral SCCs that were positive for p53 from an South Asian population. The sharp contrast of the result from this study as compared with those of other studies raises the possibility that there might have been technical problems in the staining procedures. On the other hand, it is well known that the etiological factors for oral malignancies in the South Asian population are different from those of the Western World (Banoczy, 1984; Blot et al., 1988; William, 1990). In Western countries, oral SCC only accounts for 5% of all the cancers and the main etiological factors are smoking, and alcohol or a combination of the two. In the South Asian population, oral SCCs accounts for more than 40% of all cancers (Daftary et al., 1991) and the main etiological factors are betel quid with tobacco chewing and tobacco smoking (Saranath et al., 1989; Saranath et al., 1993). It is, therefore, possible that the low percentage of p53 expression in oral SCCs from the South Asian population by Ranasinghe and his associates (1993) may reflect the differences in the etiological factors when compared to other parts of the world. It is likely that a large sample containing more SCC cases might draw more reliable conclusions. Further studies are needed to give more insight into the functional aspects of p53 in oral squamous cell carcinomas.

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Chapter 2. Hypothesis and Objectives

2.0 Hypothesis

(**a**) There will be an altered expression of epidermal growth factor receptor (EGFr) in oral dysplastic lesions and squamous cell carcinomas (OSCC). The above normal levels of the growth factor receptor may show a positive correlation with the degree of dysplasia and the appearance of OSCC. The different degrees of dysplasia may also show an increase in the intensity of staining reaction for the EGF receptor when compared to normal and hyperplastic oral tissues.

(**b**) Although normal cells in an epithelium express only very little quantities of p53 protein, the cellular changes that are taking place in the epithelium during the process of carcinogenesis makes the epithelium increasingly positive for the expression of p53 oncoprotein from a stage of premalignant lesion to squamous cell carcinoma through various degrees of dysplasia. Hence, like any other given samples, East Indian samples of oral squamous cell carcinomas may also show a higher incidence of p53 oncoprotein. However, a recent study conducted by Ranasinghe et al. (1993) showed only a low prevalence of expression of the oncoprotein p53 (11%). It is possible that the oncoprotein is necessary only at certain point during the process of carcinogenesis other than the very initial step, and that it may complement many other like-proteins which are co-expressed or over-expressed during the process. If this holds true for the process of carcinogenesis in oral squamous cell tumours, it seems that there could be some discrepancies in the results obtained by Ranasinghe et al. (1993). If the prevalence of expression of oncoprotein p53 is going to be lower than what is expected, then that points towards different factors, including genetic susceptibility that are of etiologic importance in the development of oral carcinomas in India and elsewhere.

2.1 Objectives of the study

Experiment 1. Expression of Epidermal Growth Factor Receptor in Oral SCCs.

The primary aim of the study was to investigate the expression of epidermal growth factor receptor (EGFr) in: **1**) normal and hyperplastic oral epithelium; and **2**) mild, moderate and severe dysplastic lesions, carcinoma in situ, and squamous cell carcinoma of oral mucosa. The results of the studies conducted by Shirasuna et al. (1991) and Kunikata et al. (1992) on EGFr failed to produce consistent results (Tables 3 and 4). The present study was the first of its kind to look at the expression of EGFr in Canadian samples using immunohistochemical methods. More samples of oral dysplastic lesions (Table 6) were included in the proposed study.

Experiment 2. Over-expression of p53 in oral SCCs.

Most of the studies have suggested that there is a six-fold increase in the expression of p53 oncoprotein in oral SCCs. On the contrary, one of the studies conducted by Ranasinghe et al. (1993) stated that the expression of p53 oncoprotein in oral squamous cell carcinomas is very low. Hence, the second objective of this project was to determine the extent and pattern of expression of the oncoprotein p53 in oral squamous cell carcinoma samples from Kerala, Southern India, where the population is exposed to the same etiologic factors in the same geographical location.

2.2 Staining and grading and statistical analysis of the lesions

The staining reactions were graded into moderate, strong and very strong categories by visual observation while the pattern was graded according to the presence or absence of membrane-bound staining in given area and also whether the positive cells were seen in a whole given field (cell membrane positive, CMB; Kearsley et al., 1991; Yamada, Takagi and Shioda, 1992). The staining pattern was considered to be focal cell membrane type (FCMB) when occasional positive cells were seen in a given field (Kunikata et al., 1992). The EGFR staining in different epithelial layers was recorded for each case according to the number of compartments involved and the intensity of staining into different grades by visual observation. According to the number of layers involved in the staining reaction, the slides were classified into two types: Grade 1, where only basal and a few layers of the parabasal cells were involved (Lower compartment of cells); and Grade 2, when the staining extended beyond the parabasal layers to the upper spinous layers (including both lower and upper compartments of cells). Since some slides showed variation in the staining of different epithelial layers, the grade for the staining of epithelial layers of a slide was based on the area with staining of the most epithelial layers. Since some slides showed variation in staining intensity from area to area, the grade for the staining intensity of a given slide was based on the area with the maximum staining intensity.

The intensity of p53 oncoprotein staining reaction was graded as follows: negative or equivocal (- or ±); weakly positive (+); moderately positive (++); strongly positive (+++); and very strongly positive (++++). The pattern of the staining for p53 positive cells was classified according to the number of positive cells in a given field; scattered when only a few positive single cells were seen in a given field, and diffuse when a large number of positive cells were clumped together in a given area (Zhang et al., 1993). Chi-square (χ^2) tests of independence were performed to examine the association between staining intensity (measured on a 4-point scale) and degree of dysplasia. To analyze the correlation between the expression of p53 oncoprotein and the chewing habits (frequent chewers Vs infrequent chewers), the Fisher Exact test of significance and regression statistics (for r value) was used.

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Chapter 3. Over-expression of Epidermal Growth Factor Receptor on Oral SCCs

3.0 Materials for EGFR

The human oral dysplasia specimens and SCCs were obtained from archival paraffin blocks processed in the Department of Oral Pathology at The University of British Columbia (UBC) Hospital, Vancouver, Canada. The premalignant lesions were classified into mild, moderate and severe dysplasia. The cases of severe dysplasia also contained carcinoma-in-situ (CIS) cases as the histopathological differences between these two lesions are often subjective and difficult to differentiate (Table 6). Furthermore, both the lesions tend to show similar ratings of prognosis. Samples of hyperplastic oral lesions served as positive controls for the immunohistochemical detection of EGFR. The diagnosis of the dysplastic lesions was confirmed by two Oral Pathologists from UBC, Drs. R.W. Priddy and L. Zhang, without prior knowledge of the original diagnosis. The detailed history of the patients is shown in Appendix 2, along with the results of staining reactions.

3.1 Immunohistochemical detection of EGFR

Routinely processed five-micron sections were dewaxed with xylene (3X 10 min), hydrated through serial alcohols (4X5 min), and then washed with PBS (pH 7.6) for 3X5 min. Following the endogenous peroxidase block with a 3% solution of hydrogen peroxide in water at room temperature for 30 min, the tissue sections were rinsed with PBS 2X5 min, treated with pepsin (1 mg/ml in 1N hydrochloric acid) at 37°C for 5 min for antigen retrieval (Brozman, 1978), and then rinsed again with buffer for 3X5 min. In the next step, following overnight incubation at 4°C with the prediluted primary antibody (EGFR, Biogenex, San Ramon, CA) at a further dilution of 1/2 to the buffer, the tissue sections were rinsed with buffer for 2X5 min, and then incubated with prediluted biotinylated mouse anti-immunoglobulins containing 1% human serum (Link: Ready-to-use kit, Biogenex, CA) at room temperature for 30 minutes (Hsu, Raine and Fanger, 1981). After thorough rinsing with buffer for 2X5 min, the slides were again incubated with prediluted alkaline phosphatase-conjugated streptavidin (Label: Ready-to-use kit, Biogenex, CA) at room temperature for 30 minutes and rinsed with buffer for another 10 min. The slides were then incubated with 0.04% diaminobenzidine hydrochloride solution containing 0.02% hydrogen peroxide to trap the peroxidase activity, at room temperature for 8 min, counterstained with hematoxylin, neutralized with 1% NH_4OH, rehydrated through ascending range of alcohols, cleared in xylene, and then mounted in Permount.

A known EGFR-positive case of hyperplasia of oral squamous epithelium (Fibroepithelial polyp) served as the positive control while the negative control was substituted with negative serum (non-immune serum or ascites in PBS with 0.1% sodium azide) supplied by the manufacturer.

3.2 Results

Table 7 summarizes the EGFR staining intensity in different groups of specimens. Normal oral mucosa showed moderate to strong staining (Fig. 4). The majority of hyperplastic lesions and dysplastic oral lesions also demonstrated moderate to strong EGFR staining (Figs. 5-8), and some hyperplastic and dysplastic oral lesions showed very strong EGFR staining. Although there seemed to be a tendency of stronger staining in moderately and severely dysplastic lesions as compared to normal mucosa, hyperplastic and mildly dysplastic lesions, there was no significant differences among these cases.

The majority of the oral SCCs (60%) showed a very strong EGFR staining (Fig. 9) and the average staining intensity of the oral SCCs was significantly higher than that of normal oral mucosa, hyperplasia and mild dysplasia (Table 7). The increase in EGFR content in SCCs was also demonstrated by the higher staining intensity of the cancer cells as compared to the adjacent dysplastic epithelium in 4/10 cases of SCCs (Table 8). Furthermore, when the cancer cells showed a very strong EGFR staining, it was rather diffuse compared to other cases. However, 2 cases of SCCs showed a patchy weak to moderate EGFR staining with large areas of negative staining while the adjacent dysplastic epithelium showed a stronger staining compared to the cancer cells (Table 8). There was no relationship between the staining intensity and differentiation of the tumours (Table 8). The staining was generally cell membrane type (CMB), though equivocal cytoplasmic staining was also noted, especially when the staining was strong. Although this was the case, there was no correlation between the presence of the equivocal cytoplasmic staining and the histological diagnosis of the lesions.

Table 9 summarizes the EGFR staining of the epithelial layers in different groups of specimens. The basal and parabasal layers were positive in all normal oral mucosa, hyperplastic and dysplastic lesions (Figs. 4-7). In addition to basal and parabasal EGFR staining, a large percentage of normal oral mucosa, hyperplastic and premalignant oral lesions also showed EGFR staining in the upper spinous cell layer (Table 9; Figs. 3, 4, 7). The basal layer showed the maximum intensity of staining with a gradual decline corresponding to the differentiation of the keratinocytes. The granular cell and horny cell layers were not stained by the antibody. There was no significant difference in the staining of the different epithelial layers among the different groups of specimens and

between the keratinized and non-keratinized specimens, as well as among lesions from different oral sites. In all groups of the specimens, the intensity of staining and the epithelial layer(s) stained were noted to vary from area to area in some cases (Figs. 6, 7). The possibility that such variations might be caused by staining artifact was ruled out by the presence of similar variations in serial sections. There were clear differences in the distribution of the EGF receptor on neoplastic mucosa when compared to normal mucosa. All layers of many tumours stained strongly with anti-EGFR antibody which supports the view that there is over-expression of the receptor protein in oral SCCs (Fig. 9). The intensity of staining varied from moderate to very strong and in most of the cases the cells at the periphery showed a very strong staining with a gradual decline in the intensity of staining towards the center of the keratin pearls in the case of well-differentiated tumours, paralleling the situation which normally occurs in differentiating layers of oral mucosa. The pattern of distribution of the receptor protein also showed a considerable amount of variation: In most cases (>90%), the staining was limited to the cell membranes (CMB); in some cases the tumour cells showed a strong focal cell membrane-bound staining (FCMB).

3.21 Statistical analysis

Chi-square (χ^2) tests of independence were performed to examine the association between staining intensity and the degree of dysplasia. Although the staining intensity was measured on a 4-point scale (1, weak staining; 2, moderate staining; 3, strong staining; and 4, very strong staining), most of the lesions showed a moderate-to-very strong intensity of staining. The test was done to see whether there is any linear relationship of staining intensity with that of degree of dysplasia. Since the staining intensity was measured on a 4-point scale, the tests of means were considered inappropriate for analysis of the data. As the chi-square test can handle only a few expected cell frequencies less than 5, empty cells create problems for the test.

In order to avoid problems, rows or columns were combined, including grouping 'strong' and 'very strong' staining intensity, and grouping A+B (consists of normal and hyperplasia) and C+D+E (consists of dysplasia of different degrees) to increase the cell frequencies. For this set of data the chi-square (10 df) value was calculated to be 24.32

with a p-value of 0.007 (Table 10). (No case, except one hyperplasia, reported grade 4 staining in groups A and B.) To look at the significance of the staining intensity in different degrees of dysplasia, another chi-square test was done; the results were not significant as the p-value was calculated to be 0.33. This could be due to the fact that the distinction between 'strong' and 'very strong' is lost by combining the different observations. When the staining intensity of various groups were analyzed by combining staining intensities of normal epithelium and the hyperplasia as group 1 (A+B), the dysplasias as group 2 (C+D+E) and SCCs as group 3 (F), there was a strong correlation and this test has given the most reliable result ($p = 0.0011$; Table 11). Group 3 had more observations with strong staining. When the dysplasias were compared the p value was not that significant (Table 12). In summary, there appears to be an increase in the intensity of staining with the appearance of squamous cell carcinoma when compared to normal mucosa while the dysplastic lesions do not show much variation in the intensity of staining among themselves.

3.3 Discussion

In addition to the conflicting data on EGFR expression in oral premalignant and malignant lesions, there is no agreement on the normal distribution of EGFR in human oral cavity. Although four different groups of researchers have investigated the normal immunohistochemical localization of EGFR in the oral cavity of subjects without oral SCC, their opinions vary on what epithelial layer(s) is normally stained, and on whether keratinization and site of the specimens affect the expression of EGFR (Table 13). The immunolocalization of EGFR may be restricted to basal cells (Shirasuna et al., 1991; Christensen et al., 1992) or may spread to upper spinous cell layers with a gradual decrease in the intensity of staining from basal layers to the top, the basal cells showing the maximum intensity of staining (Kunikata et al., 1992; Whitcomb et al., 1993). Partridge et al. (1988) have suggested that non-keratinized epithelium demonstrate only basal and parabasal cell staining while keratinized epithelium may show additional staining in the upper spinous layers. On the other hand, Kunikata et al. (1992) have reported negative staining of non-keratinized epithelium from buccal and lingual area. Three of the five studies, however, did not observe differences in EGFR staining between keratinized and non-keratinized epithelium (Shirasuna et al., 1991; Christensen et al., 1992; Whitcomb et al., 1993). In agreement with the the aforesaid researchers, this study also found no relationship with the intensity of staining and the presence or absence of

keratin in the epithelium. This may point towards the fact that the degree of keratinization may not be a decisive factor in the prediction of staining intensity. In other words, the degree of keratinization does not affect the intensity of staining.

In squamous cell carcinomas, the staining was seen extended to superficial layers of the epithelium and this is in agreement with the results obtained by Shirasuna and his co-workers (1991). Although this was the case, not all cell layers were involved in dysplastic lesions as explained by Christensen and his associates (1992). In contrast, only 68% of the dysplastic cases from the present study showed the involvement of all layers as opposed to Christensen and his co-workers' observation (6/6 cases). This may be due to the fact that cases used by Christensen and his associates in their study were from the epithelium lying adjacent to carcinomatous lesions. There are clear differences in the distribution of the EGF receptor in neoplastic epithelium when compared to adjacent normal mucosa. Results of this study also shows that strong staining is very likely and has been reported by many researchers (Partridge et al., 1988; Kearsley et al., 1991). Many tumour islands show a strong staining to anti-EGFR antibody but some tumours may show a weak staining pattern (Kearsley et al., 1991). Although many authors have suggested that there is over-expression of the receptor protein in oral squamous cell carcinomas, the work by Yamada, Takagi and Shioda (1992) and Sakai et al. (1990) have pointed out that not all cells stain positive for the receptor. As shown in Table 1, the former reported negative staining for EGFr in 49% (23/47) cases while the latter reported a still higher percent (24/28; 89%). This may support the fact that all tumour cells do not necessarily require the help of the receptor protein once the malignant transformation sets in and that there may be an autocrine-paracrine mechanism of regulation which controls the cell growth thereafter. Many authors have suggested the existence of an autocrine/paracrine mode of regulation for the growth factor receptor in the development of malignancy (Partridge et al., 1989; Todd et al., 1989; Shirasuna et al., 1991; Christensen et al., 1993).

The relationship between the expression of the receptor protein and the degree of differentiation is quite controversial. Although most authors have suggested that there is an inverse relationship between the degree of differentiation and the expression of the receptor protein (Kearsley et al., 1991; Christensen et al., 1992), cases have been reported where the expression of the receptor protein shows either no correlation with the degree

of differentiation (Yamada, Takagi and Shioda, 1992) or is directly related to size and local extent of the tumour (Kawamoto et al., 1991). It has also been shown that the expression of the receptor also depends on habits like tobacco chewing and drinking and that a chronic irritation from these known carcinogens may favor over-expression of EGF receptors in oral mucosa (Bergler et al., 1989). Several studies have reported that over-expression of EGF receptors has an inverse relationship with prognosis (Kawamoto et al., 1991). Cells which undergo active mitosis also express above normal levels of EGF receptors (Sakai et al., 1990). Dysplastic epithelium expresses above normal levels of mRNA transcripts for the receptor protein leading to the conclusion that there is over-expression of the receptor (Todd et al., 1991; Grandis and Tweardy, 1993).

In summary, in normal squamous cells of the oral mucosa, the staining for EGF receptor is confined to cell membranes of the basal and suprabasal layers as explained by Whitcomb, Eversole and Linderman (1993), while squamous cell carcinomas shows cell membrane positive staining as cited by Kearsley et al. (1991) and Yamada et al. (1992). The 'normal' cells adjacent to squamous cell carcinoma tend to show a cell membrane positive reaction. Hyperplastic and dysplastic lesions will be positive in the basal and suprabasal layers with a progressive decrease in the intensity of staining as it moves towards the superficial layers, that is, there is a reduction in the number of receptors with terminal differentiation (Shirasuna et al., 1991). In the present study, more than 90% of the tumour cells showed a very strong cell membrane-bound staining to the anti-EGFr antibody. In areas where the staining was either barely visible or weak, the cell membranes showed a tendency for very strong focal staining. The results from the present study show that the majority of SCCs over-express EGFR and the EGFR staining intensity in SCCs is significantly higher than that of normal oral mucosa, hyperplastic and mildly dysplastic oral lesions. In 2 cancer cases, however, large proportions of cancer cells were negative with patches of cancer cells showing a weak to moderate staining, compared to the adjacent non-neoplastic oral mucosa. There are conflicting data regarding the relationship between EGFR expression and tumour cell differentiation (Ishitoya et al., 1989; Santini et al., 1991; Dassonville et al., 1993). The present study found no correlation between cell differentiation and EGFR staining, though the sample size was small for statistical analysis.

There is difference of opinion regarding the cytological localization of EGFR in cancer cells using immunohistochemical method. In a study conducted by Sakai et al. (1990), cytoplasmic staining was found to be the primary staining pattern for oral SCCs: all 4 EGFR positive SCCs showed cytoplasmic staining and only 1 of the 4 cases showed a few focal cell membrane positive cells. In a number of other studies also cytoplasmic staining was noted in a few cancer cells, though the majority of oral SCCs demonstrated cell membrane-bound staining (Yamada et al., 1989; Yamada, Takagi and Shioda, 1992; Prime et al., 1994). Most studies have not described any cytoplasmic pattern of staining either in normal mucosa cells (Whitcomb, Eversole and Lindermann, 1993) or in oral cancer cells (Partridge et al., 1988; Shirasuna et al., 1991). Kearsley et al. (1991) observed cytoplasmic staining in regions where the staining reaction to the anti-EGF receptor antibody was intense. In agreement with their observation, this study also reports the presence of cytoplasmic pattern of EGFR distribution in areas where the staining intensity is very strong, in all groups of specimens including normal oral mucosa, suggesting an artifact in the areas of intense staining.

Over-expression of EGF receptor is considered to be a hallmark of SCC (Kamata et al., 1986; Ozanne et al., 1986; Ozawa et al., 1988; Bergler et al., 1989) and may occur independently of c-erb-B-1 activation (Field, 1992; Saranath et al., 1992). It is possible that tumours which express excessive amounts of EGF receptor may have enhanced proliferation in response to autocrine or paracrine production of TGF-α (Partridge et al., 1988; Todd et al., 1991). The production of TGF-α may also have a positive feedback-like effect and may accelerate the degradation of EGF receptor soon after binding and internalization. Ligand binding and subsequent internalization reduces the half-life of the receptor and this may, in turn, affect the amount of the receptor protein being recycled or the amount of the available (Carpenter, 1984). Recent studies have shown that the activated receptor protein acts through a class of compounds termed receptor tyrosine kinases (RTKs) (Blenis, 1991; Pelech and Sanghera, 1992; Fantl, Johnson and Williams, 1993) and that this may have some link between other mitogenic pathways including other cellular proteins responsible for cellular proliferation such as $p21^{ras}$ (Davis, 1993; Hsuan, 1993; Williams et al., 1993). Over-expression of the receptor may be due to gene amplification (Yamamoto et al., 1986; Rikimaru et al., 1992), overproduction of the protein itself (Hunts et al., 1985; Prime et al., 1994) and/or production of ligands such as TGF-α which may have an autocrine pathway of regulation (Pingree et al., 1991; Grandis and Tweardy, 1992; Christensen et al., 1993; Grandis and Tweardy, 1993).

3.4 Conclusion

The results from this study showed no differences in EGFR staining between keratinized and non-keratinized epithelium, including normal mucosa, hyperplastic and dysplastic epithelium, and no differences in staining reactivity in specimens from different anatomical locations. That genetic abnormalities play a crucial role in the multistage process of head and neck cancer is a well known fact now. The process of carcinogenesis is driven by disregulation of cellular proliferation and differentiation owing to the constant exposure of the epithelium, especially the the one of the upper aerodigestive tract, to various carcinogens. The finding of genotypic abnormalities in precancerous lesions adjacent to oral SCCs reveal that there is a strong relationship between the genetic abnormalities and the process termed 'field cancerization' (Hittelman et al., 1991; Voravud et al., 1993). Field cancerization is the phenomena by which the epithelium becomes 'condemned' or predisposed to disregulated growth as evidenced by a higher incidence of second primary lesions at the same time or following the primary carcinomas in the head and neck region. It has been shown that in patients with primary head and neck carcinomas, 4-8% tend to show synchronous second primary lesions and 4-13% tend to develop metachronous lesions (Shibuya et al., 1986).

Most of the available data (including the results of the present study) do, however, support that EGFR may be a good marker for oral SCC and that EGFR may play important roles in the maintenance of oral SCCs, as a significant percentage or the majority of oral SCCs have been found to show over-expression of EGFR, even though some SCCs express a decreased level of EGFR instead. Some studies have shown the reduction of EGFR in malignant tumours may be due to occupation and down-regulation in response to autocrine secretion of growth factors (Nazmi et al., 1990). The information on over-expression of EGFR in oral SCCs may improve diagnosis and treatment options for patients with oral SCCs. The high content of EGFR in oral SCCs may make it possible to detect metastasis of oral SCC using receptor-directed labeling technique (Soo et al., 1987; Grandis and Tweardy, 1992). Yoneda et al. (1991) reported that the growth of a cell line from an oral SCC with EGFR over-expression was dependent upon the EGFR pathway and administration of an anti-EGFR monoclonal antibody inhibited the growth of the cancer both *in vitro* and *in vivo*. Similar therapeutic use of anti-EGFR monoclonal antibody has been reported in animal studies (Masui et al., 1986) and in other human cancers (Divgi et al., 1991).

The second primary lesions are thought to arise independently after exposure to a common carcinogen by the process of field cancerization (Grandis and Tweardy, 1993; Voravud et al., 1993). The development of efficient clinical trials for the prevention of aerodigestive tract premalignant and malignant lesions depends on the identification of individuals at risk who may benefit by intervention trials (Kelloff et al., 1992). In order to make the results statistically significant large samples are very essential and so are extended study periods. It may be possible that in future, innovative therapies may be developed using monoclonal antibodies directed towards EGF receptor (Grandis & Tweardy, 1992). Monoclonal antibodies directed towards the EGF receptor may become a breakthrough in the field of Preventive Oncology and Therapeutics. Advanced techniques like polymerase chain reaction (PCR), and in-situ hybridization (ISH) and its modifications (e.g., Fluorescence in-situ hybridization, FISH), are valuable tools that would help researchers to decipher the mechanisms of tumourigenesis from a molecular viewpoint (Patel et al., 1994a).

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Chapter 4. Over-expression of p53 in oral SCCs from Kerala

4.0 Materials for p53

The tissue sections for immunohistochemical detection of p53 were obtained through Dr. Ambika (Professor, Division of Oral Pathology, Dental College, Trivandrum, Kerala, India). A detailed history of chewing, smoking and drinking habits were recorded during routine clinical examination (Tables 14 and 14a).

4.1 Patient Data

Cases for the present study were randomly selected from the formalin-fixed, paraffin-embedded archival material collected during the period of 1990-1993, in the Department of Oral Medicine/Pathology, Dental College, Trivandrum, Kerala, India. The cases include 12 females and 11 males with an average age of 58.3 (40-75). It was difficult to record the habits in some cases, either because of the patients were too shy to express themselves or because of their forgetfulness. Only one patient reported a history of occasional cigarette smoking. The socioeconomic status of the subjects was classified according to the guidelines suggested by Leete and Fox (1977): Class I, Professionals; Class II, Non-professional and Managerial; Class III, Non-manual or Manual Skilled; Class IV, Manual Unskilled. Thus, the sample for the present study showed a heterogeneous nature with respect to their cultural (Hindus, Christians and Muslims) and socioeconomic aspects.

Chewing habits. The chewing mixture generally contains the following substances: betel leaf, tobacco (processed or raw), slaked lime and areca nut. The mixture does not contain any other special ingredients as observed in other parts of the Indian subcontinent. Furthermore, all substances are used fresh. According to the frequency of chewing, the patients were classified into heavy chewers (10-15 betel quids/day), moderate chewers (5-9 betel quids/day) and occasional chewers (<5 betel quids/day). Most of the patients in this study were chronic chewers who were habitual chewers for more than 20 years. Some of the subjects also happened to be chronic smokers who smoked bidis for more than 20 years. Previous studies have reported that people in most regions of Kerala use all ingredients of the chewing material while fresh (Orr, 1933; Hirayama, 1966; Sankaranarayanan et al., 1990).

Smoking habits. According to the number of bidis/cigarettes smoked per day, the patients fell into three different groups: occasional smokers (<5 bidis or cigarettes per day), moderate smokers (5-20 bidis/day) and heavy smokers (20+/day). A majority of the patients reported having chewed the quids and smoked bidis throughout the day. As the majority of the patients in this study were from lower socioeconomic classes (III and IV, see below), they preferred bidis to cigarettes. Bidis are a form of local cigarette made by wrapping less than 0.5 g of coarse tobacco in a dry temburni (*Diospyros melanoxylon*) leaf. It is cheaper than cigarette.

Alcohol habits. Most of the patients in this study group came from a rural population, where they prefer home-brewed liquor (*Arrack/Toddy*) to whisky or other types of drinks. This home-brewed variety has been estimated to contain about 50% ethanol. Although it was difficult to extract the exact details of alcohol consumption, all patients who drank alcohol reported an approximate amount of alcohol they have consumed on a daily basis. Hence, they were classified into mild drinkers who used only less than 2 ounces of alcohol per day, moderate drinkers, where the intake was between 2-4 ounces of alcohol per day and heavy drinkers, where the alcohol consumption was over 5 ounces per day.

4.2 Immunohistochemical detection of p53 oncoprotein

After dewaxing with xylene (3X10 min) and dehydrating through serial alcohols (4X5 min), routinely processed 5μ sections were microwaved with 1% solution of $PbAc_2$ at high intensity for 1 min, followed by leaving the slides in the solution for another 9 min in order to retrieve the antigen of interest (modified from Shi et al., 1991). After rinsing with distilled water and blocking the endogenous peroxidases with 0.6% hydrogen peroxide in methanol at room temperature for 30 min, the tissue sections were rinsed with PBS (pH 7.3) for 3X5 min, and then treated with normal horse serum (Vectastain ABC kit) at room temperature for 1 hour to block background staining (Hsu, Raine and Fanger, 1981). Following an overnight incubation at 4°C with the primary p53 antibody (Dimension Labs, Mississauga, ON), the slides were washed with buffer, incubated with the secondary antibody (Biotinylated mouse immunoglobulins; Vector ABC kit) at room temperature for 1 hour. This was followed by another 1 hour incubation with avidin-biotin complex conjugated to horse radish peroxide (Vectastain ABC kit). After rinsing with buffer for 5 min, the slides were incubated with diaminobenzidine peroxidase substrate to trap the peroxidase reaction, counterstained with Gill's hematoxylin, rehydrated through ascending range of alcohols, cleared in xylene, and then mounted in Permount. A known p53-positive case of oral carcinoma served as the positive control while the negative control was substituted with PBS (Zhang et al., 1993).

4.3 Results

In this study, over-expression of p53 oncoprotein was detected in 13/23 cases of squamous cell carcinomas of the oral cavity. The immunostaining reactions for oncoprotein p53 within the tumour cell populations were confined to the cell nuclei. Well differentiated tumours showed a strong immunostaining for p53 along the periphery of the tumour nests where the cells were actively proliferating, whereas the center of the keratin pearls remained negative for the staining (Fig. 10). As shown in Table 15, among the 14 cases of positive SCCs, 5 cases showed strong staining (+++), 7 cases showed moderate staining (++), and 2 showed a weak staining (±/+). The pattern of staining also showed a significant difference amongst the 14 positive cases; most of the cases (11/14) showed a diffuse pattern of staining with the remaining cases being scattered in nature. Verrucous carcinoma samples showed weak-to-strong staining. Normal epithelium in

adjacent areas of the carcinomas remained negative for immunostaining. The present study found no significant relationship in the expression of the oncoprotein with respect to the site of lesion (Table 16). In most of the p53 positive cases, the subjects had a history of chronic chewing, chewing and smoking, and chewing and smoking coupled with drinking habits. One of the male patients with a positive history of drinking, showed a negative reaction to the anti-p53 antibody. In the case of chewers, the staining intensity showed a significant correlation between the number of the betel quids chewed per day as shown in Table 17. Over-expression of the p53 oncoprotein showed a slight predilection for the male sex and most of them had smoking habits coupled with chewing [7/11 (63%) males Vs 6/12 females, (50%)].

4.31 Statistical analysis of p53 expression

As shown in Table 18, to analyze the data, the Fisher Exact test of significance for association between two binary variables was used. It is useful replacement for the common χ^2 test in the case of 2X2 table where some of the cells have very small frequencies. The p-value for the test was recorded as 0.0011, indicating a highly significant association between the number of betel quids chewed per day and a positive p53 reaction. The positive predictive value is the probability of a positive p53 reaction test in a subject who chews 5 or more quids per day with different combinations of tobacco, lime, areca nut and betel leaf (frequent chewer); the negative predictive value is the probability of negative p53 reaction in a subject who chews less than 5 quids per day with different combinations of tobacco, lime, areca nut and betel leaf (infrequent chewer). In this sample, the positive predictive value was found to be 14/15 (93%) and the negative predictive value was 6/6 (100%). This shows that a frequent tobacco/betel quid chewer has 93% likelihood of a positive p53 compared to an infrequent chewer who has 100% likelihood of negative p53 reactions. Further analysis also showed that there is strong correlation between the number of betel quids chewed per day and the intensity of staining (Table 19). In order to overcome technical difficulties, the regression analysis was carried out using median value for the number of betel quids chewed per day for each group. The p value was calculated to be 0.0001 (r= 0.8).

4.4 Discussion

A number of studies have shown that normal p53 acts as a *molecular policeman* which monitors genetic damage, or strand breaks in the DNA (Lane and Benchimol, 1990; Montenarh, 1992). It is postulated that p53 could have both a role in regulating transcription of genes that suppress cell proliferation, perhaps those affecting passage from late G_1 to S phase of the cell cycle (Levine, Momand and Finlay, 1991), and a biological function as a G_1 checkpoint control allowing the repair of DNA damage (Lane, 1992). The wild-type of the protein has a shorter half-life when compared to mutant variety of the protein which is more stable and is, in part, responsible for malignant transformation (Nigro et al., 1989; Gusterson et al., 1991). Over-expression of the p53 oncoprotein has been reported in oral squamous cell carcinomas as shown in Table 5. The staining intensity of p53 also shows a linear correlation with increasing degrees of dysplasia and the appearance of SCC (Zhang et al., 1993). Since more than half of the cases that have been studied here shows the presence of p53 oncoprotein, it is possible that mutation of p53 plays a role in the progression of oral malignancies from a state of dysplasia to squamous cell carcinomas. It is also possible that there may be mutation in one of the alleles, and the remaining allele is lost during a later period. It has been suggested that mutation in one of the alleles and/or loss of the remaining allele leads to malignant transformation (Hinds et al., 1989; Hinds et al., 1990; Hollstein et al., 1991; Brachman et al., 1992; Sakai et al., 1992; Somers et al., 1992; Kim et al., 1993). The fact that the expression of the protein is higher for oral SCCs demonstrating more than 40% of cells with binding to proliferating cell nuclear antigen (PCNA) shows that malignancy might be promoted due to an increase in the cell cycle somehow related to p53 oncoprotein expression (Nishioka et al., 1993).

Soussi et al. (1994) have suggested that more than 90% of missense mutations account for the immunohistochemically detectable p53 whereas nonsense mutations, which arise from alterations in nucleotide sequences that convert triplet codings for given amino acids into termination codons, and/or deletions are not immunohistochemically detectable. The loss of function of the wild-type allele and/or the presence of missense mutations may, in part, explain the increased positive p53 staining in oral carcinomas. It may also be due to the fact that the present study used a special technique to retrieve the antigen. Further experimental evidence is needed to support this view. As the staining reaction of the protein is limited to the nucleus, it appears that the mutant protein that

have acquired a dominant transforming activity remain in the nucleus (Langdon and Partridge, 1992; Nishioka et al., 1993). The process of carcinogenesis is presumed to be taking place in a step-wise manner: multiple genetic changes are required before a normal cell turns fully neoplastic (Shin et al., 1994). Etiological factors such as tobacco and alcohol have been shown to induce a variety of genetic changes in human neoplasia including head and neck, and oral squamous cell carcinomas (Casson et al., 1991; Brachman et al., 1992; Sakai et al., 1992; Somers et al., 1992; Boyle et al., 1993; Caamano et al., 1993; Kim et al., 1993; Yin et al., 1993; Field et al., 1994). Tobacco has also been implicated in the causation of lung cancer (Westra et al., 1993) and bladder cancer (Habuchi et al., 1993; Zhang et al., 1994).

Several investigators have suggested that unrepaired DNA damages might lead to mutation in the p53 gene by inducing G→T transversions (Hollstein et al., 1991; Puisieux et al., 1991; Field, Spandidos and Stell, 1992; Lee et al., 1993; Field et al., 1994). Most of the carcinogens are able to induce mutations in exons 5-9 of p53 gene and the most commonly observed changes are the G→A transitions (Sakai et al., 1992; Boyle et al., 1993; Kim et al., 1993) and G→T transversions (Somers et al., 1992; Largey et al., 1993). It has also been suggested that in vitro immortalization is a rate-limiting step in carcinogenesis. Recent studies have shown that p53 is needed for immortalization and that the gene may get inactivated by factors such as HPV types 16 and 18 (Crook et al., 1991; Burns et al., 1993; Kim et al., 1993) or it may have some role in programmed cell death or apoptosis (Donhower, 1994; Lane, 1994). The wild-type p53 gene product can reduce the transforming efficiency of many oncogenes and can suppress tumour growth, whereas mutant forms can lead to malignant transformation, especially when the protein is co-expressed with cellular oncogenes such as *ras* (Hinds et al., 1989; Hinds et al., 1990; Shaulsky et al., 1990; Pierceall et al., 1992).

The results of this study although differ from that of Ranasinghe and his associates (1993), strongly suggests that p53 oncoprotein is involved in the tumourigenesis of oral SCCs. Recent studies have shown that p53 may play an important role in the causation of oral cancers in habitual tobacco/betel quid chewers, and that the expression of p53 oncoprotein shows a relationship with the number of quids/day and the frequency of chewing (Kaur, Srivastava and Ralhan, 1994). Studies have also shown that ras oncogene and p53 gene form the part of the machinery which controls cellular

proliferation and/or differentiation. In normal cells, cyclin-dependent kinases (CDK) exist in multiple active forms containing cyclins, proliferating nuclear antigen (PCNA), and p21 (Draetta, 1990). As such, p21 is considered to be an inhibitor of cellular proliferation. Studies by Xiong, Zhang and Beach (1993) have shown that there is loss of p21 protein from CDK complexes in cells obtained from patients with Li-Fraumeni Syndrome (LFS), a syndrome that represents an inherited familial disorder with a relatively high susceptibility for early onset of various tumours (Li and Fraumeni, 1969). It is known that LFS patients already have a copy of p53 mutant allele while the remaining wild-type p53 allele might undergo mutation (Srivastava et al., 1990; Lozano, Hasen and Strong, 1994) and that their cells carry no known DNA viruses (Xiong et al., 1993). Therefore, the loss of p53 gene may alter normal functioning of the cell cycle and the functions of other cell cycle-associated proteins which act to cooperate during malignant transformation (Hinds et al., 1990).

It is possible that the normal tumour suppressor function of p53 is mediated through p21 or p21 is dependent upon the p53 pathway to effect the changes in cell proliferation and CDK complexes (Werness et al., 1990; Blanton et al., 1991; Watling, Gown and Coltrera, 1992; Xiong, Zhong and Beach, 1993). Although the incidence of *ras* mutations is comparatively low in oral squamous cell carcinomas in Western countries (Rumsby et al., 1990; Chang et al., 1991) and Japan (Sakai et al., 1992), H-*ras* analysis in the Indian oral cancer patient group (Bombay) has revealed a high percentage of tumour-associated loss of heterozygosity (LOH) in about 35% of the cases (Saranath et al., 1991). A still higher percent of *ras* mutation (50-70%) has been documented in oral SCC from Kerala and this study has also shown that the ras oncogene is, in part, responsible for malignant transformation (Kannan et al., 1993). Hence, it is possible that there may be co-expression of p53 with the mutation of H-*ras* and that these mutated proteins are essential for malignant transformation or may have a synergistic effect on malignant transformation at least in some oral cancers caused by tobacco/betel quid chewing. Also, the ingredients of the betel quid may play a decisive role in the causation of p53 mutations. As the betel quid in Kerala does not contain any additional spices (Orr, 1933; Sankaranarayanan et al., 1989, 1990) as opposed to Northern India (Sanghvi, 1981) or Sri Lanka (Seneviratna and Uragoda, 1973), it may be possible that this patient group lack the protective effects, if at all they have any, from these added condiments.

The phenolic compounds in areca nut may trap nitrites and may exhibit a strong anticlastogenic and antimutagenic effect (Stich and Rosin, 1984, 1984a), but these compounds may show chromosome damaging effects when used in combination with other ingredients of the betel quid such as tobacco, catechu, betel leaf, lime, and spices and flavoring agents (Nair et al., 1985; Nair et al., 1991; Dave, Trivedi and Adhavryu, 1992). Studies have shown that the exposure time to the carcinogens in tobacco and the age at the very first exposure is quite significant in the causation of oral cancers (Nair et al., 1985; Dave, Trivedi and Adhvaryu, 1991; Kayal et al., 1993). Such factors as the duration of exposure to tobacco, the amount of tobacco, the age at the very first exposure are also important as the areca nut-related phenolics stay in the saliva of the habitual chewers only for a limited time (Warnakulasuriya et al., 1984; WHO, 1984; IARC, 1985; Stich and Anders, 1989); it usually disappears soon after expectoration of the macerated betel quid mixture (Stich and Tsang, 1988). There is epidemiological evidence that the tobacco/quid chewers of Kerala usually keep the chewing mixture for prolonged periods in their mouth and most of them start the chewing habits at an early age (Sankaranarayanan, 1990; Sankaranarayanan et al., 1990). This may increase the exposure time to the carcinogens when compared to subjects from other places such as Sri Lanka (10-30 min., Seneviratna and Uragoda, 1973). Unlike other places, in Kerala, the habitual chewers prefer slaked lime to stone lime (WHO, 1984) which obviously will increase the pH along with tobacco thereby inflicting more damage onto the tissues (Stich and Anders, 1989). Reactive oxygen species generated from the chewing mixture is known to cause genetic damage in a sequential dose-dependent manner (Stich et al., 1988; Nair et al., 1992) and cell death (Thomas and McLannen, 1992). But there is no conclusive evidence to show that the presence of excess lime and the lime-induced tissue injury would affect the p53 expression.

It appears that some carcinogens in tobacco act only at certain sites of the gene (Brachman et al., 1992; Sakai et al., 1992; Somers et al., 1992; Boyle et al., 1993) and that moderate to heavy smokers, unlike nonsmokers, are likely to have multiple mutations (Field et al., 1994). It is also likely that there exists a synergistic effect between the various etiologic factors and the incidence of genetic damage (BEIR IV, 1988; Yu et al., 1988; Kayal et al., 1991; Thomas and Kearsley, 1992; Hoffmann et al., 1994). It has been shown that nutritional deficiencies, and tobacco chewing and smoking habits are more prevalent in the rural population of India, which constitutes more than 80% of the total population (Jayant, Notani and Sanghvi, 1983). Chili, a main ingredient of the diet

in the southern parts of India, is known to contain mutagenic and tumour promoting agents (Toth, Rogen and Walker, 1984; Agrawal et al., 1986). Notani and Jayant (1987) have shown that there is a 11-13 fold increase in the incidence of oral cancer in habitual chewers who do not prefer a vegetarian diet. There is epidemiological evidence that the relative risk for *bidi* is much higher when there are other habits such as tobacco chewing and alcohol drinking (Notani, 1988; Sanakaranarayanan et al., 1989; Kuriakose et al., 1992). The fact that most of the male subjects in the present study had chronic tobacco/betel chewing and bidi smoking habits may account for the increase incidence of genetic damage which, in turn, predispose them to oral cancer. As reported by many investigators, alcohol may have a synergistic effect since most people in the rural areas in Southern India use home-brewed alcohol which contains about 40-50% ethanol (Sankaranarayanan et al., 1990). In effect, the aforesaid etiologic factors may act in a sequential manner to bring about the malignant changes.

Recent studies have shown that UV rays play a major role in DNA damage, repair and malignant transformation at least in some cases of SCCs of the lip (Berner et al., 1993) and skin (Brash et al., 1991; Kastan et al., 1991; Sim, Slater and McPhee, 1992). Being a tropical region, the coastal areas of Kerala tend to show a relatively high UV index. But, whether this would affect the incidence of oral cancers in other sites, remains to be proved. Secondly, this region of India is one of the five major naturally radioactive zones of the world. The background radiation in this region has been calculated to be as high as 1500 mRads which is well above the maximum permissible dose of 500 mRads for the general population (Rajendran et al., 1992). There is experimental evidence that natural radiation can induce mutations and this might occur outside exons 5-11, the highly conserved regions of the gene (Vähäkangas et al., 1992). This may also be true for oral cancers induced by tobacco/betel quid chewing (Ranasinghe et al., 1993a).

The number of betel quids chewed per day, exposure to the carcinogens and the presence of more than one habits is important in the process of carcinogenesis. As shown in Tables 14 and 14a, 19/20 (95%) of the patients from the present study were chewers when compared to the Sri Lankan population (32/38; 84%). Even though the Sri Lankan population had smoking habits, the number of bidi smokers were less when compared to the present study (Table 20). Above normal levels of natural radiation, the carcinogens in the tobacco/betel quid mixture, and the bidi smoke alone or in combination with one

another may play a role in the incidence of mutation outside the 'conserved regions' of p53 gene and this may be that there is a higher incidence of mutations when compared to Sri Lankan samples. Further studies using advanced techniques such as polymerase chain reaction (PCR) coupled with direct DNA sequencing may help elucidate the molecular aspects of oral cancers in tobacco/betel quid chewers.

It has been suggested that over-expression of tumour suppressor genes is likely to be associated with dysfunction of a common regulatory pathway in the case of proliferation and differentiation (Werness et al., 1990; Blanton et al., 1991; Li et al., 1992). Many researchers have shown that keratinocytes transfected with HPV types 16 and 18 tend to undergo malignant transformation on exposure to tobacco-related carcinogens alone or in combination with tumour promoters such as NMU and phorbol esters (Li et al., 1992a; Miller et al., 1992; Garette et al., 1993). It is possible that the tumour suppressor gene p53 may be inactivated by the papilloma virus and that E6 protein of the virus can bind to and promote degradation (Brachman et al., 1993; Garette et al., 1993; Woods et al., 1993). As the gene acts to control transcription, its inactivation may predispose the affected tissues to malignant transformation. Cases have been reported where there is a negative relationship between the suppressor gene inactivation and the presence of HPV (Scheffner et al., 1990; Crook et al., 1991). Virus-induced changes along with the tobacco-related carcinogen-induced damage may sequentially make oral keratinocytes more prone to malignant transformation (Davidson, Hsu and Schantz, 1993; Kim et al., 1993; Kim et al., 1993a).

The ingredients of the betel quid also play a major role. Unlike other places, in Kerala, all the substances are used fresh and most patients have a tendency to leave the chewing material in their mouth for longer periods, and sometimes even during sleep (Orr, 1933; Hirayama, 1966). This may, in turn, increase the time of exposure to the carcinogens (Sankaranarayanan et al., 1989, 1990). Lime is known to contain radioactive substances which may emit α-particles ($_2He^4$) as high as 0.35 pCi which is equivalent to the amount of alpha-particles emitted from 0.53 mg/Kg U^{238} (Chakravarti, Dhiman and Nagpaul, 1981). Thus, it could be hazardous for people who chew continuously or who are exposed to the excess amounts of lime as it may cause damage to the tissues and the genetic material. Yet another possibility is that the hot and spicy food items, a habitual action in southern parts of India, may give rise to a variety of carcinogens. Although this

is true, a detailed dietary analysis is required to establish the exact role of diet in oral, and head and neck cancers. Also, southern parts of India is one of the areas where there is relatively high inbreeding which may, in part, account for the increased rate of genetic susceptibility.

In summary, it is possible that the duration of exposure to the carcinogen, the amount of carcinogen, the rate of metabolism (whether slow or active) and the age at first exposure, all these factors play a major role in the incidence of oral cancers in habitual betel quid/tobacco chewers in Kerala. On the other hand, the Sri Lankan population may not be exposed to carcinogens as that of the Keralites or their carcinogen metabolic pathways may be different from that of the Keralites. The genetic susceptibility of the present study group, more or less heterogeneous in its socioeconomic and cultural aspects, may be more than that of the Sri Lankan population which is rather a homogeneous group consisting of mostly Sinhalese (Seneviratna and Uragoda, 1973). Most of the patients in the present study had a history of chronic chewing and they reported having started the habits while very young (Dr. Ambika, personal communication). The genotoxic effects of tobacco, alcohol and diet-related factors may have a field cancerization effect on the mucosa of the upper aerodigestive tract and may thereby predispose the whole epithelium to malignant transformation (Hittelman et al., 1991; Voravud et al., 1993). However, the results of work done by Kannan et al. (1993) taken together with the present study suggests that a correlation between the over-expression of p53 protein and *ras* mutation is very likely. Further analysis of these cases at the molecular level might give some insight into functional aspects of the etiological factors in the development of oral cancers in habitual tobacco/betel quid chewers.

4.5 Conclusion

The results of the study on the levels of p53 expression shows that there is over-expression of p53 oncoprotein in oral a high percent of SCCs from Kerala. The over-expression of p53 in oral carcinoma samples from Kerala is strongly associated with the number of betel quids chewed and the number of bidis smoked per day. Other factors such as diet-related factors, natural radiation, relatively high genetic susceptibility, and/or the presence of transforming proteins of viruses may act sequentially to make the cells more prone to malignant transformation. p53 oncoprotein is likely to be involved in the promotion and/or progression of the squamous cell malignancies, and the discovery of p53 mutations in human SCCs suggests that alterations in tumour suppressor genes play an important role in some oral cancers (Yin et al., 1993). Although the protein is implicated in cell proliferation and/or differentiation, more investigations are needed to decipher the molecular mechanisms involved. The differences in the expression of p53 in oral cancers of habitual tobacco/betel quid chewers from that of the Sri Lankan population may be attributed to the various carcinogens and their metabolism, susceptibility, DNA repair systems, and/or genetic predisposition. In order to prove this it will be required to study a variety of oral neoplasms from a number of different populations exposed to different risk factors.

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Appendix 1.
Table 1. Increase in EGFR expression in Oral SCCs.

Author	**Over-expression of EGF receptor protein**	Weak Points	Method
Kamata et al. (1986)	**2/5** cases showed a 2-fold increase when compared to normal epidermal keratinocytes ($1x10^5$ binding sites/cell). The sensitivity to the inhibitory effect of EGF correlated well with the elevated level of EGFR.	This study did not mention the EGFR level in the remaining cases with respect to normal keratinocytes.	IP
Yamamoto et al. (1986)	**1/5** cases showed x30 gene amplification and a 3-5 fold increase in the expression of the protein. **1/5** cases showed the same level of EGFR as normal keratinocytes	No information on the EGFR level in the other 3 cases.	IP
Sakai et al. (1990)	**4/28** cases showed a positive staining while the rest of the cases remained negative. (1) EGFR staining was often localized at periphery of the tumour cell nest. (2) In 1 case, cells in the mitotic phase stained stronger than those under other phases.		IH
Kawamoto et al. (1991)	**10/14** culture cases showed a 2-10 fold increase in EGFR number compared to normal gingival tissue. (1) There is a positive correlation between EGFR numbers and increasing size &/or local extent of primary tumours. (2) A linear correlation between EGFR number and poor prognosis was also established in 2 cases of oral SCC.		LAB
Kearsley et al. (1991) (head & neck)	**All 46 (24 oral SCC)** cases were positive for the anti-EGFR staining reaction. The intensity of the reactions showed a considerable amount of variation: (1) Diffusely positive varying from weak to strong. (2) Maximum staining was always located on the peripheral invading islands. (3) All 5 poorly differentiated SCC showed strong staining. (4) In several well differentiated SCCs, more intense staining was noted in the peripheral cells of the tumour islands while the central part of the islands remained negative for the staining reaction.	No summary of results was given regarding the staining intensity of all SCCs.	IH
Shirasuna et al., 1991	**6/6** SCC cases showed diffuse strong staining while normal epithelium showed moderate basal staining.		IH
Kunikata et al. (1992)	**28 SCCs** showed varying intensity of anti-EGFR staining: Highly keratinized tumour cells were unstained with EGFR.	This study failed to give a proper classification for the staining reactions.	

Yamada et al. (1992)	**24/47** cases were positive for anti-EGFR staining: (1) The positivity rate of the EGFR was not related to tumour differentiation (2) In a given positive tumour, well differentiated SCC showed higher proportion of positive cells (31%) as compared to poorly differentiated SCC (2%). (3) The receptors were localized mainly on the cell membranes of normal mucosa in comparatively well-differentiated regions of the tumour mass. The expression of the receptor decreased in cancer when compared to that of normal mucosa. The staining was limited to prickle-like keratinizing tumour cells; basal and parabasal non-keratinizing cells within the tumour remained low in the level of the receptor.		IH

(Abbreviations: IH, Immunohistochemical methods; IP, Immunoprecipitation; LAB, Labelled Avidin-Biotin peroxidase method)

Table 2. Other Studies on EGFR

Yamamoto et al., 1986	**1/6** Cases showed amplification of EGFR on Southern Blot analysis (12.5%)
Eisburch et al., 1987	**1/17** Cases showed a 16-fold increase in the protein when analyzed with Northern Blot, but none of them showed amplification or rearrangement on Southern Blot analysis (**0/17**).
Ishitoya et al., 1989	**8/15** Cases showed over-expression of the receptor protein on Western Blot analysis (53.33%).
Ishitoya et al., 1989	Southern Blot analysis showed an 8-fold amplification of the gene in **1/21** cases (4.7%).
Weichselbaum et al., 1989	**5/11** Cases showed a many-fold increase in the expression of the protein transcripts on Northern Blot analysis, but only **1/6** cases showed gene amplification on Southern Blot analysis (16.7%).
Kearsley et al., 1991	**3/27** Cases showed gene amplification on Southern Blot analysis (7%).
Leonard et al., 1991	**3/12** Cases showed a 5 to 10-fold amplification on Southern Blot analysis (25%).
Rikimaru et al., 1992	**3/27** Cases showed gene amplification when analyzed with Southern Blot (7%).
Saranath et al., 1992	**19/66** Cases showed gene amplification on Southern Blot analysis (29%).
Yamada et al., 1992	**1/25** Cases showed a 4-fold gene amplification on Southern Blot analysis (4%).
Grandis and Tweardy, 1993	**3/6** Cases showed a 3 to 19-fold increase in the expression of mRNAs (50%).
Irish and Bernstein, 1993	**6/47** Cases showed gene amplification on Southern Blot analysis (12.7%).
Summary	**Avg: 18.49%**

Table 3 Intensity of EGFR Staining and The Pattern of EGFR in Different Epithelial Layers in Normal, Hyperplastic and Dysplastic Oral Epithelium

Only four papers, two included dysplastic lesions:

S: Shirasuna et al. (1991) (dysplasia)

K: Kunikata et al. (1992) (dysplasia)

P: Partridge et al. (1988)

W: Whitcomb et al. (1993)

	Basal cells	Prickle cell layer	Superficial layers
Normal	P: √ S: ++ K: + (other areas) - (buccal & tongue) W: +++	P: - (non-keratinized epithelium) + (keratinized epithelium) S: - K: +/- W: ++	P: - S: - K: - W: +
Hyperplasia	P: √ S: ++ K: +	P: √ S: ++ K: +	P:- S: - K: -
Dysplasia	S: ++ K: Not mentioned	S: ++ (all dysplasia of varying degrees showed similar results) K: No epithelial layer was mentioned. Reduced staining was noted. Hyperkeratinized lesions were particularly devoid of EGFR staining	S: - K: Not mentioned

√ Did not mention the staining intensity

-, Negative; +, Weakly positive; ++, Moderate staining; +++, Strong staining

Table 4. The pattern of distribution of EGFR in Cell Membrane vs Cytoplasm in Normal, Hyperplastic and SCCs

Seven papers on SCC, 2 papers on dysplasia and 2 on normal

S: Shirasuna et al. (1991) (dysplasia)

K: Kunikata et al.(1992) (dysplasia)

Diagnosis	Pattern of staining
Normal	6 papers: did not mention 4 papers: CMB
Hyperplasia	7 papers: did not mention 1 paper: CMB
Dysplasia	K: CMB, FCMB S: Not mentioned
SCCs	3 papers: did not mention 1 paper: CMB 2 paper: Mostly CMB; but in cases with strong staining, the pattern was more that of the CYTP type. 1: Mainly CYTP with Focal CMB

CMB: Only the cell membrane are positive
FCMB: A positive reaction in cell membrane here and there in a given field
CYTP: No cell membranes are positive but the whole cytoplasm is positive

Table 5. Expression of p53 in oral SCCs.

References	p53 expression
Field et al., 1991	49/73 (67%)
Gusterson et al., 1991	16/47 (34%)
Ogden et al., 1991	18/30 (60%)
Langdon and Partridge, 1992	12/15 (80%)
Sakai and Tsuchida, 1992	14/15 (93.3%) (cell-lines)
Warnakulasuriya and Johnson, 1992	13/37 (35%)
Watling et al., 1992	8/19 (42%)
Burns et al., 1993	6/17 (35%) 5/11 (45%) (cell lines)
Caamano et al. 1993	7/17 (41%)
Girod et al., 1993	46/85 (54%)
Nishioka et al., 1993	21/40 (52%)
Zhang et al., 1993	7/10 (70%)
Kaur et al., 1994	24/32 (75%)
Shin et al., 1994	15/33 (45%)
Average	53%

Table 6. Number of samples used for immunohistochemical detection of EGFR

Category	Number of samples
Normal oral epithelium	10
Epithelial hyperplasia	10
Mild dysplasia	11
Moderate dysplasia	12
Severe dysplasia/CIS	10
SCC	11

Table 7. Results of EGFR Staining Reactions

Diagnosis	# of cases	Staining intensity (%)		
		++	+++	++++
Normal	9	3 (33%)	6 (67%)	0
Hyperplasia	12	5 (42%)	6 (50%)	1 (8%)
Mild dysplasia	11	6 (54.5%)	5 (45.5%)	0
Moderate dysplasia	12	2 (16.7%)	9 (75%)	1 (8.3%)
Severe dysplasia & CIS	10	2 (20%)	6 (60%)	2 (20%)
SCC	10	2 (20%)	2 (20%)	6 (60%)

Table 8. Staining Intensity of SCC as Compared to Adjacent Epithelium

	Differentiation of SCC	SCC/adjacent epithelium
Case 1	Well differentiated	⇓ (++/+++)
Case 2	Moderately differentiated	Same (++++/++++)
Case 3	Poorly differentiated	⇓ (++/+++)
Case 4	Well differentiated	++ (++++/++)
Case 5	Well differentiated	Same (++++/++++)
Case 6*	Very well differentiated	Same (+++/+++)
Case 7*	Very well differentiated	Same (++/++)
Case 8	Poorly differentiated	++ (++++/++)
Case 9	Well differentiated	++ (++++/++)
Case 10	Well differentiated	+ (++++/+++)

* Verrucous carcinoma

Table 9. EGFR Staining in Different Epithelial Layers

Diagnosis	# of cases	Epithelial layers stained with EGFR (%)	
		Basal + parabasal (Grade 1)	Basal + parabasal + upper spinous cells (Grade 2)
Normal	9	2 (22)	7 (78)
Hyperplasia	12	4 (33)	8 (67)
Mild dysplasia	11	4 (36)	7 (64)
Moderate dysplasia	12	5 (42)	7 (58)
Severe dysplasia & CIS	10	2 (20)	8 (80)

Table 10. Correlation Between Staining Intensity Among Hyperplasia, Dysplasia and Squamous Cell Carcinoma

Staining intensity	Group A	Group B	Group C	Group D	Group E	Group F	Total
Moderate (Grade 2)	3	5	6	2	2	2	20
Strong (grade 3)	6	6	5	9	6	2	34
Very strong (Grade 4)	0	1	0	1	2	6	10
Total	9	12	11	12	10	10	64

(Group A, Normal mucosa; Group B, Hyperplasia; Group C, Mild dysplasia; Group D, Moderate dysplasia; Group E, Severe dysplasia; Group F, SCC)

Comment:

χ^2 (10 df) = 24.32; p value = 0.007. The value seems significant, but the bottom row is very sparse.

Table 11. Correlation Between Staining Intensity, Degree of Dysplasia and the Appearance of Carcinoma

Staining intensity	Group A + Group B	Group C + Group D + Group E	Group F	Total
Moderate (Grade 2)	8	10	2	20
Strong (Grade 3)	12	20	2	34
Very strong (Grade 4)	1	3	6	10
Total	21	33	10	64

(Group A, Normal mucosa; Group B, Hyperplasia; Group C, Mild dysplasia; Group D, Moderate dysplasia; Group E, Severe dysplasia; Group F, SCC)

χ^2 (4 df) = 18.30; p value = 0.0011

Comment:

Significant: Group F has more 'very strong' staining scores. This is the most reliable result.

Table 12. Correlation Between Staining Intensity, Degree of Dysplasia and Carcinoma (Grade-3 and Grade-4 staining reactions grouped together)

Staining intensity	Group A + Group B	Group C + Group D + Group E	Group F	Total
Moderate (Grade 2)	8	10	2	20
Strong + very strong (Grade 3 and 4 together)	13	23	8	44
Total	21	33	10	66

(Group A, Normal mucosa; Group B, Hyperplasia; Group C, Mild dysplasia; Group D, Moderate dysplasia; Group E, Severe dysplasia; Group F, SCC)

χ^2 (2 df) = 1.06; p value = 0.59

Comment:

The results are not significant. The distinction between 'strong' and 'very strong' staining is lost by combining the groups.

Table 13. Immunohistochemical localization of EGFR on normal oral mucosa

Authors	Basal cells (site of specimen)	Parabasal cells	Upper spinous layer	Antibody used
Partridge et al., 1988	+ (site not specified)	+	Non-keratinized epithelium: - Keratinized epithelium: +	MAbs EGFR1 & F4 (No source was mentioned)
Shirasuna et al., 1991	++ (Site was not specified)	-	-	MAb from Oncogene Science
Kunikata et al., 1992	- (buccal & lingual mucosa) + (retromolar and palate)	- +	- +	MAb from human A-431 cells (Bio-Markor, Israel)
Whitcomb et al., 1993	+++ (buccal, lingual, gingiva, lip, floor of mouth, palate)	++	+ (No difference in staining pattern between keratinized and non-keratinized epithelium was noticed.)	MAb 3 to EGFR & MAb 1 to EGFR from Oncogene Science

Table 14. Patient data: Tobacco Chewing and Smoking, and Drinking Habits of Patients from Kerala

Case #	Age/ gender	Socioeconomic class	Chewing (pan per day)	Smoking (Bidis[1] per day)	Alcohol (ounces per day)	Contents of pan[2]
1	40/F	I	5-9	0	0	BNTL
2	45/F	IV	10-15	0	0	BNTL
3	50/F	IV	10-15	0	0	BNTL
4	52/M	III	0	0	10+	0
5	52/F	NI	NI	NI	NI	NI
6	55/M	III	5-9	20+	5-9	BNTL
7	55/M[3]	III	<5	5-9	0	BNT
8	57/M	IV	10-15	20+	5-9	BNTL
9	57/M	IV	5-9	<5	<5	BNTL
10	59/F	III	5-9	0	0	BNTL
11	60/F	IV	5-9	0	0	BNTL
12	60/F	NI	NI	NI	NI	NI
13	60/M	III	<5	0	0	BNT
14	60/M	III	5-9	5-9	0	BNTL
15	60/F	IV	10-15	0	0	BNTL
16	61/F	IV	<5	0	0	BNTL
17	62/M	IV	<5	<5	0	BNTL
18	62/F	IV	5-9	0	0	BNTL
19	64/M	IV	10-15	20+	5-9	BNTL
20	64/M	III	10-15	5-20	0	BNTL
21	65/M	III	5-9	5-20	0	BNTL
22	66/F	IV	10-15	0	0	BNTL
23	75/F	IV	<5	0	0	BNTL

[1]Bidis: Local cigarettes made by wrapping less than 0.5 g of coarse tobacco in a dry temburni (*Diospyros melanoxylon*) leaf.

[2] Pan: The most common masticatory available in India which is formed by wrapping up bits of tobacco and small slices of areca nut in a betel leaf smeared with lime.

Abbreviations: B, Betel leaf; N, areca Nut; L, Lime; T, Tobacco; NI, No Information available

[3]Smoking cigarettes instead of bidis.

Socioeconomic class: Class I, Professionals; Class II, Non-professional and Managerial; Class III, Non-manual or Manual Skilled; Class IV, Manual Unskilled (Modified from Leete and Fox, 1977)

Table 14a. Tobacco Habits of Patients with Oral Cancer from Kerala: A Percent Breakdown

Ingredients of the betel quid			
	Male	Female	Total
Group I: BTNL	8	10	18 (78%)
Group II: BTN	2	0	2 (9%)
Group III: NI	1*	2	3 (13%)
			23

Chewing/Smoking/Drinking Habits			
Habit	Male	Female	Total
Group I: Chewing only	1	10	11 (48%)
Group II: Chewing and Smoking	5	0	5 (22%)
Group III: Chewing, Smoking and Drinking	4	0	4 (17%)
Group IV: Drinking only	1	0	1 (4%)
Group V: NI	0	2	2 (9%)
			23

* This patient had heavy drinking habits.

(Abbreviations: B, Betel leaf; N, areca Nut; L, Lime; T, Tobacco; NI, No information recorded)

Table 15. Patients' Data and p53 Staining Results

Age/ gender	Socioeconomic class	Chewing (pan per day)	Smoking (Bidis[1] per day)	Alcohol (ounces per day)	Contents of pan[2]	p53 staining intensity	p53 staining pattern
40/F	I	5-9	0	0	BNTL	+	Scattered
45/F	IV	10-15	0	0	BNTL	++	Diffuse
50/F	IV	10-15	0	0	BNTL	+++	Diffuse
52/M	III	0	0	10+	0	-	-
52/F	NI	NI	NI	NI	NI	-	-
55/M	III	5-9	20+	5-9	BNTL	+++	Diffuse
55/M[3]	III	<5	5-9	0	BNT	-	-
57/M	IV	10-15	20+	5-9	BNTL	+++	Diffuse
57/M	IV	5-9	<5	<5	BNTL	++	Diffuse
59/F	III	5-9	0	0	BNTL	-	-
60/F	IV	5-9	0	0	BNTL	±	Scattered
60/F	NI	NI	NI	NI	NI	-	-
60/M	III	<5	0	0	BNT	-	-
60/M	III	5-9	5-9	0	BNTL	++	Diffuse
60/F	IV	10-15	0	0	BNTL	++	Scattered
61/F	IV	<5	0	0	BNTL	-	-
62/M	IV	<5	<5	0	BNTL	-	-
62/F	IV	5-9	0	0	BNTL	++	Diffuse
64/M	IV	10-15	20+	5-9	BNTL	+++	Diffuse
64/M	III	10-15	5-20	0	BNTL	++	Diffuse
65/M	III	5-9	5-20	0	BNTL	++	Diffuse
66/F	IV	10-15	0	0	BNTL	+++	Diffuse
75/F	IV	<5	0	0	BNTL	-	-

(+, weak staining; ++, Moderate staining; +++, Strong staining; Negative, No staining; Diffuse, A group of positive cells; Scattered, Occasional positive single cells only; 0, No pattern)

[1]Bidis: Local cigarettes made by wrapping less than 0.5 g of coarse tobacco in a dry temburni (*Diospyros melanoxylon*) leaf.

[2] Pan: The most common masticatory available in India which is formed by wrapping up bits of tobacco and small slices of areca nut in a betel leaf smeared with lime.

Abbreviations: B, Betel leaf; N, areca Nut; L, Lime; T, Tobacco; NI, No Information available

[3]Reported having smoked cigarettes instead of bidis.

Table 16. Relationship Between Over-expression of p53 and Site of Lesion

Site/Total number of cases	Intensity of staining			
	-	+	++	+++
Buccal (5/9, 55%)	4	0	3	2
Tongue (4/9, 44%)	5	1	1	2
Gingiva (3/4, 75%)	1	0	1	2
Palate (1)	0	1	0	0

Table 17. Expression of p53 and Chewing Habits (number of quids/day)

Number of pans chewed per day	Intensity of p53 Staining			
	-	+	++	+++
<5 quids/day	6£	0	0	0
5-9 quids/day	2*	1	4	1
10+ quids/day	0		3	4

£ Includes a case with no history of chewing

* Includes a very weakly stained verrucous carcinoma

Table 18. Statistical Analysis of p53 Expression and Chewing Habits in Oral Cancer Patients from Kerala

	>5 quids/day	<5 quids/day	Total
p53 positive cases	14	0	14
p53 negative cases	1	6	7
Total	15	6	21

Fisher Exact test (p value = 0.0011).

Table 19. Correlation Between Number of Betel Quids Chewed and the Intensity of p53 Staining Reactions

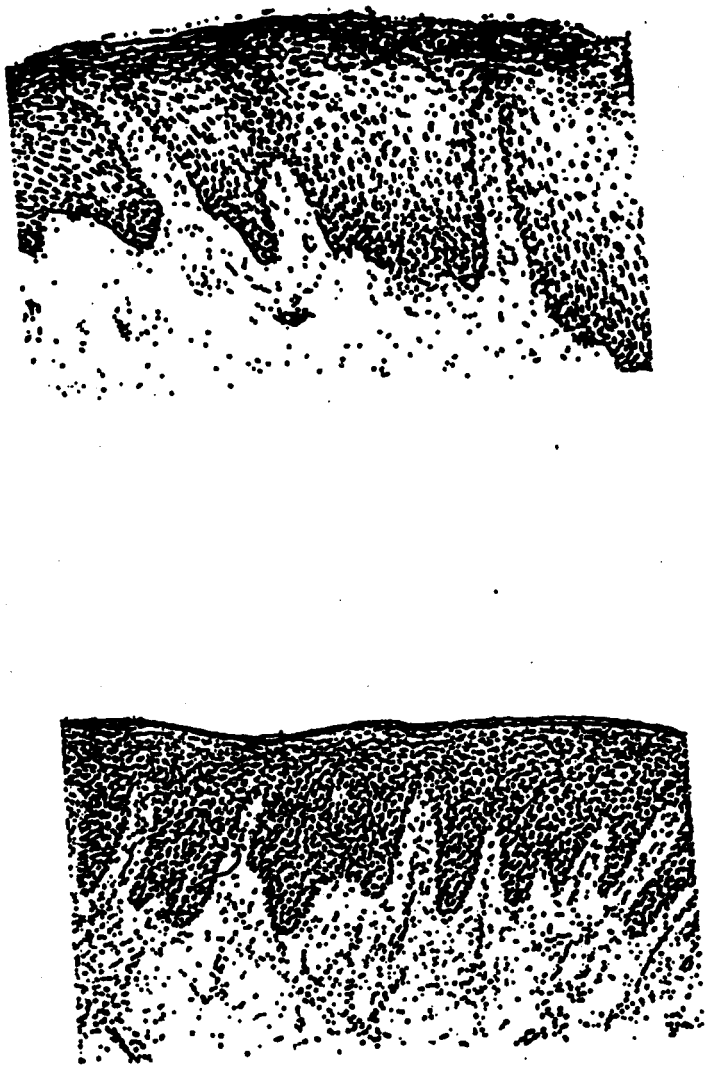
Number of quids chewed/day	Intensity of p53 staining
7 (5-9)	1
13 (10-15)	2
13 (10-15)	3
7 (5-9)	3
3 (0-5)	0
13 (10-15)	3
7 (5-9)	2
7 (5-9)	0
7 (5-9)	0
3 (0-5)	0
7 (5-9)	2
13 (10-15)	2
3 (0-5)	0
3 (0-5)	0
7 (5-9)	2
13 (10-15)	3
13 (10-15)	2
7 (5-9)	2
13 (10-15)	3
3 (0-5)	0

(Multiple R, 0.794; R Square, 0.630; Adjusted R Square, 0.609; SE, 2.514; Observations, 20; p-value, 0.00016)

Table 20. Tobacco Chewing Habits of Patients with Oral Cancer from Sri Lanka (Ranasinghe et al., 1993)

	Male	Female	Total
Betel Chewing			
With tobacco	27	5	32 (84%)
Without tobacco	1	2	3 (8%)
Smoking			
Cigarettes	6	0	6
Cigars	2	0	2
Bidi	4	0	4
Mixed Smoking			24 (63%)
Chewing and Smoking			21 (55%)

Fig. 1. Normal non-keratinized and orthokeratinized stratified squamous oral epithelium (Bhaskar, 1986).



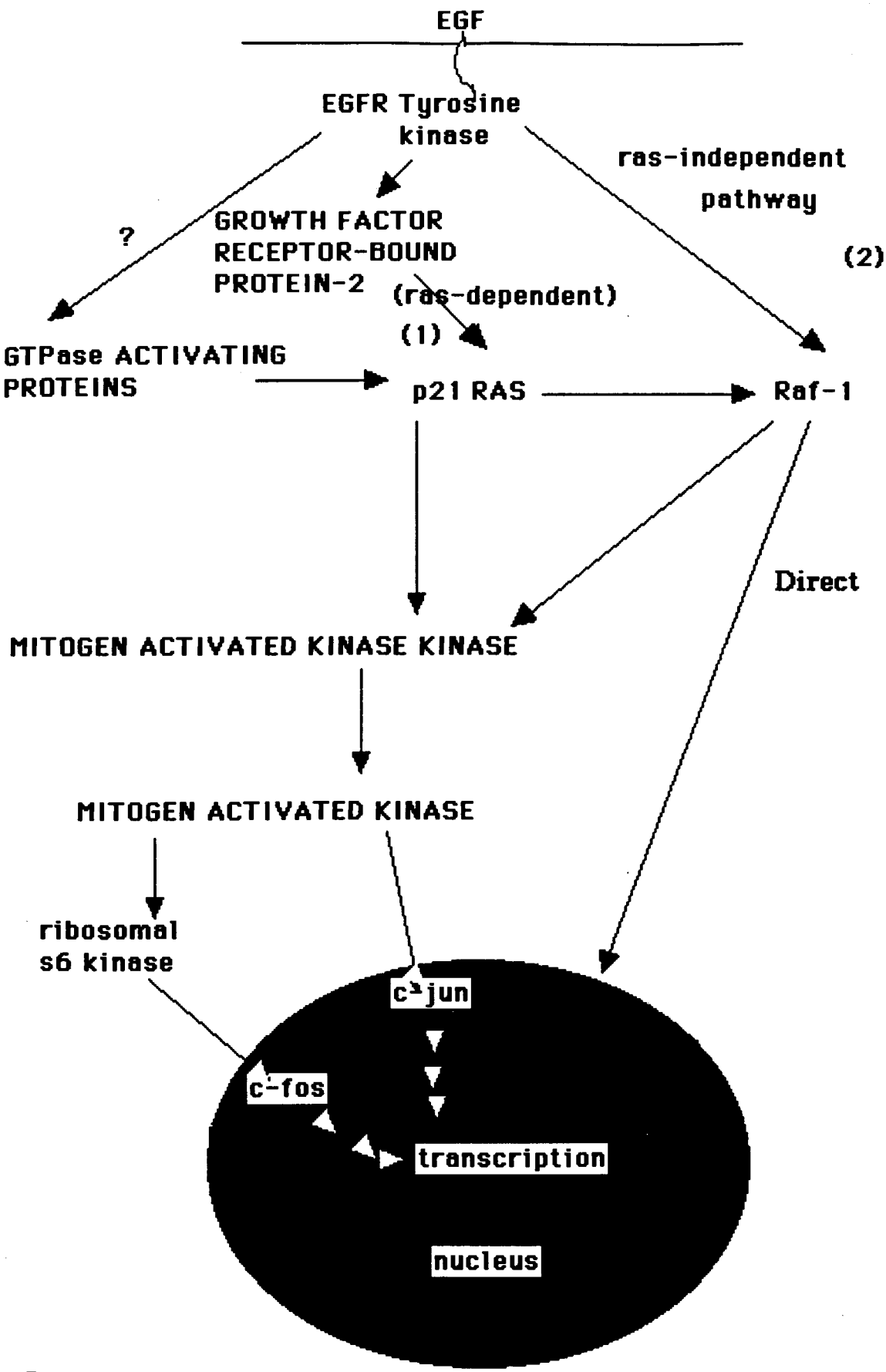


Fig. 2.

A signal chain of events (modified from Hsuan, 1993)

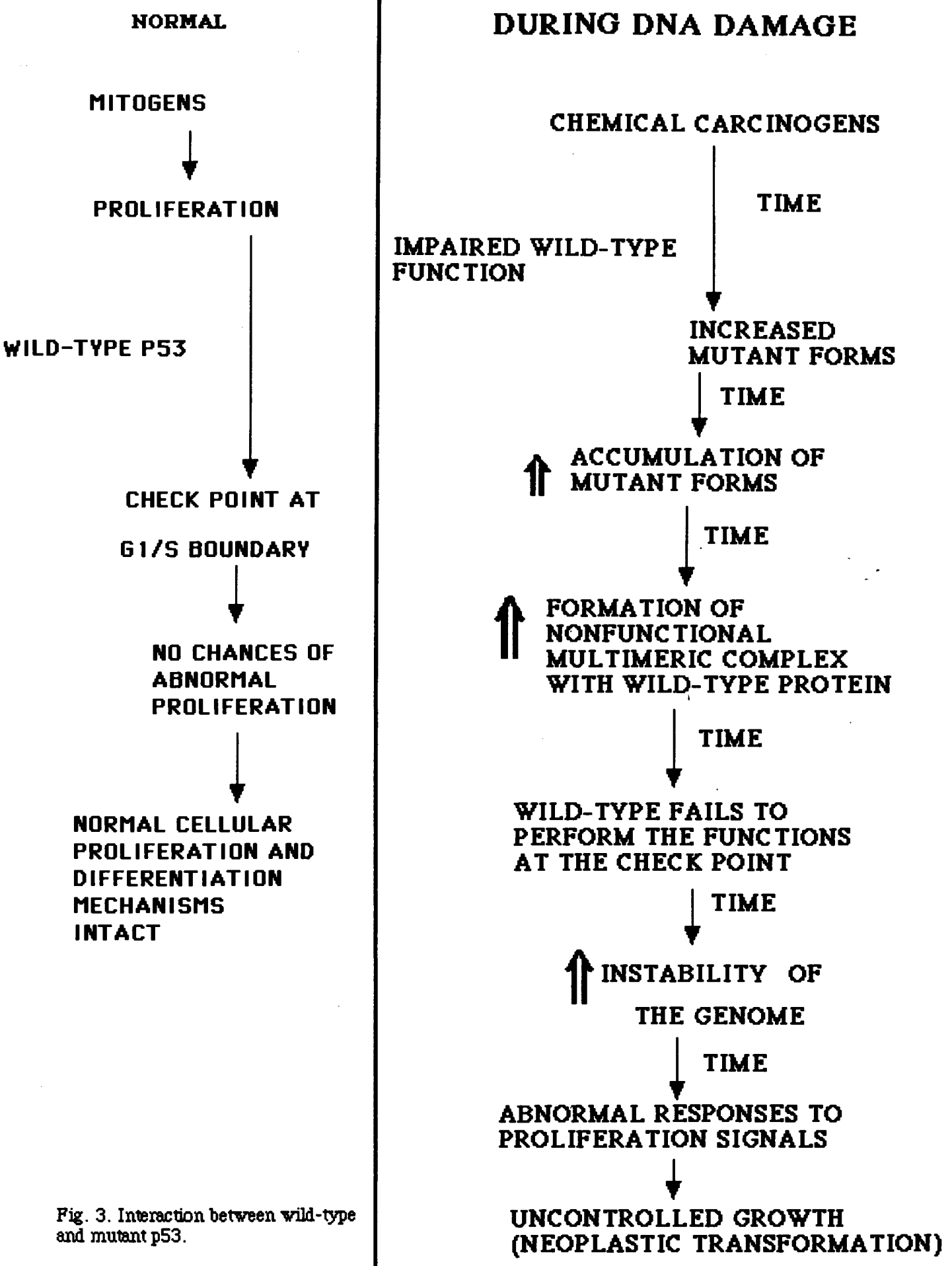


Fig. 3. Interaction between wild-type and mutant p53.

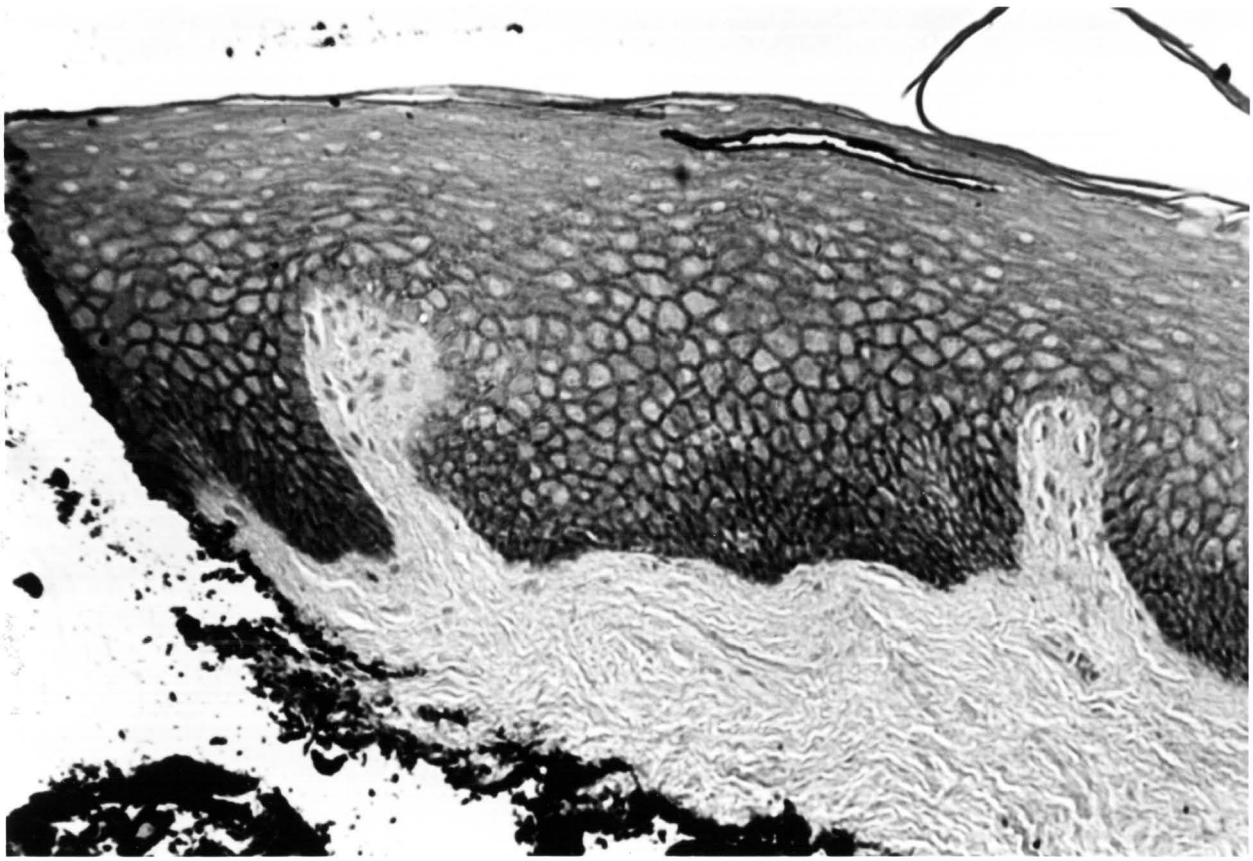


Fig. 4. Photomicrograph of normal oral mucosa showing strong EGFR staining in basal, parabasal and upper spinous cell layers. (Original magnification 80)

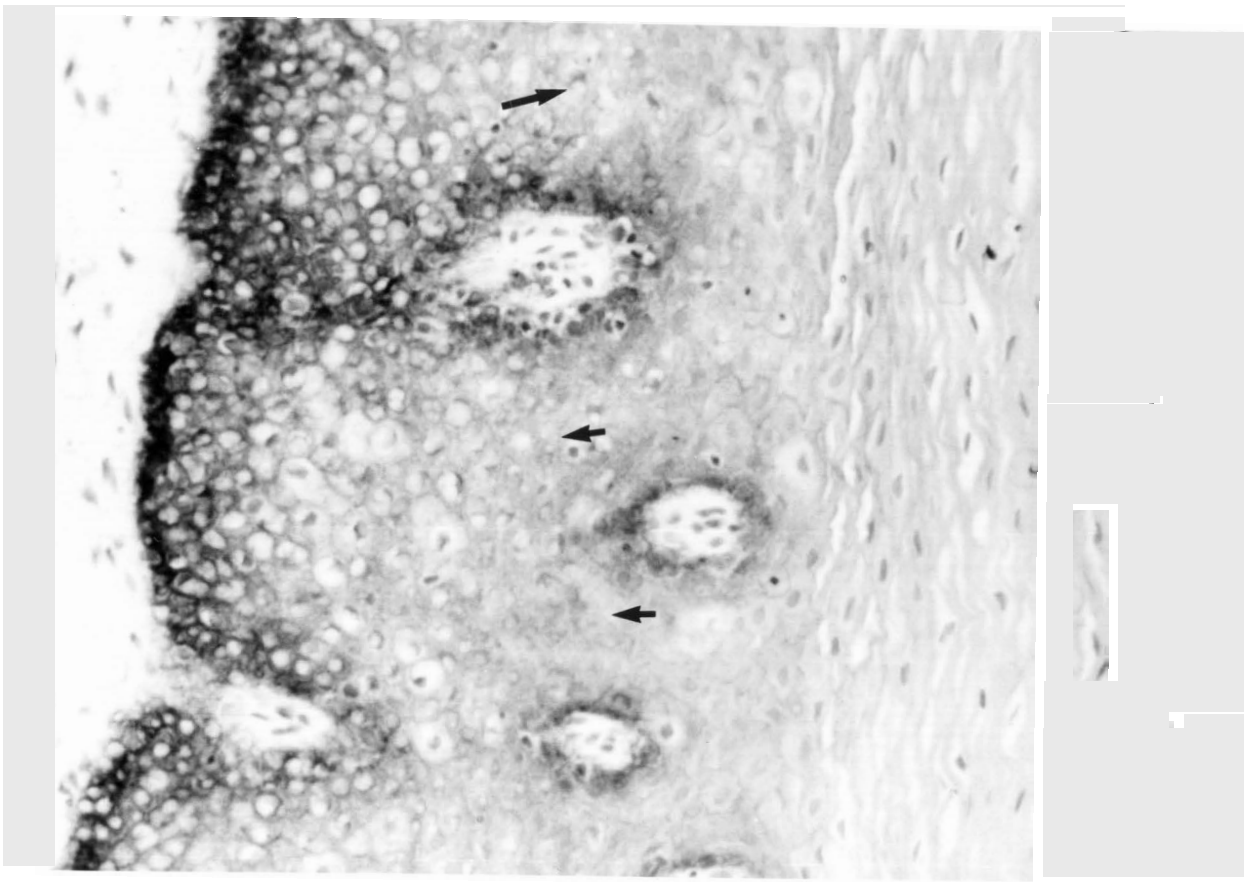


Fig. 5. Photomicrograph of a hyperplastic oral lesion demonstrating strong basal and parabasal EGFR staining and a weaker staining in the upper spinous cells (Black arrows). (Original magnification 80)

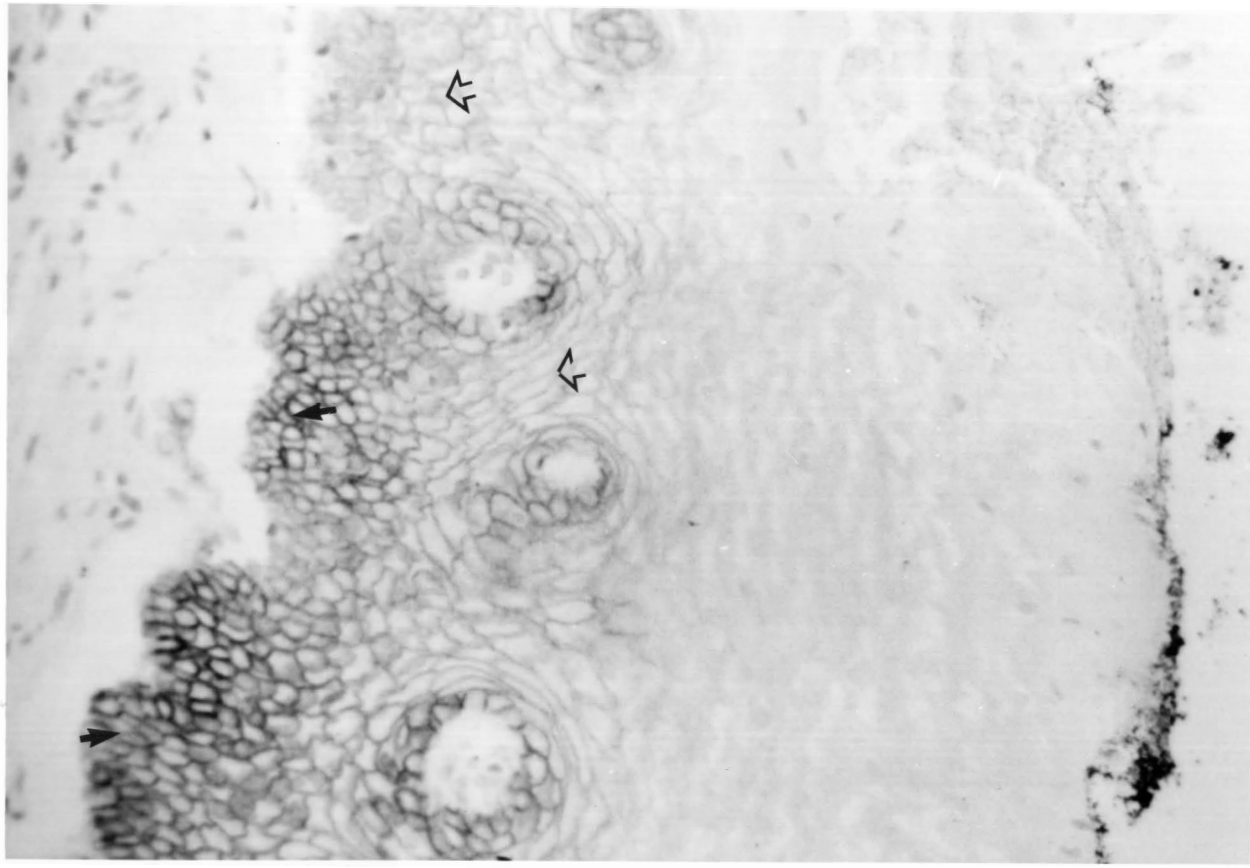


Fig. 6. Photomicrograph of a mildly dysplastic oral lesion demonstrating moderate basal and parabasal EGFR staining in some areas (Black arrows), but negative or equivocal staining in other areas (Hollow arrows). (Original magnification 80)

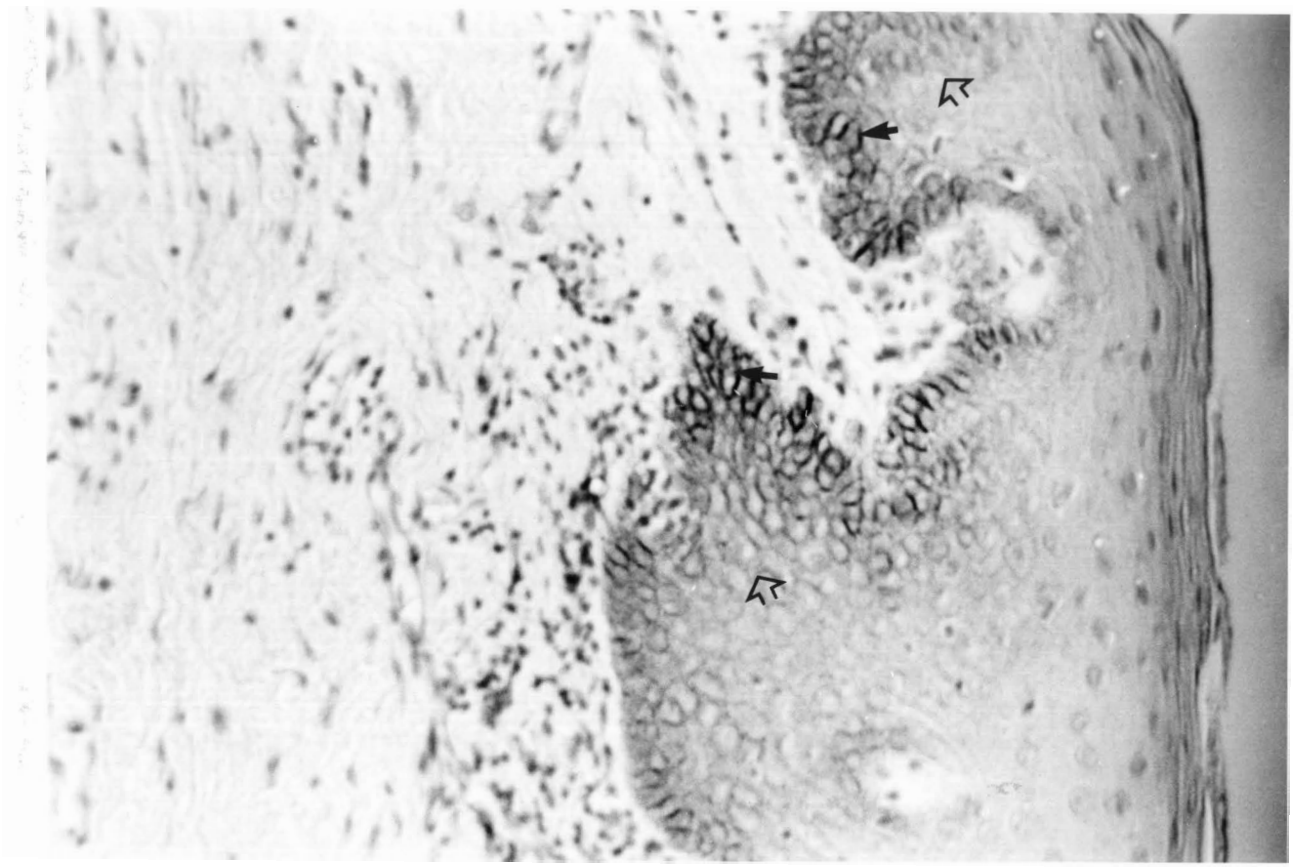


Fig. 7. Photomicrograph of a moderately dysplastic oral lesion demonstrating strong EGFR staining in basal and parabasal cells on the left side of the field (Black arrows), but weak or equivocal staining in other areas of the field (Hollow arrows). (Original magnification 80)

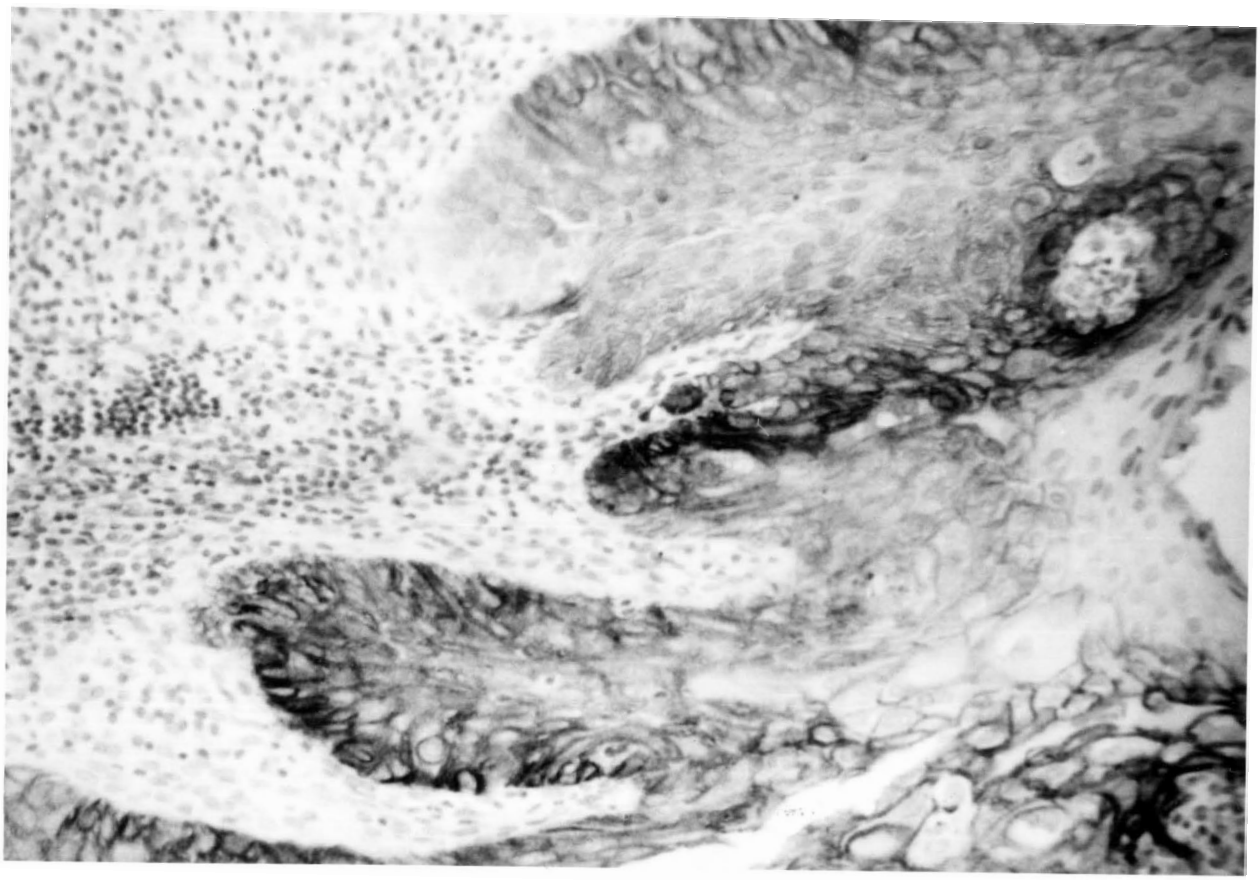


Fig. 8. Photomicrograph of a severely dysplastic oral lesion demonstrating very strong EGFR staining in basal, parabasal and upper spinous cell layers. (Original magnification 80)

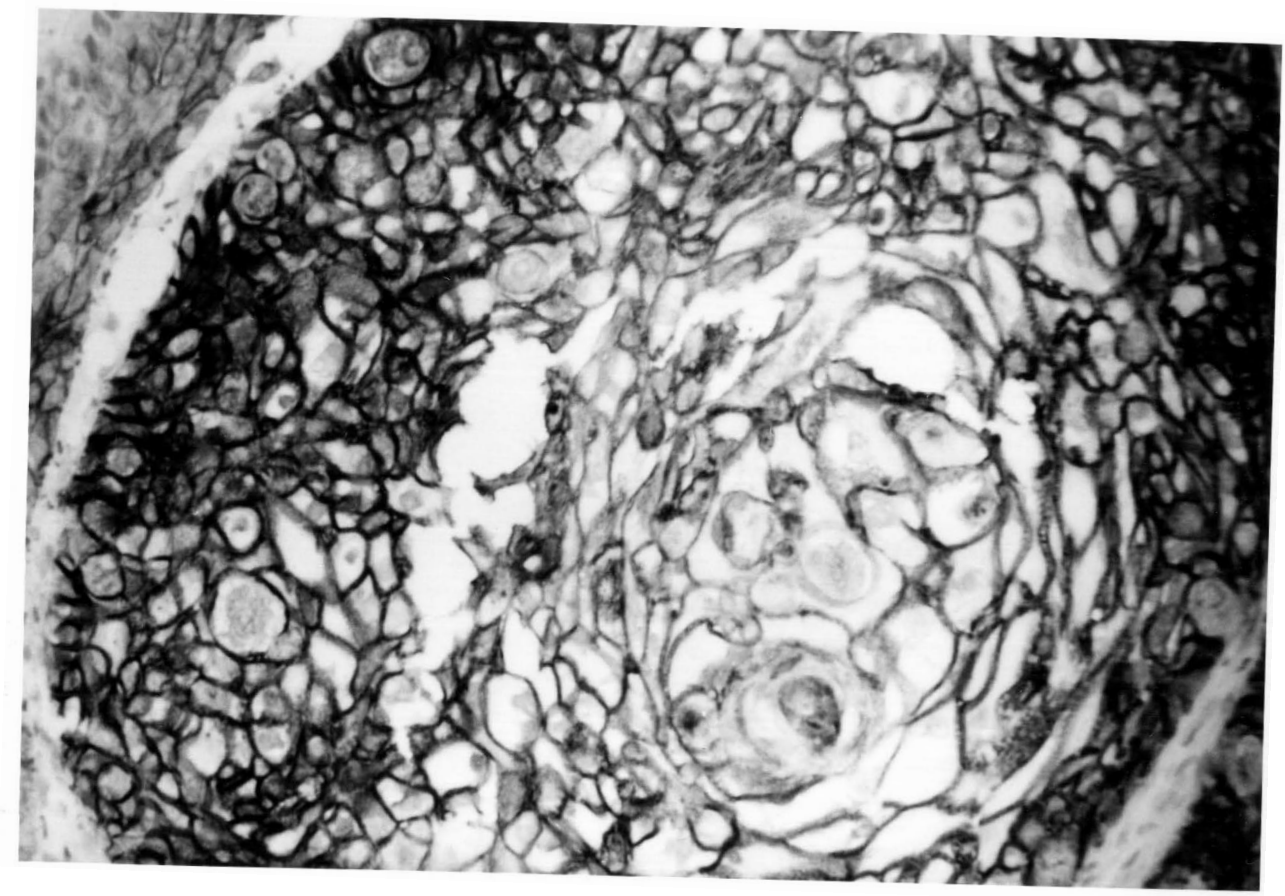


Fig. 9. Photomicrograph of an oral squamous cell carcinoma demonstrating very strong EGFR staining. (Original magnification 80)

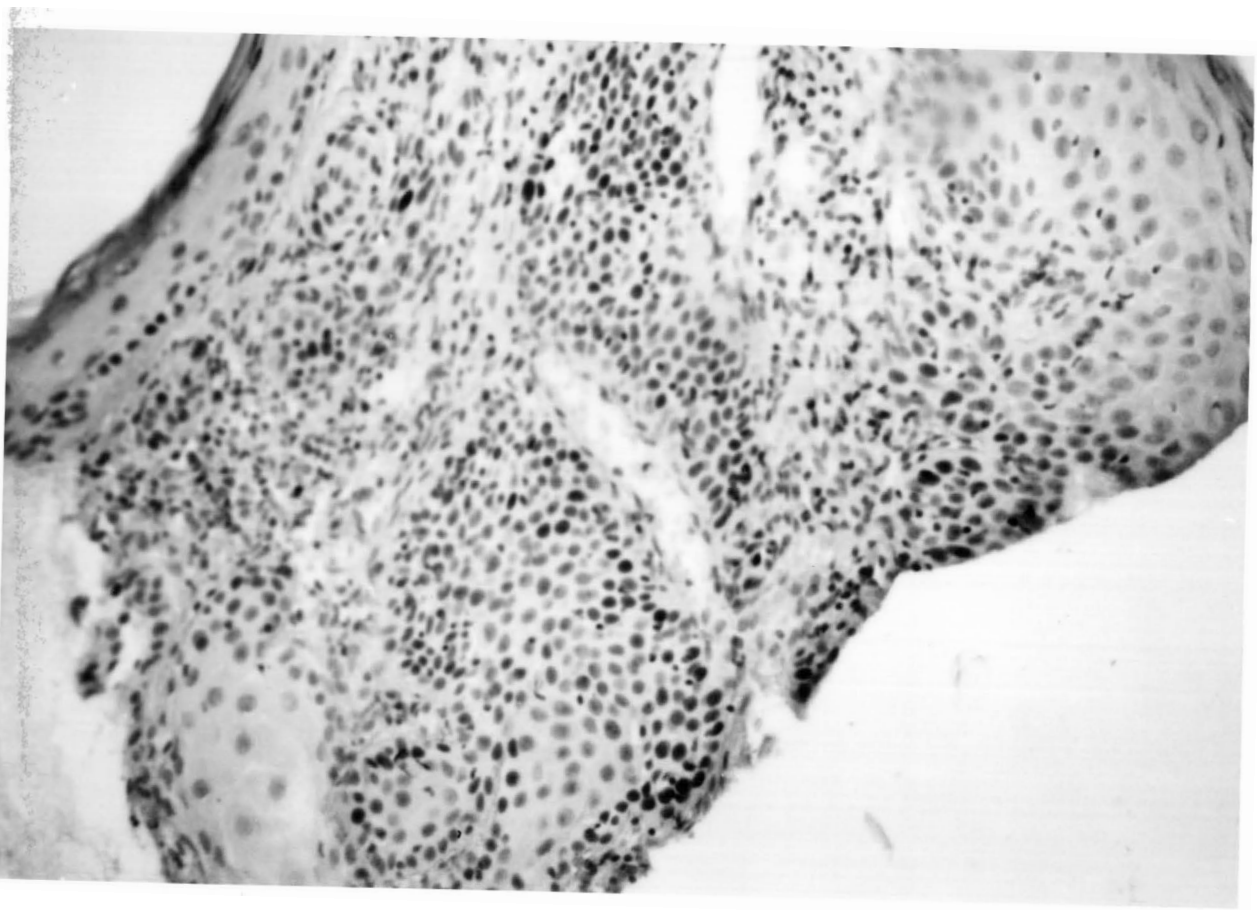


Fig. 10. Photomicrograph showing the expression of p53 oncoprotein on an oral squamous cell carcinoma. (Original magnification 80)

Appendix 2. EGFR: Patient Data and Results of Staining Reactions

Number of cases, age & gender	Degree of dysplasia	Site	Habit	Staining Pattern	Grades of EGFR staining(S) and the number of compartments (C) involved	EGFR expression in adjacent non-neoplastic or 'normal' epithelium (S'=intensity of staining; C'=Compartments involved).	Comments
1. 6044-92 57/M	Mild	Antr. Flr of the mouth		CMB	S=2 ——— C=1		Grade 2 Layers: Basal and Suprabasal Some areas showed a staining of moderate intensity
2. 4176-92 57/M	Mild	Postr Right Palate		CMB	S=2 ——— C=2		Grade 2 Layers: Basal, suprabasal and spinous layers Focal areas showed a moderate staining

3. 9857-92 57/F	Mild	Ventral surface of Tongue		CMB	S=3 ——— C=2		Grade 3 Layers: Basal, suprabasal and lower spinous Some areas showed a weak staining
4. 6922-92 54/M	Mild	Rt. l atl. Border of Tongue		CMB	S=3 ——— C=2		Grade 3 Layers: Basal, suprabasal and spinous layers Some areas remained negative to the staining reaction.

5. 8151-92 59/M	Mild	Lt. Hard Palate	T	CMB	S=3 ——— C=1		Grade 3 Most of the areas showed a moderate staining. In certain regions, focal strong staining was evident in basal and suprabasal layers of the epithelium.
6. 6615-92 34/F	Mild	Lt. Flr of the Mouth	T	CMB	S=3 ——— C=2		Grade 3 Layers: Basal, suprabasal and lower spinous layers Certain areas show a strong basal and suprabasal staining.

7. 11284-91 45/M	Mild	Lt. Buccal Mucosa	T	CMB	S=2 ——— C=2		Grade 2 Layers: Basal, suprabasal and lower spinous layers Occasional moderate staining and negative basal staining were evident in some areas.
8. 2858-92 63/M	Mild	Rt. Border of Tongue		CMB	S=2 ——— C=2		Grade 2 Layers: Basal, suprabasal and lower spinous layers
9. 10167-92 56/M	Mild	Lt. Buccal Mucosa		CMB	S=3 ——— C=3		Most areas showed a strong staining involving basal, suprabasal and lower spinous layers

10. 9458-92 52/F	Mild	Rt. Border of Tongue		CMB	S=2 ——— C=1		Grade 2 Both basal and suprabasal layers were involved in the staining reaction.
11. 9578-92 41/F	Mild	Rt. Flr of the mouth		CMB	S=2 ——— C=1		Grade 2 Certain regions showed a weak to moderate intensity of staining.
1. 723-92 54/M	Moderate	Floor of the Mouth		CMB	S=3 ——— C=1		Grade 3 Uniform staining in the basal and suprabasal cells was noticed.

2. 992-92 48/F	Moderate	Rt. Buccal Mucosa	T	CMB	S=3 ——— C=2		Good for picture Grade 3 Layers: Basal, suprabasal and lower spinous layers Focal strong staining was evident in some areas.
3. 6426-92 30/F	Moderate (lichenoid)	Rt. Ventral Surface of Tongue		CMB	S=4 ——— C=2		Grade 4 Layers: Basal, suprabasal, and lower and upper spinous layers
4. 11454-91 49/F	Moderate	Lt. Floor of the Mouth		CMB	S=3 ——— C=2		Grade 3 Layers: Basal and suprabasal

5. 1823-92 38/F	Moderate	Lt. Tongue		CMB	S=2 ——— C=3		Grade 2 'Normal' areas showed a weak staining; in certain areas, the dysplastic areas were weakly stained than the 'normal' cells. Layers: Basal, suprabasal and lower spinous layers
6. 11439-91 35/F Good slide	Moderate	Lt. Lower Lip		CMB	S=3 ——— C=2		Grade 3 Layers: Basal, suprabasal and lower spinous layers are involved

*7. 3540-92 61/M	Moderate	Lower Lip		CMB	S=2 ——— C=2		Grade 2 Focal weak to moderate staining was evident in some sections with the involvement of basal, suprabasal and lower spinous layers.
8. 2992-92 73/F	Moderate			CMB	S=3 ——— C=2		Grade 3 Layers: Basal, suprabasal and lower spinous layers 'Normal' cells showed a negative staining whereas dysplastic areas showed a moderate staining.

9. 4671-91	Moderate			CMB	S=3 ——— C=1		Grade 3 Layers: Basal and suprabasal
10. 3634-93 59/M	Moderate	Buccal mucosa		CMB	S=3 ——— C=1		Grade 3 Occasional moderately stained areas were present. Basal and suprabasal layers showed a positive staining.

11. 9412-88 57/F	Moderate	Antr. Flr. of mouth		CMB	S=3 ——— C=1		Grade 3 Layers: Basal and suprabasal Less dysplastic areas showed a weak positive staining whereas more dysplastic areas remained negative to staining reaction.
12. 1736-93 50/F	Moderate	Ventral Surface of Tongue		CMB	S=3 ——— C=2		Grade 3 Basal, suprabasal and lower spinous layers stained positive to the antibody with focal areas of strong staining.

1. 9524-92 37/M	Severe (CIS)	Rt. Ventral Surface of Tongue		CMB	S=4 ——— C=2		Grade 4 Some areas showed a very strong staining with the involvement of upper spinous layers, but certain areas showed a weak basal staining.
2. 1130-91 55/M	Severe	Antr Flr. of the Mouth and Vestibule		CMB	S=3 ——— C=2		Grade 3 Staining extended beyond suprabasal layers with occasional strong staining. Certain areas show a negative basal staining

3. 8215-92 88/M	Severe	Rt. Antr. Flr. of the Mouth		CMB	S=4 ——— C=2		Grade 4 Layers: Basal+Suprabasal+ Upper Spinous layers Certain areas were very strongly stained. Basal cells remained negative in some areas.
4. 2628-92 63/M	Severe (CIS)	Marginal Gingiva		CMB	S=2 ——— C=1		Grade 2 The same section showed a variation in staining intensity. 'Normal' cells showed a weak staining compared to the dysplastic cells.

5. 3598-91 69/F	Severe (CIS)	Rt. Ventral Surface of Tongue		CMB	S=4 ——— C=2		Grade 4 Layers: Basal+Suprabasal+ Upper Spinous In certain areas the staining reaction was very strong.
6. 7039B-90 77/M	Severe (CIS)	Rt. Flr. of the Mouth			S=2 ——— C=1		Grade 2 Both basal and suprabasal layers remained positive to anti-EGFR staining.
*7. 1600A-92 61/M	Severe	Lower Lt. Lip		CMB	S=3 ——— C=2		Grade 3 Cells beyond suprabasal layers were involved in the staining reaction.

8. 7530-90 75/F	Severe	Rt. Antr. Flr. of the Mouth		CMB	S=3 ——— C=2		Grade 3 Tissue sections showed marked variation in the degree of staining intensity and in the number of layers involved in the staining reaction. Superficial spinous layers were involved in certain areas.

9. 11785-92 78/F	Severe	Lt. Latl. Border of Tongue		CMB	S=3 ——— C=2		Grade 3 The staining reaction showed an extension beyond the suprabasal layers, but basal cells remained negative in some areas.
10. 11935-92	Severe (CIS)			CMB	S=3 ——— C=2		Grade 3 Some areas showed a moderate-to-strong staining reaction and the staining reaction involved cells beyond suprabasal layers.

1. 4005-92 80/F	SCC	Upper Lt. Tuberosity		Mainly CYTP	S=2 ——— C=2	S'=3 ——— C'=2	Grade 2 The tumour cells showed a moderate positive staining reaction in all layers of the epithelium while all layers of the adjacent 'normal' epithelium showed a strong staining.
2. 2564-92 36/M	SCC	Lt. Latl. Border of Tongue		Both	S=4 ——— C=2	S'=4 ——— C'=2	Grade Both the tumour cells and the adjacent epithelium showed a positive reaction in all layers of the epithelium.

3. 6265A-92 66/M	SCC	Antr. Lower Alvr. Ridge		Not stained	S=2 ——— C=2	S'=3 ——— C'=2	Grade 3 Tumour cells showed a staining reaction of moderate to strong intensity while almost all layers of the adjacent epithelium showed a strong staining reaction.
4. 5254-91 84/M	SCC	Rt. Latl. Border of Tongue		Both	S=3 ——— C=2	S'=2 ——— C'=2	The adjacent epithelium showed a staining reaction of weak to moderate intensity.

5. 2519-92 49/F	SCC	Buccal Mucosa (3.6 region)		Some areas showed CYTP type of staining	S=4 C=2	S'=4 C'=2	Grade 4 Tumour nests (all layers) showed a very strong staining reaction and so also the adjacent epithelium.
6. 2602-91 73/F	Verrucous Carcinoma with micro-invasion	Buccal Mucosa (3.4 and 3.5 region)		Both	S=3 C=2	S'=3 C'=2	Grade All layers of both the tumour nests and the adjacent epithelium showed a moderate-to-strong staining.

7. 5151-92 60/M	Verrucous Carcinoma	Antr Mandible		Mostly CMB	S=4 ——— C=2	S'=4 ——— C'=2	Grade: 4 Both the tumor tissue and the adjacent epithelium showed a very strong staining reaction.
8. 1431-91 74/M	SCC	Floor of the mouth		Mostly CMB	S=4 ——— C=2	S'=1 ——— C'=2	Grade 4 The adjacent epithelium showed a weak staining pattern involving cells up to the lower spinous layer.

9. 7435-91 60/M	Verrucous Carcinoma	Rt. latl. tongue		Both	S=4 C=4	S'=3 C'=3	Grade 4 Both the tumor nests and the adjacent epithelium showed the same intensity of staining.
10. 7154-91 46/M	SCC	Rt Flr. of the mouth		FCMB CYTP	S=4 C=2	S'=3 C'=2 The adjacent epithelium showed a weak cell membrane positive staining with focal strong cytoplasmic staining	The adjacent epithelium showed a less intense staining reaction when compared to the tumor cells.

Hyperplasia

Case Number	Diagnosis	Biopsy site	Staining Pattern	Grade	Layers involved
7464 S 92 40/M	Hyperortho-keratosis and acanthosis	Rt. buccal mucosa	CMB	S=2 C=1	Basal and suprabasal
9706 S 92 26/M	Marked hyper-parakeratosis and acantl osis	Rt. buccal mucosa	CMB	S=3 C=2	Basal, suprabasal and lower spinous cells
10026 S92 15/F	Mucocele	Lower lip	CMB	S=2 C=1	Basal and suprabasal
10023 S92 54/F	Healing pyogenic granuloma	Antr. mandible (lingual side)	CMB	S=4 C=2	Basal, suprabasal and lower spinous cells
3367 S92 42/M	Pyogenic granuloma	From 3.2 and 4.1 region	CMB	S=4 C=2	Cells beyond spinous layers are involved. Ulcerated areas showed the same intensity of staining as that of the hyperplastic areas

7511 S92 51/M	Hyper- parakeratosis	Latl. border of tongue	CMB	S=3 C=2	Basal, suprabasal and lower spinous cells
10124 S 92 58/F	Pyogenic granuloma	From 3.1 and 4.1 region	CMB	S=2 C=1	Basal and suprabasal
10130 S92 9/C	Ulcerated fibroepithelial polyp	Rt. buccal mucosa	CMB	S=2 C=2	Basal, suprabasal and lower spinous cells. Ulcerated areas showed the same intensity of staining as that of the hyperplastic areas
8923 S 92 27/F	Fibroepithelial polyp	Lt. buccal mucosa	CMB	S=2 C=1	Basal and suprabasal layers showed a moderate staining
5933 S 93 52/F	FEP	Buccal Mucosa	CMB	S=3 C=2	Certain areas showed strong staining with the involvement of cells up to upper spinous layers

5313 S 93 75/F	FEP	Antr Maxilla	CMB	S=4 C=2	The cells showed a very strong staining with the involvement of all four layers
4027 S 93	Ulcerated FEP	Mand. Gingiva	CMB	S=3 C=2	Certain areas showed weak to moderate staining

Normal Oral mucosa

Case #, age & sex	Biopsy site	A/T	EGFR staining: Layers involved and the pattern of staining	Grade
(7654-92) 29/M	Palate		Basal, suprabasal and lower spinous cells. CMB	S=2 C=2
(3337-92) 55/M	distal to 2.6		Basal, suprabasal and lower spinous cells. CMB	S=3 C=2

(4661-92) 53/F	Buccal to maxillary right third molar		All layers CMB	S=3 C=2
(10371-92) 84/F	Mandibular ridge(4.5)		Basal, suprabasal and lower spinous cells. CMB	S=2 C=2
(844-92) 34/F	Ventral tongue		Basal, suprabasal and lower spinous cells. CMB	S=3 C=2
(5078-92) 42/M	Right lower lip		Basal and suprabasal	S=2 C=1
(6829 -92) 36/M	2.2, 2.1 and 1.1 region		Basal, suprabasal and lower spinous cells. CMB	S=3 C=2
11631-92	Not Recorded		Basal, suprabasal and lower spinous cells. CMB	S=3 C=1
(3256-92) 59/F	Buccal mucosa		Basal and suprabasal CMB	S=3 C=1

Abbreviations: A, Alcohol; T, Tobacco; B, Basal cells; SB, Suprabasal cells; SPF, Superficial cells; CMB, Cell membrane positive type of staining; FCMB, Focal cell membrane positive type of staining; CYTP, Cytoplasmic positive type of staining; FEP, Fibroepithelial polyp

Staining intensity and layer involvement:

1) Layer involvement: Grade 1, Basal + Suprabasal cells; Grade 2, Basal+Suprabasal+Upper Spinous layers

2) Staining intensity: Grade1, weak staining; Grade 2, moderate staining; Grade 3, strong staining; Grade 4, very strong.