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Invited Review

Epidermal growth factor receptor (EGFR) biology and human oral cancer

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Summary. Dysregulation of the epidermal growth factor receptor (EGFR) is one of the most frequently studied molecular events leading to oral carcinogenesis. Over-expression of EGFR is a common event in many human solid tumors. Elevated levels of EGFR mRNA in human cancer occur with and without gene rearrangement. Structural alterations in the receptor can also result in the dysregulation of the EGFR pathway. EGFR over-expression without gene re-arrangement is frequently observed in human oral cancers. However, little is known whether structural alterations in the receptor or perturbations in the EGFR pathway contribute to oral carcinogenesis. Several preliminary studies suggest that EGFR-targeted therapeutic approaches might be successful in controlling oral cancer.

Key words: Growth factor receptor, Squamous cell carcinoma, Oral, Biomarker

Introduction

Cancer of the oral cavity represents the sixth most common malignancy worldwide (Sidransky, 1995; Silverman, 1998). In some populations, oral cancer accounts for greater than 50% of all newly diagnosed malignancies. Approximately half of the patients afflicted will die within five years of diagnosis, while surviving patients may be left with severe esthetic and/or functional compromise (Silverman, 1998). Carcinomas account for 96% of all oral cancers, and 91% of which are squamous cell carcinomas (Parkin et al., 1988). Therefore, the oral cancer problem primarily consists of the biology, diagnosis and management of squamous cell carcinomas. Advances in understanding the underlying mechanisms of oral carcinogenesis will likely be necessary to improve patient survival curves which, despite better early detection of oral cancer, have plateaued over the past two decades and remain among the worse of all cancer sites (Parkin et al., 1988; Schantz, 1993, Kim and Shin, 1997). While the molecular mechanisms of oral carcinogenesis are poorly understood, recent advances in understanding the epidermal growth factor receptor (EGFR) may have important implications in the biology, diagnosis and management of oral cancer.

The EGFR pathway is among the most extensively studied models in tumor biology, providing one of the first links between an activated oncogene and human tumor formation (Downward et al., 1984). EGFR is a member of the tyrosine kinase receptor superfamily (Fig. 1). These tyrosine kinases are widely believed to play an important role in embryonic development, wound healing and carcinogenesis (Aaronson, 1991). Human EGFR is highly homologous to the viral oncogene v-*erbB*, which is carried by the avian erythroblastosis virus (Downward et al., 1984). The EGFR or *erb*B subfamily of the tyrosine kinase receptors is characterized by an extracellular ligand binding domains, transmembrane domains and tyrosine kinase-bearing cytoplasmic domains. The *erb*B subfamily includes *erb*B1 (EGFR), *erb*B2 (HER2 or *neu*), *erb*B3 and *erb*B4 (Downward et al., 1984; Schechter et al., 1985; Kraus et al., 1989) (Fig. 2A). EGFR has been found to be mutated and/or over-expressed in a variety of human tumors concomitantly with overexpression of one or more of its ligands (Gullick, 1991). Recent investigations have focused on clinical use of EGFR as a biomarker and target for immunotherapy.

Epidermal growth factor receptor (EGFR) biology and human cancer

Gene structure and regulation

The EGFR gene has been mapped to the 7p13-q22 region, contains 28 exons and spans 75-kb (Kondo and Shimizu, 1983; Callaghan et al., 1993). The EGFR promoter is typical of many "house-keeping" genes in

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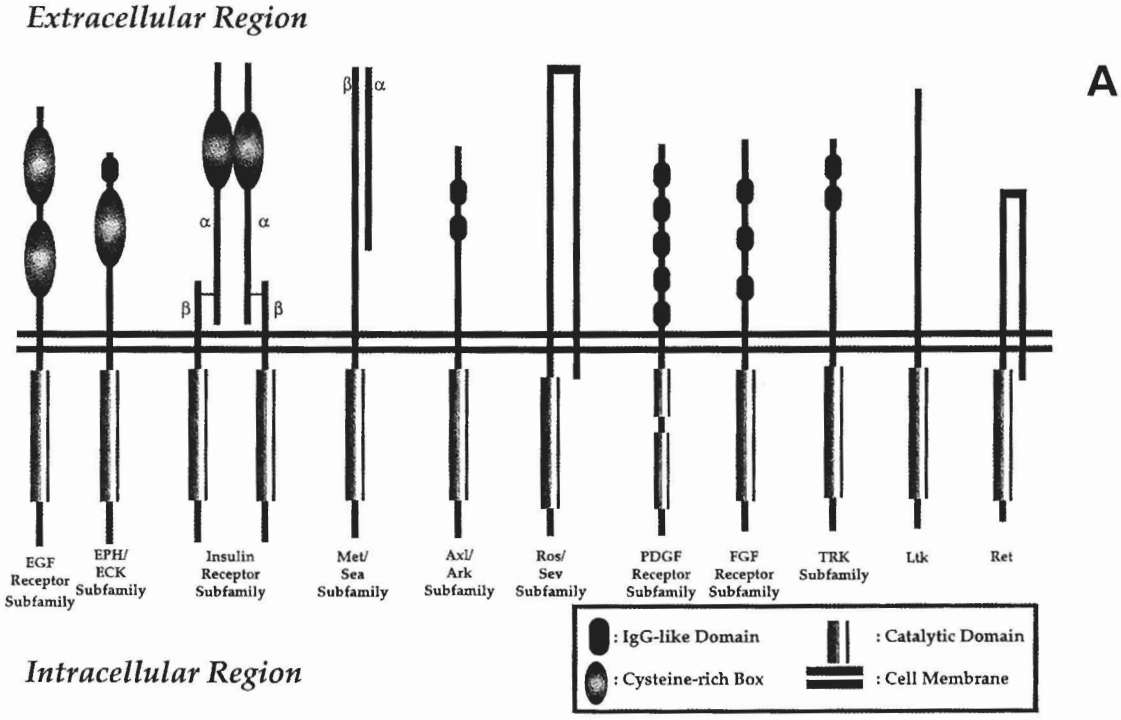


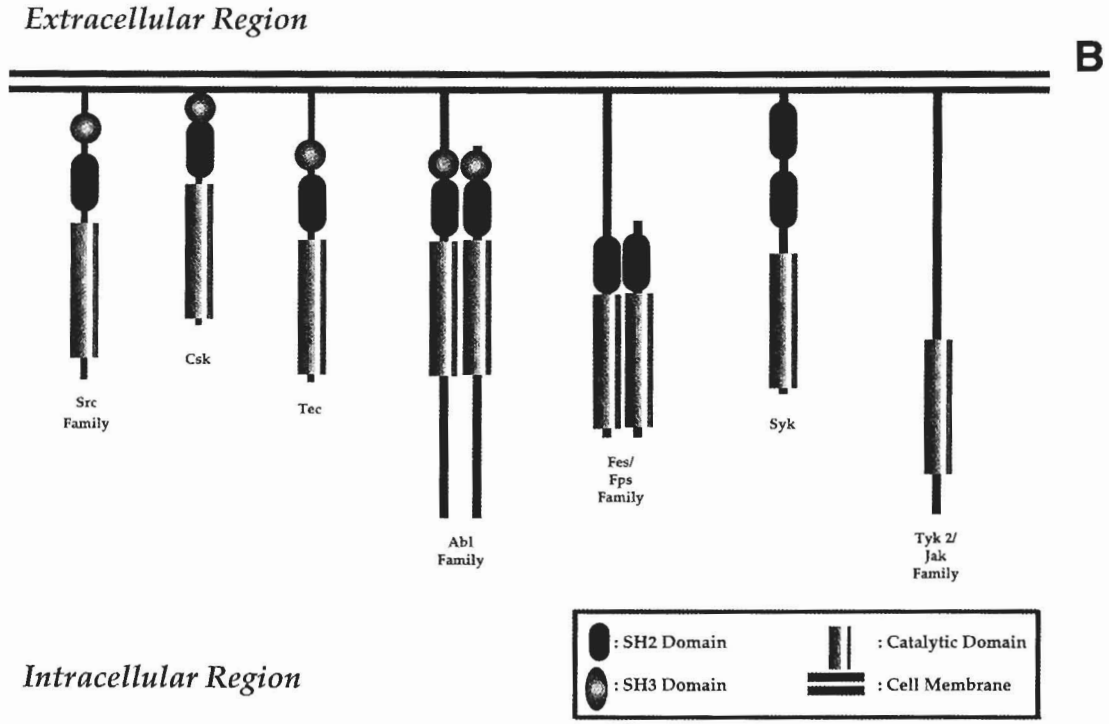
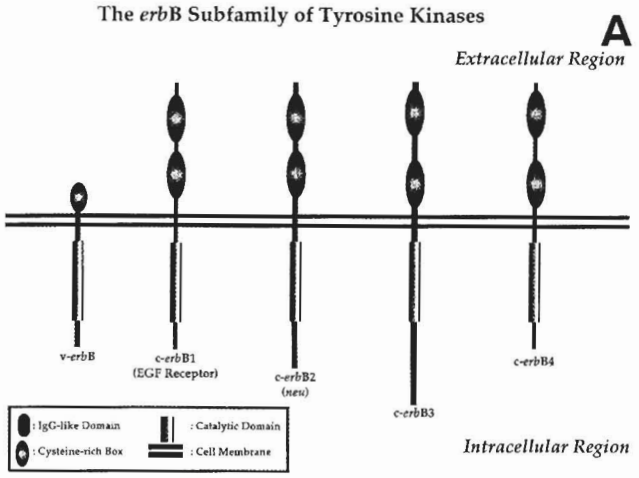


Fig. 1. A. The membrane-spanning tyrosine kinase receptors. Each subfamily has an extracellular, transmembrane, and cytoplasmic portion. While each receptor has at least one kinase domain, other regions, such as an IgG-like domain and cysteine-rich box, are shared by many of the subfamilies of tyrosine kinase receptors. **B.** The non-membrane-spanning tyrosine kinase receptors. Several cytoplasmic polypeptides share the same tyrosine kinase domain. The *src* family is the original member of this group.



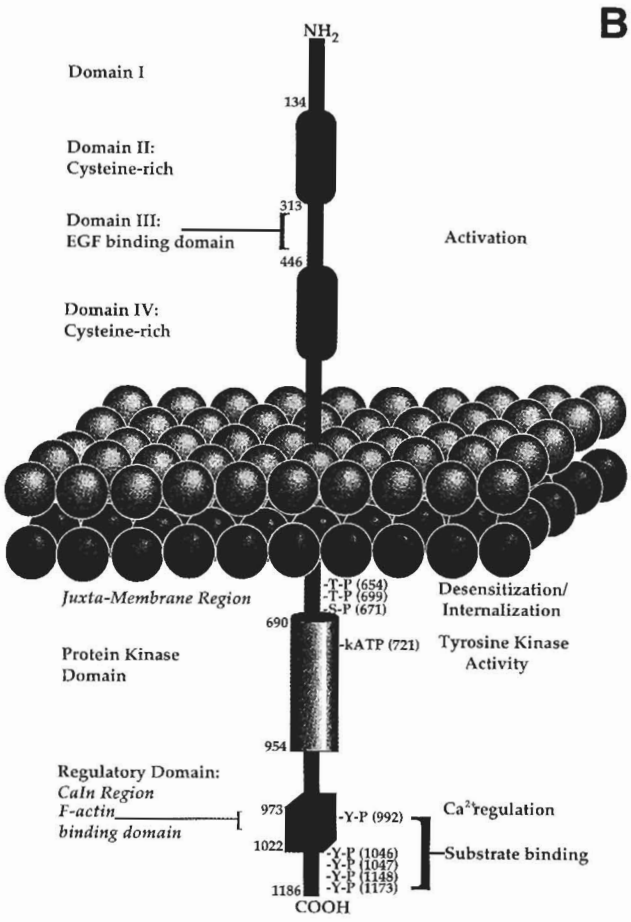


Fig. 2. A. The erbB subfamily of tyrosine kinases. Four known human receptors belong to this group: c-*erb*B1 (EGFR or HER1), c-*erb*B2 (*neu* or HER2), c-*erb*B3 (HER3) and c-*erb*B4 (HER4). v-*erb*B is the viral homologue initially isolated from the avian erythroblastosis virus. **B.** The structure of EGFR. The extracellular portion of EGFR is comprise of four domains. Domain III (aa 313-446) is responsible for ligand binding. EGFR has a single hydrophobic domain (aa 622-644). The cytoplasmic portion of EGFR begins with a juxta-membrane region (aa 645-690) which is followed by the protein kinase domain (aa 690-954) and regulatory domain (aa 955-1186).

that it is extremely GC rich and contains no TATA or CAAT box (Ishii et al., 1985; Merlino, 1990). The 5' flanking region does contain ETF1 and TCF binding sites (Ishii et al., 1985; Kageyama et al., 1988).

Mutations of EGFR in human malignancies usually consist of overexpression with or without amplification and/or coding sequence alterations. Differential splicing and structural alterations of the EGFR gene, which result in changes of the peptide domain structure, will be discussed in the section below. In epithelial tumors, EGFR amplification occurs at a frequency of 10-15% and without re-arrangements (Saint-Ruf et al., 1992). Amplification has been reported in breast tumors (Ro et al., 1988), squamous epidermoid tumors (Hollstein et al., 1988; Ishitoya et al., 1989; Saranath et al., 1992), and urogenital tumors (Ishikawa et al., 1990). Re-arrangement and amplification frequently occur in high-grade brain tumors. Approximately 50% of glioblastoma multiforme tumors contain double minutes (Bigner et al., 1990a,b). About half of glioblastomas demonstrating amplification also have a normal copy of the gene, which is also amplified (Carter and Kung, 1994).

Non-transformed human cell lines express two major species of EGFR mRNA (10-kb and 5.6-kb). In some tumor cells, a 2.8-kb transcript corresponding a truncated form of the receptor is also expressed (Ullrich et al., 1984). The level of mRNA usually correlates with the amount of EGFR protein. EGF and TGF-α and -ß, triiodothyronine, and retinoic acid increase EGFR mRNA levels (Fernandez-Pol et al., 1989). Elevated EGFR expression in many cancers, most notably breast, are associated with disease recurrence, reduced survival, and presence of metastasis (Davies and Chamberlin, 1996). High EGFR expression in bladder cancers occurs predominantly in invasive, but not superficial tumors (Gullick, 1991). EGFR overexpression in colon cancer has been associated with a higher rate of liver metastasis (Radinsky and Ellis, 1996).

EGFR domain structure

EGFR is a 170-kDa transmembrane glycoprotein composed of a single polypeptide chain of 1186 residues (Fig. 2B). It is synthesized as a 160-kDa precursor which is N-glycosylated at 12 potential sites (Mangelsdorf-Soderquist and Carpenter, 1986). The primary structure was originally determined by cloning and sequencing human cDNAs from the human vulval carcinoma cell line A431 and placenta (Lin et al., 1984; Ullrich et al., 1984). EGFR contains a ligand-binding extracellular domain anchored by a single transmembrane domain. Intracellulary, EGFR consists of a juxta-membrane region, protein kinase domain, and a carboxy-terminal region.

Extracellular domain

The 621 amino acid extracellular domain of EGFR binds to ligand with high and low affinities (Boonstra et al., 1985). Four subdomains (I to IV) are based on repetitive sequence motifs and the organization of exons in the human gene (Carter and Kung, 1994). Subdomains II and IV are cysteine rich) (Carpenter and Zendegui, 1986). Subdomain III is sufficient to bind EGF with high affinity (Kohda et al., 1993). In addition to signal pathway activation by ligand binding, receptor occupancy also results in feedback inhibition by receptor internalization and degradation (Ullrich and Schlessinger, 1990; Carter and Kung, 1994). The most common structural mutation associated with cancer that affects EGFR is the deletion of the extracellular domain. Deletion commonly results in a 267 amino acid truncation of the receptor in the extracellular domain. Amino-terminal truncations are seen in other tyrosine kinase receptors during tumorigenesis, such as v-*ros*, v-*kit*, and *neu* oncogenes (Carter and Kung, 1994). In glioblastomas, a 83 amino acid deletion in subdomain IV does not appear to interfere with ligand-dependent control of the receptor (Bigner et al., 1990a). Deletion of the ligand-binding domain of the receptor is believed to release tyrosine kinase inhibition in unoccupied receptors (Hsu et al., 1990).

Transmembrane domain

The transmembrane region consists of a 23-amino acid sequence (residues 622-644). The domain spans the cellular membrane once. Mutations of the transmembrane region do not effect the function of EGFR (Kashles et al., 1988; Carpenter and Wahl, 1991). Deletion of the transmembrane domain does not abate transforming activity of v-*erb*B (Privalsky, 1987).

Juxta-membrane region

The juxta-membrane region, spanning between the cellular membrane and the kinase domain, is a regulatory region of EGFR. Substrate choice and cell-specific growth promotion differs between EGFR and *erb*B2, in part, due to different amino acid residues at position (Di Fiore et al., 1992). Alteration of EGFR tissue specificity regions has been associated with *in vitro* transformation of fibroblasts and *in vivo* sarcomagenic potential (Shu et al., 1991). Kinase activity, high affinity ligand binding, cell shape changes and induction of DNA synthesis are inhibited by phosphorylation in this region (Hunter, 1984; Felder et al., 1992; Carter and Kung, 1994).

Protein kinase domain

The receptor's intrinsic catalytic activity resides in the kinase domain. Crystallographic data of the kinase region suggests a continuous region composed of two subdomains separated by a cleft (Knighton et al., 1991). Ligand binding appears to cause a conformational shift, which leads to an increased affinity of EGFR for its neighbors, dimerization, and subsequent activation of the tyrosine kinase (Boonstra et al., 1995). Alteration of conserved amino acids in this region eliminates biologic activity, but not normal synthesis, processing, and high affinity ligand binding (Chen et al., 1987; Honegger et al., 1987; Moolenaar et al., 1988; Carter and Kung, 1994). Large carboxyl-terminal truncations, internal deletions, and a variety of amino acid substitutions in either the juxta-membrane or the protein kinase domains result in a loss of inhibitory signals. These functional alterations appear in a tissue specific fashion (Carter and Kung, 1994).

Carboxyl-terminal region

The carboxyl-terminal region contains five tyrosine phosphorylation sites and a 48-amino acid CaIn region. The five tyrosine residues (at positions 992, 1068, 1086, 1148, and 1173) are regulated by auto-phosphorylation, *src*-family tyrosine kinases, protein phosphatases and/or SH2-bearing proteins (Selva et al., 1993). The significance of phosphorylation of these residues is controversial but may serve to alter the conformation of the kinase domain, limit access of exogenous substrates to the kinase active site or act as a binding site for SH2-bearing proteins (Downward et al., 1985; Walton et al., 1990; Rotin et al., 1992). EGFR lacking all five phosphorylation sites cannot cause morphologic changes in response to EGF even though mitogenesis is unaffected (Decker, 1993). Mutations in this region have been associated with angiosarcoma formation (Carter and Kung, 1994). The CaIn region (residues 974-1021) is thought to function in ligand-induced Ca^{2+} influx and receptor internalization (Chen et al., 1989). Internalization motifs occur at residues 973-977 and 996-1000. The CaIn region which also binds adaptin, α-actinin, and F-actin may be important in growth factor induced signal transduction (Sorkin et al., 1992; Boonstra et al., 1995). In glioblastomas, deletions of the CaIn and tissue specificity regions co-existed with extracellular domain deletions (Matsui et al., 1990).

Ligands

Six growth factors bind EGFR: the epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), amphiregulin (AR), heparin-binding EGF-like growth factor, betacellulin, cripto, and epiregulin (Prigent and Lemoine, 1992). Each ligand has a distinct expression pattern during development and in adult tissues, suggesting multiple functions of EGFR (Alroy and Yarden, 1997). The members of the EGFR ligand family share three conserved intramolecular disulfide bonds believed important both for receptor binding and resistance to proteolytic degradation (Campbell et al., 1989). Differences in ligand structure accounts modify

the response of EGFR-bearing cells. EGFR ligands have widely different isoelectric points, varying from 4.6 (EGF) to 7.8 (amphiregulin), which may account, in part, for local environment modulation of response to a particular ligand (Davies and Chamberlin, 1996). Varying lengths of N- and C-termini also modify the biologic response between EGFR ligands (Shoyab et al., 1989).

To achieve malignant transformation, tissue must co-express ligand with high levels of EGFR (Di Marco et al., 1989; Derynck, 1992). Frequently, tumor cells over-express both EGFR and its ligands (Derynck, 1988). In such cells, blocking EGFR activation by use of an antibody against the ligand binding site prevents proliferation (Ennis et al., 1989; Modjtahedi et al., 1994). Cells expressing normal levels of EGFR, which are exposed to continuous ligand, undergo hyperproliferation but not transformation (Messing, 1990; Menard and Pothier, 1991; Aida et al., 1994).

Signaling pathways

The signal transduction cascade initiated by EGFR begins with receptor occupancy. EGF receptors are present in two affinity classes, high and low. The former is responsible for ligand-induced effects; a role for the latter is presently unclear (Boonstra et al., 1995). Ligand binding to the extracellular domain results in an altered three dimensional conformation and dimerization of the receptor (Boonstra et al., 1995; Chrysogelos and Dickson, 1994). The driving force for homo- as well as heterodimer formation with other *erb*B family members is the higher stability of the ternary complex formed between a ligand and two receptors, as compared with a monomeric receptor (Alroy and Yarden, 1997). Receptor dimerization appears essential for receptor tyrosine kinase activation (Ullrich and Schlessinger, 1990). Autophosphorylation of several C-terminal residues is an early event following tyrosine kinase activation (Chrysogelos and Dickson, 1994).

Activated EGFR interacts directly with signaling proteins containing SH2 domains (Carpenter, 1992). Originally identified by homology to *src*, SH2 domains allow protein-protein complexes by binding to peptides that contain phosphorylated tyrosine residues (Panayotou and Waterfield, 1993). The multiple component complexes recruit enzymes from the cytoplasm, select proteins for EGFR-mediated phosphorylation, and modify the activity of signaling enzymes (Carter and Kung, 1994). Intracellular proteins associated with activated EGFR include *crk* (Birge et al., 1992), GAP (Zhou et al., 1993), *grb2/sem5* (Songyang et al., 1994), *nck* (Park and Rhee, 1992), p91 (Fu and Zhang, 1993), PLC-γ (Rhee and Choi, 1992) and *src* (Luttrell et al., 1994). Pathways induced by EGFR activation include the PIP2 cascade and *ras* pathways (Moran et al., 1990; Buday and Downward, 1993; Soler et al., 1994). Downstream protein phosphatase pathways, in turn, have been shown to regulate EGFR (Griswald-Prenner et al., 1993; Case et al., 1994). Cellular events believed to be mediated by receptor signaling protein complexes include ion fluxes, additional phosphorylation events, gene expression, DNA synthesis and malignant transformation (Chrysogelos and Dickson, 1994).

EGFR and human oral carcinogenesis

Gene regulation

EGFR overexpression has been estimated at 50-98% of all oral cancers (Ishitoya et al., 1989; Kawamoto et al., 1991; Santini et al., 1991; Scambia et al., 1991; Christensen et al., 1995). Variation in detection is likely due to method of receptor extraction used, differing calibration standards, stromal contamination, tumor heterogeneity, and choice of normal control (Christensen et al., 1995). Upregulation of gene expression, rather than gene dosage or mRNA stability, appears to be the primary mechanism of EGFR overexpression in oral cancers (Rubin Grandis et al., 1997). Amplification has been found in several malignant oral epithelial cell lines but is rarely reported in fresh samples (Yamamato et al., 1986; Eisbruch et al., 1987; Weichselbaum et al., 1987; Ishitoya et al., 1989). This discrepancy may be explained be cell culture artifact. EGFR expression appears to increase proportionally to the degree of epithelial dysplasia (Rubin Grandis et al., 1998; Todd et al., 1991) (Fig. 3). In fresh samples, EGFR mRNA has been estimated to be increased 69-fold in malignant over normal (Rubin Grandis and Tweardy, 1993). TGF-α is overexpressed 5-fold in malignant over normal (Rubin Grandis and Tweardy, 1993). In cell lines, EGFR and TGF-α mRNA are elevated 77- and 10-fold respectively (Rubin Grandis and Tweardy, 1993). Overexpression of EGFR has been correlated to increased matrix metalloproteinase-3 expression and is thought to contribute to invasion and metastasis (Kusukawa et al., 1996).

Clinical significance

The use of EGFR overexpression as a prognostic biomarker in head and neck cancer continues to be debated. Recent reports support EGFR overexpression as a biomarker for the malignant conversion of pre-malignant oral lesions in head and neck cancers (Rubin Grandis et al., 1998). Several reports suggest decreased survival rate with shorter relapse-free survival, while other studies find no correlation between EGFR over expression and prognosis (Mukaida et al., 1991; Dassonville et al., 1993; Frank et al., 1993; Miyaguchi et al., 1993; Itakura et al., 1994). EGFR overexpression has also been associated with advanced tumor stage and metastasis (Santini et al., 1991; Shirasuna et al., 1991; Yano et al., 1991; Storkel et al., 1993).

Therapeutic agents which interfere with the EGFR pathway in oral cancer have begun to be investigated. Recent studies have shown that EGFR tyrosine kinase

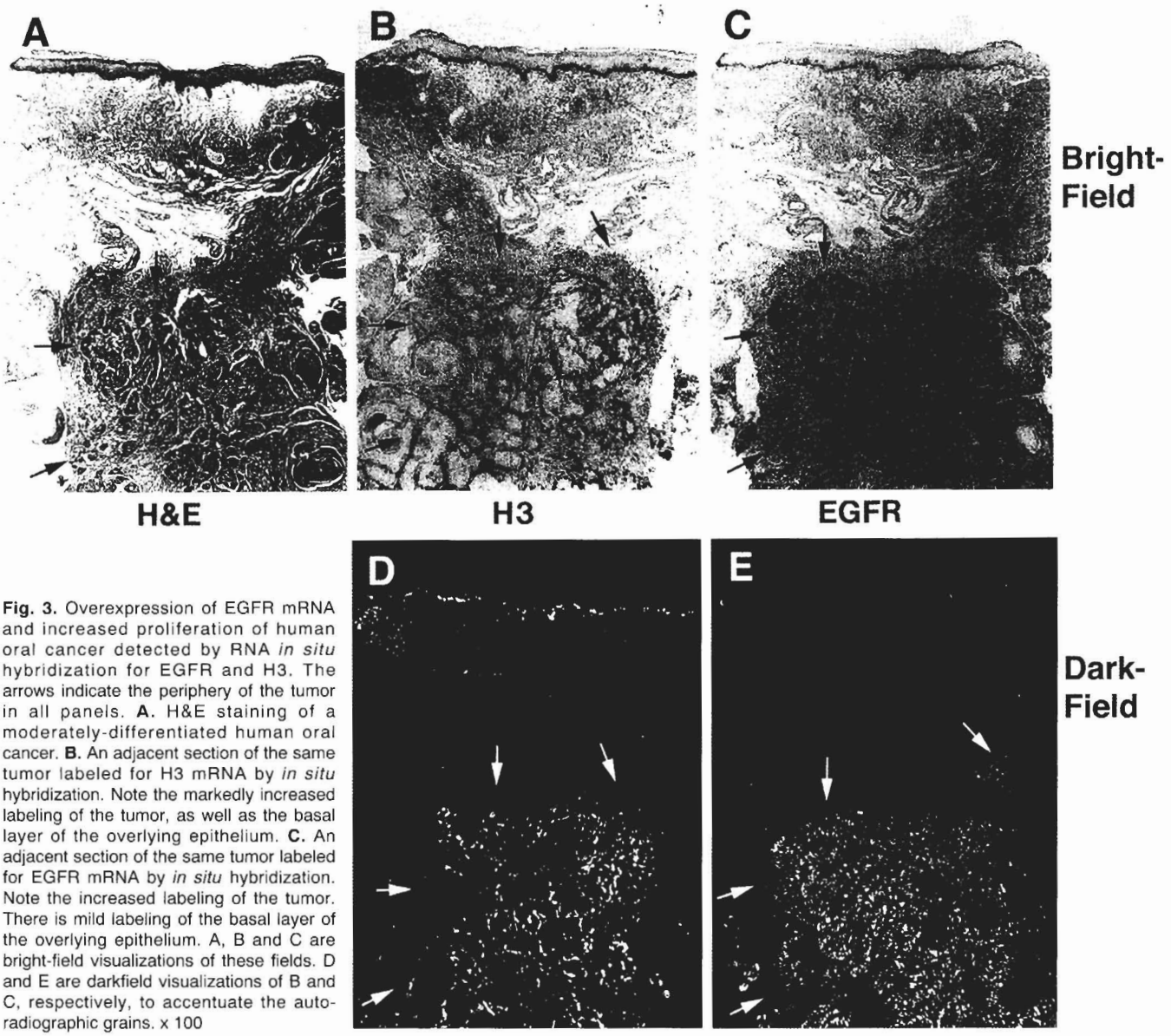


Fig. 3. Overexpression of EGFR mRNA and increased proliferation of human oral cancer detected by RNA *in situ* hybridization for EGFR and H3. The arrows indicate the periphery of the tumor in all panels. **A.** H&E staining of a moderately-differentiated human oral cancer. **B.** An adjacent section of the same tumor labeled for H3 mRNA by *in situ* hybridization. Note the markedly increased labeling of the tumor, as well as the basal layer of the overlying epithelium. **C.** An adjacent section of the same tumor labeled for EGFR mRNA by *in situ* hybridization. Note the increased labeling of the tumor. There is mild labeling of the basal layer of the overlying epithelium. A, B and C are bright-field visualizations of these fields. D and E are darkfield visualizations of B and C, respectively, to accentuate the autoradiographic grains. x 100

inhibitors may be potent cancer chemotherapeutic agents (Kelloff et al., 1996). Treatment of tumors overexpressing EGFR with monoclonal antireceptor antibodies may induce growth inhibition and terminal differentiation, as well as enhancing chemotherapeutic efficacy (Gutowski et al., 1991; Yoneda et al., 1991; Modjtahedi et al., 1994; Perez-Soler et al., 1994; Rubin Grandis et al., 1997). Antibodies and antisense constructs directed against EGFR do not inhibit normal oral keratinocyte proliferation (Rubin Grandis et al., 1997). Retinoic acid, which has been well established to resolve oral dysplastic lesions and prevent secondary tumors, may owe some of its therapeutic effect to its downregulation of both EGFR and TGF-α mRNA (Vokes et al., 1993; Rubin Grandis et al., 1997).

Conclusions

EGFR biology continues to be an active effort in the study of tumor development. Because of the time of the discovery of EGFR and its linkage to autocrine stimulatory loops, a wealth of literature exists linking EGFR dysregulation with human cancers at a wide variety of anatomic sites, including breast and brain. EGFR overexpression has been well documented in human oral cancer and its models, but the mechanism and significance to the biology of oral malignancies remains to be defined. Little is known about the spectrum of EGFR gene mutation(s) or upstream/downstream events contributing to oral carcinogenesis. While EGFR blocking strategies, including antibodies and

antisense constructs, have been applied to the oral cancer problem, these studies remain in their early stages. Prior to EGFR becoming a significant biomarker or therapeutic target in human oral cancers, controversies and gaps in our understanding of its role in oral epithelial biology must be better defined.

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