Dysregulation of Epidermal Growth Factor Receptor Expression in Premalignant Lesions during Head and Neck Tumorigenesis

Dong M. Shin, Jae Y. Ro, Waun Ki Hong, and Walter N. Hittelman

ABSTRACT

The development of head and neck cancer, believed to result from field carcinogenesis and a multistep process of tumorigenesis, is often associated with an accumulation of genotypic and phenotypic alterations. The phenotypic changes could be the result of dysregulation of growth control genes such as epidermal growth factor receptor (EGFR). With the goal of identifying a potential biomarker of the multistep process of tumorigenesis, we studied specimens of 36 head and neck squamous cell carcinomas from 5 different sites that contained normal epithelial and/or premalignant lesions adjacent to the tumors. Almost all of the individuals from whom these specimens were obtained had been exposed to first-hand smoking and/or alcohol consumption. Using a monoclonal anti-EGFR antibody for immunohistochemical analysis on paraffin-embedded sections with attached 886 cells for internal control, the levels of EGFR expression were assessed by image analysis. The relative staining intensity of EGFR in normal epithelium adjacent to tumors was 2-fold higher than that in normal control epithelium ($P = 0.021$), suggesting that, even in histologically normal epithelium, EGFR was already upregulated in tissues surrounding tumors. These findings supported the theory of field carcinogenesis in head and neck tumorigenesis. As tissue progressed from normal tissue adjacent to tumor to hyperplasia and to dysplasia, EGFR expression remained elevated. However, in the step from dysplasia to squamous cell carcinoma, EGFR expression was further and dramatically upregulated ($P = 0.01$). Therefore, these results indicate that EGFR dysregulation happens in two steps, the moderate up-regulation of EGFR expression in normal epithelium adjacent to tumor and the further up-regulation of EGFR expression in the change from dysplasia to squamous cell carcinoma. In summary, the studies presented here indicate that EGFR dysregulation might be a useful marker for identifying individuals at risk of tumor development and an intermediate end point in chemoprevention trials.

INTRODUCTION

The development of head and neck cancer has been suggested to occur as a result of “field carcinogenesis,” the exposure of an entire field of tissue to repeated carcinogenic insult, which predisposes the field to the development of multiple cancers (1, 2). Furthermore, these cancers often develop through a series of recognizable stages, known as the multistep process of tumorigenesis (2). This theory holds that the accumulation of genetic alterations during the multistep process initiates phenotypic changes in the tissue. These phenotypic changes may be associated with dysregulation of growth control genes such as the EGFR or transforming growth factor alpha genes (3–6).

EGFR is a glycoprotein ($M_r = 170,000$) with an intrinsic tyrosine-specific protein kinase activity that regulates cell growth in cell lines and in a variety of human tumors (7–10). Many studies have demonstrated that EGFR has protein kinase activity specific for tyrosine residues. Upon binding their respective ligands, the tyrosine kinase activity becomes stimulated, as indicated by enhanced autophosphorylation of the receptor, increased phosphorylation of exogenous substrates, and elevated phosphorylation of the tyrosine residues of several proteins in vitro and in vivo (7, 8, 11, 12). High numbers of EGFR genes have been found in primary brain tumors of glial origin (13) and in several other types of malignancies including bladder tumors (14), breast carcinomas (15, 16), squamous cell carcinomas of the lungs (17, 18) and head and neck, and their cell lines (19–23).

To examine whether levels of EGFR amplifications and/or overexpression could be useful clinical values in head and neck squamous cell carcinomas, EGFR gene amplification and overexpression were studied in head and neck cell lines and tumors (24, 25). Three cell lines demonstrated EGFR gene amplification and 10 lines showed an increase in EGFR mRNA compared to normal keratinocytes (24). Santini et al. (25) also measured EGFR levels in head and neck squamous cell carcinomas and showed overexpression in 59 of 60 samples. They found a significant correlation between EGFR levels and tumor size and stage. However, few data are available on the frequency of an association between growth regulation and EGFR expression in “normal” tissue adjacent to tumors and premalignant lesions in head and neck areas in humans.

We hypothesized that only those premalignant lesions that express high levels of EGFR progress to frank malignancies during the tumorigenesis pathway. Thus, if one were trying to assess the level of risk of tumor development in a carcinogen-exposed field, biomarkers that measure specific changes in this hypothesized field would be important tools. If these biomarkers were modulated by chemopreventive agents, e.g., retinoids, they could serve as intermediate markers of response or progression in clinical trials. With the goal of identifying a potential biomarker in the multistep process of head and neck tumorigenesis, we studied EGFR expression in 36 head and neck squamous cell carcinoma specimens that contained adjacent normal tissue and premalignant lesions. Through these experiments, we hoped to develop a better understanding of the role of EGFR expression in the head and neck tumorigenesis process.

MATERIALS AND METHODS

Selection of Tumor Samples. Thirty-six formalin-fixed, paraffin-embedded tissue specimens of head and neck squamous cell carcinomas from five different sites were obtained from the Department of Pathology at The University of Texas M. D. Anderson Cancer Center in 1990 and 1991. All tumor specimens were selected because they also contained adjacent normal tissue and/or premalignant lesions. Hematoxylin and eosin-stained histological slides were reviewed, and the diagnoses were confirmed by a pathologist (J. Y. R.). The normal, hyperplastic, dysplastic, and tumor areas were identified according to criteria described previously (26). Biopsy specimens of oral epithelium obtained from four normal individuals (i.e., cancer-free, nonsmoking volunteers) were used as normal controls. Four-μm sections were mounted on amine-lysine-coated slides (Histology Control System, Glen Head, NY). Cell pellets were generated from a head and neck cancer cell line that expresses EGFR (886 cell line), fixed in formalin, and embedded in paraffin. Four-μm sections from this paraffin block were placed on slides beside the tumor sections to serve as internal controls for measurements of EGFR expression.

Immunohistochemical Analysis. Anti-EGFR monoclonal antibody (Clone E30) was obtained from BioGenex, Inc. (San Ramon, CA). Immunohistochem-
methylation analysis used a modification of the avidin-biotin immunoperoxidase method described previously (27). Briefly, after deparaffinization by xylene and rehydration with graded alcohols, endogenous peroxidase activity was blocked by incubating the slides in 3% H2O2 with methanol for 10 min. After washing in PBS, the slides were incubated with nonimmune horse serum to decrease the background signal, rinsed in PBS, and incubated with a 1:1 dilution of prediluted anti-EGFR mouse monoclonal antibody for 2 h at 37°C per the manufacturer’s recommendation. The slides were subsequently washed with PBS, incubated with biotinylated secondary antibody for 45 min at room temperature, and then incubated with biotin-avidin peroxidase conjugate (ABC Kit Vector Laboratories, Burlingame, CA) at a dilution of 1:1000 for 30 min at room temperature. After washing in PBS, the EGFR antigen was visualized with a 0.1% 3,3′-diaminobenzidine solution (Sigma-Cambridge, MA) in 1X solution of PBS and hydrogen peroxide (0.01%). The slides were not counterstained to allow quantitation of the EGFR expression by image analysis.

Image Analysis of EGFR Expression. The degree of EGFR expression in the epithelial layers was quantitated on digitized images of the immunohistochemically stained slides using a Magiscan Image Analysis System (Joyst-Loebl, Ltd., Dukesway, England) and a Nikon light microscope with a computer-controlled stage. The first step of the analysis involved visual identification of the epithelial layer of premalignant lesions or tumors (using the adjacent hematoxylin and eosin-stained slides as a template). Regions of the epithelium that contained approximately equivalent levels of EGFR expression were circled with a light pen, and each circled region was characterized by its total integrated optical density, area (in pixels), and the relative coordinates of its center of gravity on particular staining areas. The specific intensity of each region was calculated as the total integrated absorbance divided by the area of that region, minus the contribution of integrated absorbance of an equivalent area of background. To overcome the variations in degree of staining from slide to slide and to be able to compare one slide to another, all values measured on the specimen were normalized to that measured on the 886 cell sections that were placed on each slide and thus stained under identical conditions. Thus, the normalized value of EGFR expression or the RSI of EGFR expression represents the level of EGFR expression relative to that of 886 cells.

\[
\text{RSI of EGFR} = \frac{OI - KB \times OA}{mean (OI - KC \times OA)} \text{ 886 cells}
\]

where \(OI\) is the integrated signal, \(OA\) is the integrated area, \(KB\) is the mean absorbance of the background of connective tissue, and \(KC\) is the mean absorbance of 886 cells. Since each circled region was characterized for both staining intensity and its relative position in the epithelial layers, we were able to construct, using software developed in our laboratory, a topological map of EGFR expression in the tissue wherein the levels of EGFR expression were displayed on a relative pseudocolor scale.

RESULTS

Patient and Tissue Characteristics. Tumor specimens were obtained by surgical resection from 36 patients with head and neck squamous cell carcinoma. As shown in Table 1, men were predominant (\(n = 25\)), and the patients ranged in age from 25 to 76 years. Tumor specimens were obtained from five different head and neck sites including the larynx, hypopharynx, floor of the mouth, base of the tongue, and oropharynx. All four tumor stages were included, with a predominance of Stage II and IV tumors. Moderately well-differentiated tumors were predominant. All but three of the patients had a documented smoking history with a median 60 pack-year history. All patients had been untreated before surgery. Following surgery, 19 patients received radiotherapy and 11 received radiotherapy and chemotherapy.

The 36 primary tumor specimens and one block per each case were chosen because they had adjacent normal tissue and/or premalignant lesions in the same section. No metastatic or recurrent tumors were included in this study. Of the 36 cases considered, 28 exhibited histologically normal adjacent squamous mucosa, 15 hyperplasia, and 24 dysplasia. Four biopsy specimens from the oral mucosa of non-smoking, cancer-free individuals served as “normal” controls.

EGFR Dysregulation in Normal Epithelium Adjacent to Tumors. Before we performed immunohistochemistry for EGFR expression in tissue samples, we calibrated monoclonal anti-EGFR antibody titers using paraffin-embedded 886 cells with one representative tissue sample. The 886 cells and tissue samples had a good linear relationship by image analysis according to the different concentrations of the antibody. Therefore, we used a 1:2 dilution of prediluted solution of this antibody for all experiments. We also defined the membrane-associated expression as positive staining of EGFR in each experiment. When normal oral mucosa (control) specimens from nonsmoking, cancer-free individuals were immunohistochemically analyzed, minimal but detectable levels of membrane-associated EGFR were observed in all four samples; the EGFR expression was limited to the basal layer (Fig. 1B), whereas tumor tissue without adding anti-EGFR antibody was used for the negative control for each experiment (Fig. 1A). In contrast, histologically normal epithelium adjacent to tumors exhibited somewhat increased levels of EGFR expression, and the EGFR expression expanded into parabasal layers (Fig. 1C). To quantitate the EGFR expression detected by the immunohistochemical analysis, we used image analysis to determine the mean RSI over the histologically normal epithelium. As shown in Table 2, when quantitated as a whole, histologically normal epithelium adjacent to head and neck tumors exhibited about twice the degree of EGFR expression than normal control epithelium from nonsmokers (RSI, 0.78 versus 0.38, respectively; \(P = 0.021\)). Nevertheless, some degree of heterogeneity of expression was observed from specimen to specimen. As shown in Table 3, the histologically normal epithelium adjacent to tumors showed higher EGFR expression in 22 of 28 (79%) samples (i.e., RSI \(\geq 0.50\)) than four control epithelium (RSI < 0.50). More strikingly, 8 of 28 samples (29%) had very high levels of expression of EGFR (RSI > 1.00). These results suggest that, despite histological similarity to control
epithelium, normal epithelium in the field of head and neck tumors shows increased EGFR expression.

Increased EGFR Expression during Tumorigenesis. Because high levels of EGFR expression have been reported in head and neck tumors, we studied the pattern of EGFR expression during tumorigenesis. As illustrated in Fig. 1, EGFR levels increased significantly from control epithelium to normal epithelium adjacent to tumors and remained elevated as the tissue progressed from normal mucosa (Fig. 1C) to hyperplasia (Fig. 1D) to dysplasia (Fig. 1E) and then dramatically increased as the tissue changed to squamous cell carcinoma.

Fig. 1. Immunohistochemical demonstration of EGFR expression in a representative case of head and neck tumorigenesis. A, negative control, tumor tissue with no added anti-EGFR monoclonal antibody. B, low expression of EGFR in the basal layer in a control normal epithelium. C, increased expression of EGFR in basal (b) and parabasal (pb) layers, not in superficial (s) layers in normal epithelium adjacent to tumor. D, further increase in EGFR expression and extension into the parabasal area in hyperplasia. E, dysplastic lesion shows heterogeneous EGFR expression with an intensity similar to that of hyperplasia. F, markedly increased EGFR expression in squamous cell carcinoma.
Table 2 Overall RSI of EGFR expression in head and neck tumorigenesis

<table>
<thead>
<tr>
<th>Histological type</th>
<th>No. of samples</th>
<th>Mean RSI (±SE)</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>CON</td>
<td>4</td>
<td>0.38 ± 0.07</td>
<td>0.021</td>
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<tr>
<td>ANL</td>
<td>28</td>
<td>0.78 ± 0.06</td>
<td>0.81</td>
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<tr>
<td>HYP</td>
<td>15</td>
<td>0.64 ± 0.17</td>
<td>0.92</td>
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<tr>
<td>DYP</td>
<td>24</td>
<td>0.71 ± 0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>SCC</td>
<td>36</td>
<td>1.16 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

* CON, control epithelium; ANL, normal epithelium adjacent to tumor; HYP, hyperplasia; DYP, dysplasia; SCC, squamous cell carcinoma.

Table 3 Incidence and degree of EGFR expression during head and neck tumorigenesis

<table>
<thead>
<tr>
<th>Histological type</th>
<th>No. of samples</th>
<th>Degree of RSI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤0.50</td>
</tr>
<tr>
<td>CON</td>
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<td>4 (100)</td>
</tr>
<tr>
<td>ANL</td>
<td>28</td>
<td>6 (21)</td>
</tr>
<tr>
<td>HYP</td>
<td>15</td>
<td>6 (40)</td>
</tr>
<tr>
<td>DYP</td>
<td>24</td>
<td>7 (29)</td>
</tr>
<tr>
<td>SCC</td>
<td>36</td>
<td>4 (11)</td>
</tr>
</tbody>
</table>

* CON, control epithelium; ANL, normal epithelium adjacent to tumor; HYP, hyperplasia; DYP, dysplasia; SCC, squamous cell carcinoma.
DISCUSSION

The purpose of this study was to better understand the role of EGFR expression in head and neck tumorigenesis. A variety of clinical evidence, including oral leukoplakia, had suggested that head and neck tumor development involves a multistep process occurring in a field exposed to repeated carcinogenic insult. Because head and neck tumors had already been shown to express high levels of EGFR with great frequency, we were interested in determining when these changes in expression occurred and whether this process could be correlated with growth regulation through EGFR in the affected tissue regions. To this end, we studied EGFR expression in histologically normal epithelium and premalignant lesions adjacent to carcinomas of the head and neck and found that up-regulation of EGFR expression occurred in two stages; the first occurred with the transition from control epithelium of nonsmokers to normal epithelium adjacent to tumors and the second with the transition from dysplastic lesions to squamous cell carcinomas. The finding that EGFR expression in the histologically normal-appearing epithelium adjacent to the tumors was twice as high as that in the normal control epithelial tissues in individuals never exposed to first-hand smoking or alcohol suggests that these changes represent a field cancerization whereby the whole epithelial tissue in the aerodigestive tract has been repeatedly exposed to carcinogenic insult. Therefore, the increased of expression of EGFR in the normal adjacent epithelial tissue might be a result of factors expressed by the tumors that cause phenotypic changes in surrounding normal tissues.

Although it is certainly possible that EGFR is expressed in wounded epithelium as part of a regenerative process, there is increas-
ing evidence that genetic events also accompany this process. For example, we recently demonstrated polysomies of chromosomes 7 and 17 in this histologically normal field by nonisotopic in situ hybridization by applying chromosome-specific centromeric DNA probes to the same samples studied in this study (29). The same normal control epithelium showed no chromosome polysomies (i.e., with \( \geq 3 \) copies of the chromosome), whereas one-third of histologically normal epithelia adjacent to tumors showed polysomies of both chromosomes 7 and 17. Moreover, the frequency of polysomy increased as the tissues progressed from histologically normal epithelium to hyperplasia to dysplasia to squamous cell carcinoma (29). The EGFR gene, which is located on chromosome 7p12–13 (30), increased EGFR expression at the mRNA (31, 32) and protein levels in a majority of head and neck cancers (20, 21). Thus, alteration of the number of copies of chromosome 7 in conjunction with up-regulation of the EGFR gene in this tissue field might play a critical role in an early stage of the tumorigenesis pathway. Similarly, Sozzi et al. (33) have demonstrated that bronchial epithelial cells derived from histologically normal tissue adjacent to bronchogenic carcinomas had structural abnormalities of chromosomes in 6 of 19 patients. In that study, chromosome 7 was involved in four cases. In addition, EGFR and HER-2/neu were overexpressed in 6 of 13 normal bronchial epithelium in patients with lung cancer (33). Their findings are consistent with our observation that early genetic lesions could be present in this affected field and be a target of further complex and multiple genetic changes during the pathogenesis of aerodigestive tract tumors.

The second important observation of this study is that EGFR expression remained elevated through all of the premalignant stages of tumorigenesis and further increased in the change from dysplastic lesions to squamous cell carcinomas. This finding supports the concept of multistep tumorigenesis in the head and neck region. However, in some cases, as shown in Fig. 3C, the transitional area from hyperplasia to dysplasia also had a dramatic increase in EGFR expression. In the same area in this sample, we also observed a sudden increase in proliferating cell nuclear antigen expression (31), indicating that EGFR dysregulation may control cell proliferation in this particular situation. That is, progression toward the malignant phenotype is accompanied by an accumulation of genetic damage in the exposed tissue that culminates in an autocrine loop stimulation necessary for cell proliferation (34). It may take long periods of time for the cells to transform from the genetically altered but histologically normal epithelium to the frank malignant phenotype. In the schema of the multistep process of tumorigenesis, many genotypic and phenotypic alterations may be involved. Among these, a specific gene alteration may give a “hit” to the premalignant cells to transform toward the malignant phenotype. EGFR amplification and/or overexpression may play this role in some cases; in other cases, the cells may be transformed at transcriptional regulation during the carcinogenesis process.

One of the goals of this study was to identify regulatory markers that might be useful for assessing the risk of tumor development in histologically normal but carcinogen-exposed tissue and in premalignant lesions. The results reported here were based on studies of premalignant lesions in individuals with 100% risk of developing tumors (i.e., they had premalignant lesions adjacent to squamous cell carcinomas). A working hypothesis for future studies would be that individuals whose premalignant or histologically normal epithelium exhibits the greatest degree of dysregulation of EGFR expression might be expected to be at highest risk for tumor development (or second tumor development in those whose primary tumors were apparently cured).

The effectiveness of chemoprevention trials of aerodigestive tract tumors is dependent on the identification of individuals at risk who may benefit most by such intervention. At present, the subjects of such trials are chosen on the basis of known risk factors such as the presence of premalignant lesions (e.g., oral premalignant lesions, bronchial metaplasia/dysplasia, and Barrett’s esophagus), a history of significant tobacco/alcohol exposure, or a history of potentially curative resection of primary aerodigestive tract tumors to prevent second primary cancer development (35, 36). However, because these subjects have only a relatively low risk of developing tumors, these clinical trials require large samples to be able to detect a statistically significant effect. Furthermore, they require extended periods of intervention if second cancer development is the primary end point. The studies presented here suggest that a determination of EGFR dysregulation might be not only a useful marker for the identifying individuals at risk of tumor development but also a marker for an intermediate end point at which EGFR expression may be regulated by chemopreventive agents. Other biomarkers, associated with EGFR dysregulation during this complex pathway of tumorigenesis including proliferating cell nuclear antigen (37), p53 expression and mutations (38), and genetic alterations (29), are currently being explored to better understand the tumorigenesis of upper aerodigestive tract tumors. It is to be hoped that these biomarkers can be used as intermediate end points in chemoprevention trials in the near future.

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