Expression of Transforming Growth Factor β Type II Receptors in Head and Neck Squamous Cell Carcinoma

Carlos A. Muro-Cacho, 2 Meredith Anderson, 2 Joehassin Cordero, and Teresita Muñoz-Antonia 3

Molecular Oncology Program, H. Lee Moffitt Cancer Center and Research Institute, Departments of Biochemistry and Molecular Biology [M. A., T. M-A.], Pathology [C. A. M-C.], and Surgery [J. C.], University of South Florida, Tampa, Florida 33612

Abstract
Transforming growth factor (TGF)-β is a potent regulator of growth and differentiation in normal squamous epithelium. TGF-β exerts its antiproliferative effect via the TGF-β type II receptor (TβR-II). A decrease in TβR-II expression is believed to be responsible, in part, for the resistance of squamous cell carcinoma (SqCC) to the antiproliferative effects of TGF-β. In the present study, we used immunohistochemistry and in situ hybridization to analyze the expression of TβR-II along the successive oncogenic stages of head and neck squamous neoplasia, from normal epithelium to dysplasia to carcinoma. Quantitation of TβR-II expression in 38 SqCCs was assessed on a visual scale ranging from negative (absence of staining) to 3+ (strong staining). Normal squamous epithelium and squamous epithelium in the vicinity of the tumors showed homogenous receptor expression with moderate intensity. Dysplastic epithelium and carcinoma in situ showed a mild decrease in receptor expression intensity. Well-differentiated to moderately differentiated carcinomas showed heterogeneous expression of variable intensity, and poorly differentiated carcinomas were completely devoid of TβR-II. In every tumor, the superficial component showed more intense receptor expression than the invasive component. These results indicate that TβR-II expression inversely correlates with disease aggressiveness and suggest that aberrant TβR-II expression is a contributing factor to the pathogenesis of SqCC.

Introduction
The molecular progression model for colorectal cancer, which was first described by Fearon and Vogelstein (1), is based on the premise that cancer is the result of accumulations of genetic alterations. This model has become a paradigm for other human solid tumors, including head and neck SqCC. Like colorectal cancer, head and neck SqCCs are thought to progress through a series of well-defined clinical and histopathological stages. Recently, Califano et al. (2) correlated the histopathology of 87 lesions of the head and neck with allelic losses determined by microsatellite analysis. Based on their findings, they proposed a molecular progression model for head and neck SqCC that includes progressive losses of 9p (benign squamous hyperplasia); 3p and 17p (dysplasia); 11q, 13q, and 14q (carcinoma in situ); and 6p, 8, and 4q (invasive carcinoma). Therefore, as many other malignancies, head and neck tumors are not the result of alterations or mutations in a single oncogene or tumor suppressor gene but the consequence of multiple alterations of several different genes.

Most malignant cells, including those of SqCC, are resistant to the antiproliferative effects of TGF-β. Elucidation of the processes involved in the acquisition of this resistance is important to understand the mechanisms involved in early stages of malignant conversion. Although immortalization per se does not render cells resistant to TGF-β, many malignant epithelial cells are refractory to the antiproliferative effect of TGF-β (3). For instance, rat liver epithelial cells are normally inhibited by TGF-β, but transformation by carcinogens or oncogenes can render them unresponsive to TGF-β (4, 5). Also, TGF-β can inhibit the growth of cells derived from well-differentiated colon adenocarcinomas. However, as tumors develop into poorly differentiated and aggressive neoplasias, their cells lose sensitivity to inhibition by TGF-β (5).

Head and neck SqCC cell lines are resistant to the antiproliferative effects of TGF-β due to either the lack of the receptor or the presence of mutations in the coding region that render the kinase inactive (6, 7). Several groups have examined the expression of TGF-β receptors in head and neck SqCCs (8) and found a decrease in the number of TβR-IIs. However, no correlation has been reported between expression of TGF-β receptors and degree of tumor differentiation and tumor aggressiveness. In the present study, we examined 38 head and neck SqCCs for the expression of TβR-II protein by immunohistochemistry and its mRNA by in situ hybridization. Our results indicate that TβR-II expression decreases as tumors become less differentiated and more biologically aggressive, suggesting that aberrant TβR-II expression is a contributing factor to the pathogenesis of SqCC.

1 This study was supported in part by American Cancer Society Grant RPG-98-039-01-CNE.
2 C. A. M-C. and M. A. contributed equally to this work and should both be considered first authors.
3 To whom requests for reprints should be addressed, at H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612. Phone: (813) 979-3884; Fax: (813) 979-3893; E-mail: antoniaT@moffitt.usf.edu.

Received 12/2/98; revised 3/1/99; accepted 3/1/99.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: SqCC, squamous cell carcinoma; TGF, transforming growth factor; TβR-II, TGF-β type II receptor; TβR-I, TGF-β type I receptor; RT, room temperature; FOM, floor of the mouth.
Expression of TβR-II in SqCC of the head and neck. Well-differentiated SqCC (×400): homogeneous, intense cytoplasmic expression of TβR-II protein (A) and TβR-II mRNA (B). Moderately differentiated SqCC (×400): heterogeneous, moderate cytoplasmic expression of TβR-II protein (C) and TβR-II mRNA (D). Poorly differentiated SqCC (×400): weak expression or no expression of TβR-II protein (E) and TβR-II mRNA (F). Superficial component of the tumor: intense expression of TβR-II protein (G) and TβR-II mRNA (H). Deeper infiltrating component of tumor: weak expression or no expression of TβR-II protein (I) and TβR-II mRNA (J). Squamous epithelium adjacent to the tumor, expression of TβR-II protein (K). Moderately differentiated SqCC, cytoplasmic expression of TβR-I protein (L). Negative control, no TβR-II protein expression after competition with specific TβR-II immunizing peptide (M).
Materials and Methods

Selection of Cases. Thirty-eight head and neck SqCCs were selected from the archives of the Pathology Department at the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL). Cases were selected if they fulfilled all of the following criteria: (a) the presence of areas of nonneoplastic squamous epithelium adjacent to the tumor; this epithelium lacks the histopathological features of dysplasia or malignancy; (b) the presence of areas of dysplasia and/or in situ carcinoma; (c) the presence of superficial and infiltrating components; (d) the presence of at least two different degrees of differentiation; (e) specimens were properly processed (no delayed fixation or poor handling) and free of necrosis; (f) patients did not receive any previous treatment (with the exception of a diagnostic biopsy); and (g) patients were initially diagnosed and finally treated at our institution to ensure collection of clinical data, proper processing of tissues according to Institutional Review Board-approved protocols, and patient confidentiality.

Diagnosis of SqCC. The diagnosis of SqCC was made by a head and neck pathologist using standard criteria that included the presence of intracellular bridges, keratinization, keratin pearl formation, various degrees of cytological atypia, mitotic rate, and architectural abnormalities. The degree of differentiation was assigned as follows: (a) well-differentiated, large cells with obvious keratinization, intercellular bridges, or keratin pearl formation; (b) moderately differentiated, heterogeneous population of cells of moderate size with only focal keratinization, poorly preserved intercellular bridges, and a higher degree of cytological atypia; and (c) poorly differentiated, anaplastic population lacking keratinization and intercellular bridges and with minimal resemblance to squamous epithelium. Superficial areas were defined as the portion of the tumor closest to the mucosal surface still retaining a noninfiltrating border. Infiltrating areas were defined as the deepest regions of the tumor and/or areas of the tumor with infiltrating borders. Clinicopathological staging was assigned following the recommendations of the American Joint Committee on Cancer (9).

Immunohistochemical Analysis of TβR-II and TβR-I. Sections (5 μm) from formalin-fixed, paraffin-embedded tissues were cut and placed on poly-L-lysine-coated slides. Slides were subjected to deparaffinization in xylene and hydration through a series of decreasing alcohol concentrations, following standard procedures. Endogenous peroxidase was quenched with a 3% solution of H₂O₂ for 20 min at 37°C, and the slides were washed in deionized water for 5 min. Antigen retrieval was performed by placing the slides in a clear plastic container with a vented top containing citrate buffer (4.5 ml of 0.1 M citric acid, 21.5 ml of 0.1 M sodium citrate, and 225 ml of deionized water) and microwaving in a microwave oven set on high (two times for 5 min). The slides were allowed to cool for 10–20 min and were rinsed in deionized water, placed in PBS for 5 min, and drained. Blocking serum was applied, and the slides were incubated in a humid chamber for 20 min at RT. After blocking, the following primary antibodies were applied at RT at a 1:100 dilution: (a) anti-TβR-II (COOH-terminal domain); and (b) anti-TβR-I (Santa Cruz Biotechnology, Santa Cruz, CA). After 1 h, the slides were rinsed with PBS and placed in PBS for 5 min. For detection, the Vectastain ABC Kit, Rabbit IgG, Elite series (Vector Laboratories, Inc., Burlingame, CA) was used following the manufacturer’s specifications. The biotinylated secondary antibody was applied, for 20 min at RT in a humid chamber. At the end of this incubation, the slides were rinsed and placed in PBS for 5 min, followed by the addition of the ABC complex. The slides were incubated in a humid chamber for 30 min at RT and then rinsed and placed in PBS for 5 min. We prepared 3,3′-diaminobenzidine following the manufacturer’s specifications, and applied it to the slides, and color development was monitored. When the desired intensity was reached (2–5 min), the slides were rinsed in water and counterstained with modified Mayer’s hematoxylin for 30 s. The slides were then washed in running water for 10 min, dehydrated, cleared, and mounted with resinous mounting medium. The specificity of staining for TβR-II was ascertained by abolishing the signal after competition with TβR-II-specific immunizing peptide (Fig. 1M).

Quantitation of Receptor Expression. In each case, the percentage of superficial and invasive areas and the percentage of tumor area corresponding to each degree of differentiation were recorded. The intensity of receptor expression was visually estimated as follows: 0, no expression; 1+, <25% of cells; 2+, 25–50% of cells; and 3+, >50% of cells. In each tumor, scoring of receptor expression was recorded independently in adjacent squamous epithelium; dysplasia; carcinoma in situ; well-, moderately, and poorly differentiated areas; and superficial and invasive regions (Table 1).

In Situ TβR-II mRNA Hybridization. Paraffin-embedded samples were incubated at 60°C for 20 min and deparaffinized twice with xylene for 10 min each time. Sections were rehydrated in decreasing concentrations of alcohol and rinsed three times in Millipore water. Tissue sections were then incubated in 0.2% pepsin at RT for 30 min. The TβR-II sense and antisense (negative control) biotinylated oligonucleotide probes (50 ng/μl) were diluted to their final concentration in hybridization buffer, denatured by incubation at 90°C for 8 min, and added to the sections. The sections were cover-slipped, placed in a humid chamber, and incubated at 42°C overnight. At the end of the incubation, the coverslips were soaked with 2× SSC (0.3 M sodium chloride/0.03 M sodium citrate) and removed. The tissue slides were then washed in 2× SSC/0.2% BSA for 20 min at 42°C, followed by two rinses in TBS for 3 min each. Streptavidin/alkaline phosphatase diluted 1:100 in Tris/MgCl₂/BSA was applied to each slide and incubated for 30 min at RT, followed by three rinses in TBS for 3 min each. The slides were then washed in TBS/MgCl₂ at pH 9.5 for 5 min before the addition of the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate and incubated in the dark for 1 h until the color developed. Slides were then rinsed with deionized water and counterstained with Nuclear Fast Red for approximately 10 min (depending on the intensity of the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reaction). In situ mRNA hybridization studies were limited to 10 selected SqCCs that contained representative areas of all parameters of interest. The detection of TβR-II mRNA was specific because no signal was observed with the antisense probe.
Expression of TβR-II Inversely Correlates with Tumor Aggressiveness

The age of the patients ranged from 32–85 years, with an anatomical location of the tumors was as follows: (a) 4 cases (10.5%) in the FOM; (b) 15 cases (36.1%) were stage I; (c) 6 cases (15.8%) were stage II; (d) 19 cases (30%) were stage III; and (e) 1 case (2.6%) was stage IV.

The clinicopathological staging was as follows: (a) 6 cases (15%) were stage I; (b) 1 case (2.6%) was stage II; (c) 12 cases (31.6%) were stage III; and (d) 19 cases (50%) were stage IV. Normal head and neck squamous epithelium obtained in surgical procedures not associated with squamous neoplasia has consistently revealed moderate to intense homogenous cytoplasmic expression of TβR-II (data not shown).

Images corresponding to the different tumor areas are depicted in Fig. 1. Nonneoplastic squamous epithelium lacking the histopathological features of dysplasia or malignancy and located in the proximity of the tumors analyzed has also shown moderate to intense homogenous cytoplasmic expression of TβR-II (Fig. 1K). TβR-II expression was also intense in well-differentiated, keratinizing SqCC (Fig. 1A). However, in moderately differentiated SqCC, we observed heterogeneity in the expression of TβR-II, with areas of high, moderate, and low intensity (Fig. 1C). A decrease in the levels of TβR-II expression as the tumor becomes less differentiated was more evident in poorly differentiated tumors, which were completely devoid of TβR-II expression (Fig. 1E). The specificity of staining for TβR-II was ascertained by abolishing stain in the presence of the immunizing specific peptide (Fig. 1M). In contrast to TβR-II expression, we observed no reduction in the expression of TβR-I (Fig. 1L), suggesting that in head and neck SqCC, ex-
pression of TβR-I is not directly related to the degree of tumor differentiation or to local aggressiveness.

The intensity of TβR-II expression in different areas of the tumors is summarized in Table 2. Receptor expression is progressively lost from superficial to infiltrating components and from well- to poorly differentiated areas. Thus, all superficial, well-differentiated regions show strong receptor expression (scores, 2+ and 3+), whereas all infiltrating poorly differentiated areas have no or low receptor expression (scores, 0 and 1+). The majority of poorly differentiated areas in superficial component and the well-differentiated areas in infiltrating regions show strong receptor expression, suggesting that a loss of receptor is associated with highly aggressive lesions that are both poorly differentiated and infiltrating. Expression of the type II receptor in superficial versus infiltrating areas, in the context of differentiation, is graphically represented in Fig. 2.

To investigate whether the decrease in TβR-II expression observed in the poorly differentiated tumors was due to a lack of transcription or to posttranslational mechanisms, we performed in situ mRNA hybridization in 10 selected SqCCs. The levels of mRNA in the different areas correlated with the protein content (Fig. 1, A–J). Thus, abundant TβR-II mRNA was detected in the cells of well-differentiated and superficial regions (Fig. 1H). However, as tumors lost differentiation and became invasive, the amount of mRNA progressively diminished to be almost undetectable in the most deeply invasive and poorly differentiated areas (Fig. 1J). These results indicate that abnormal TβR-II mRNA transcription is responsible for the decrease in protein levels. The detection of mRNA was specific because no signal was observed with an antisense probe (data not shown).

**Discussion**

TGF-β is a potent regulator of proliferation, growth, and differentiation in normal squamous epithelium. TGF-β exerts its antiproliferative effect through the TβR-II. In tumor cells, a decrease in TβR-II expression is believed to be responsible, in part, for the resistance of SqCCs to the antiproliferative effects of TGF-β (10–13). Recently, Grady et al. (14) reported that mutations in TβR-II coincide with the transformation of benign adenomas to malignant carcinomas in colon cancers with microsatellite instability. These observations have led to the hypothesis that escape from TGF-β-mediated negative growth control is an important aspect of the malignant phenotype in many epithelial neoplasms.

In the present study, we have analyzed the expression of TβR-II in SqCC of the head and neck along the successive oncogenic stages from normal epithelium to dysplasia to carcinoma. Our results indicate that expression of TβR-II inversely correlates with local disease aggressiveness and suggest that aberrant TβR-II expression is a contributing factor to SqCC development. To investigate whether anomalies in TβR-II expression are specific for this receptor, we have also analyzed the expression of the TβR-I, another component of the TGF-β signal transduction pathway. Normal squamous epithelium, squamous epithelium adjacent to carcinoma, and dysplastic areas had moderate to high levels of both receptors. Furthermore, TβR-I was consistently expressed in all tumors analyzed and in all areas within the tumors.

The distribution of TβR-II expression was markedly different. In general, well- and moderately differentiated areas showed moderate to high levels of receptor expression in both superficial and infiltrating regions. However, in the majority of poorly differentiated carcinomas, particularly in infiltrating regions, TβR-II expression was either markedly reduced or completely absent. The in situ hybridization studies strongly suggest that abnormal gene transcription is responsible for the decrease in protein levels.

The mechanisms of resistance to the antiproliferative effects of TGF-β are unknown. However, a decrease in the number of TβR-II binding sites has been associated with a loss of responsiveness to TGF-β in a number of cases (10–13, 15–20). This decrease in TβR-II expression correlates with tumorigenicity in many cell lines. For example, the breast carcinoma cell line MCF7(−) is TβR-II negative, resistant to TGF-β antiproliferative effects, and tumorigenic. Transfection of this cell line with TβR-II cDNA increases the number of TβR-IIs, restores TGF-β sensitivity, and decreases tumorigenicity (19). The marked decrease of TβR-II that we have observed would make tumors unresponsive to the inhibitory control of TGF-β. Furthermore, an abnormal TGF-β signal transduction pathway could contribute to the progressive loss of differentiation in carcinomas.
Expression of TβR-II Inversely Correlates with Tumor Aggressiveness

References

Expression of Transforming Growth Factor \( \beta \) Type II Receptors in Head and Neck Squamous Cell Carcinoma

Carlos A. Muro-Cacho, Meredith Anderson, Joehassin Cordero, et al.