ARTICLES

Levels of TGF-α and EGFR Protein in Head and Neck Squamous Cell Carcinoma and Patient Survival

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Background: **The most accurate predictor of disease recurrence in patients treated for head and neck squamous cell carcinoma is, at present, the extent of regional lymph node metastasis. Since elevated levels of epidermal growth factor receptor (EGFR) and of its ligand, transforming growth factor-α (TGF-α), have been detected in primary tumors of patients with head and neck squamous cell carcinoma, we determined whether tumor levels of these proteins were of prognostic importance.** ***Methods:*** **Monoclonal antibodies specific for EGFR and TGF-α were used for immunohistochemical detection of each protein in tissue sections of primary tumors from 91 patients who were treated by surgical resection. Levels of immunoreactive EGFR and TGF-α were quantified by use of a computerized image analysis system and were normalized to appropriate standards. The logrank test and proportional hazards regression analysis were used to calculate the probability that EGFR and TGF-α levels were associated with disease-free survival (i.e., no recurrence of cancer) and cause-specific survival (i.e., patients do not die of their disease). All *P* values were two-sided.** ***Results:*** **When tumor levels of EGFR or TGF-α were analyzed as continuous variables, disease-free survival and cause-specific survival were reduced among patients with higher levels of EGFR (both *P* = .0001) or TGF-α (both *P* = .0001). In a multivariate analysis, tumor site, tumor level of EGFR, and tumor level of TGF-α were statistically significant predictors of disease-free survival; in a similar analysis, regional lymph node stage and tumor levels of EGFR and of TGF-α were significant predictors of cause-specific survival.** ***Conclusion:*** **Quantitation of EGFR and TGF-α protein levels in primary head and neck squamous cell carcinomas may be useful in identifying subgroups of patients at high risk of tumor recurrence and in guiding therapy. [J Natl Cancer Inst 1998; 90:824–32]**

Head and neck squamous cell carcinoma is an epithelial cancer arising in the mucosa of the upper aerodigestive tract. Potential anatomic sites affected by this cancer include the oral cavity, oropharynx, hypopharynx, and larynx. Approximately half of all patients with head and neck squamous cell carcinoma are cured of their initial tumor. Factors such as age, sex, tumor site, tumor–lymph node–metastasis (TNM) stage, and histologic grade may help to guide treatment decisions but are not useful predictors of outcome *(1)*. The most accurate predictor of disease recurrence at present is the lymph node stage. Identification of a better marker, perhaps one that would identify patients with a poor prognosis, would provide a much needed opportunity to target patients at risk for recurrence of the initial tumor and/or development of a secondary cancer.

In addition to the TNM stage, previous studies have reported a concordance between the presence of p53 (also known as TP53) gene mutations at the histologically normal tumor margins and local recurrence *(2)*. There was also an association between p53 mutation status and disease progression *(3)*. The development of a method to identify patients at high risk for disease recurrence following standard therapy (surgery or radiation treatment) would enable clinicians to offer these individuals further treatment, including external-beam radiation therapy and/or brachytherapy, chemotherapy, and biologic response modifiers. Although these adjunctive therapies remain experimental for this cancer, reports *(4–6)* suggest that they may prolong survival. The failure to achieve better results with adjunctive treatment may be attributed either to insufficient efficacy of the treatments now being administered or to a lack of methods to identify patients who are at high risk for disease recurrence.

Many human tumor cells express high levels of growth factor receptors, raising the possibility that receptor-directed therapies may be useful as anticancer strategies. A series of murine monoclonal antibodies (MAbs) directed against human epidermal growth factor receptor (EGFR) have been evaluated in clinical

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See ''Notes'' following ''References.''

trials. These reagents, in general, have limited antitumor activity, although development of ''chimeric'' and ''humanized'' MAbs may improve their antitumor activity by abrogating the host immune response *(7)*. On the basis of this experience, the development of alternative therapeutic approaches is warranted.

We showed that the EGFR ligand, transforming growth factor-α (TGF-α), and EGFR messenger RNA (mRNA) and protein levels were greater in tumors and histologically normal mucosa located several centimeters away from the primary tumor site in patients with head and neck cancer than in control normal mucosa from patients without cancer *(8)*. Moreover, TGF-α and EGFR protein levels in the tumor were quantified by immunohistochemical staining with MAbs followed by computerized image analysis of the staining intensity *(9)*. To address the role of enhanced levels of EGFR and its ligand in head and neck squamous cell carcinoma, we showed that growth of tumor-derived cell lines but not of normal mucosal epithelial cells was inhibited by down-modulation of TGF-α with antisense TGF-α oligonucleotides *(10)*. In addition, EGFR levels and activity could be blocked by antisense oligonucleotides, anti-EGFR MAbs, and specific inhibitors of EGFR kinase activity. Again, all of these treatments reduced tumor cell line proliferation, suggesting that TGF-α and EGFR are participating in an autocrine growth activation pathway in head and neck squamous cell carcinoma *(11)*. A TGF-α–EGFR autocrine pathway provides a plausible biologic mechanism for continued growth of head and neck cancers that express high levels of TGF-α and EGFR. Thus, enhanced TGF-α and EGFR levels may be associated with decreased survival.

Currently, identification of head and neck cancer patients who are at high risk for disease recurrence relies primarily on clinicopathologic criteria, including tumor location, tumor size, and the presence and extent of regional lymph node metastases. To determine if TGF-α and EGFR levels could be of greater prognostic value than current methods, we examined TGF-α and EGFR protein levels in primary tumors from patients with head and neck squamous cell carcinoma.

Materials and Methods

Patients and Tissue Samples

Criteria for patient participation in the study were as follows: 1) a histologically confirmed diagnosis of head and neck squamous cell carcinoma of the upper aerodigestive tract (oral cavity, oropharynx, hypopharynx, or larynx), 2) primary surgical resection with curative intent occurring during the period from November 1990 through February 1993, and 3) absence of distant metastases as assessed by chest x ray and/or computed tomography scan. Patients were excluded from the analysis for the following reasons: 1) They had a history of head and neck squamous cell carcinoma, 2) they had received prior chemotherapy, or 3) they were previously treated for cancer (i.e., non-head and neck squamous cell carcinoma). One hundred nine patients met the entry criteria. Of the participating patients, four tissue samples were unavailable and 14 patients had inadequate clinical follow-up, leaving 91 patients for analysis.

Archival tissue samples (paraffin-embedded) from the 91 head and neck squamous cell carcinomas were obtained from the diagnostic histopathology laboratories at the University of Pittsburgh Medical Center. Clinical follow-up was available for all patients until October 1996. Pertinent patient information was abstracted from the computerized head and neck tumor registry.

All patients were treated with curative intent. Each underwent complete surgical resection of the primary tumor with negative surgical margins, and 77 (84.6%) of the 91 patients underwent dissection of the cervical lymph nodes with pathologic staging of the regional lymphatics (N stage). Clinical staging was conducted in accordance with the American Joint Committee on Cancer tumor–lymph node–metastasis (TNM) classification at the time of surgery *(12)*. Of the 91 patients, 56 (62%) received postoperative external-beam radiation and 16 (18%) were treated with adjunctive chemotherapy (13 on a protocol for extracapsular spread of the tumor in the cervical lymphatics and three with inoperable recurrent or metastatic disease). Patients were classified according to disease status as follows: alive without evidence of disease, dead of disease, or dead of other causes.

Tissue Pathologic Examination and Computerized Analysis of TGF-α and EGFR Staining

Diagnosis of squamous cell carcinoma was based on conventional morphologic examination of formalin-fixed, paraffin-embedded specimens. Immunohistochemical staining was performed on 4-μm-thick tissue sections by use of MAbs specific for TGF-α (Ab2; Calbiochem/Oncogene Science, Cambridge, MA) and EGFR (clone $EGFR_1$; Genosys/Cambridge Research, The Woodlands, TX) as described previously *(9)*. Antigen retrieval techniques (e.g., microwave heating) were not employed. A sample of normal skin, which demonstrates abundant TGF-α expression *(13)*, was used as a positive control reference standard for TGF-α expression. Cytospin preparations of A431 (25 000 cells per slide), a well-characterized vulvar squamous cell carcinoma cell line that overexpresses EGFR *(14)*, were fixed in formaldehyde without saponin and used as a positive control reference standard for EGFR expression. Negative controls for immunohistochemical staining consisted of replacement of the primary antibodies with an isotype-matched irrelevant murine immunoglobulin G subclass antibody. Within tumor cells, TGF-α was localized primarily in the cytoplasm and immunoreactive EGFR protein was detected primarily on the cell membrane.

The intensity of immunohistochemical staining as a reflection of the number of positive granules per cells (mean labeling concentration = mean optical density) was evaluated under 40× magnification by use of a SAMBA 4000 Image Analysis System (Image Products International, Chantilly, VA) as described previously *(9)*. Twelve representative high-power fields of each section were analyzed, and each result was reported as the mean of the optical density of the 12 values. Tumor heterogeneity was also determined by computerized image analysis and reported as concentration heterogeneity, defined as the concentration variation coefficient between cells and structures (concentration standard deviation/mean concentration). The microscope slide-mounted tissue sections were coded, and the pathologists performing the computerized image analysis were blinded to the clinical outcome of the patients. Human skin samples from three different cancer-free individuals were incubated with TGF-α antibody and analyzed on seven separate occasions to assess the variability of this TGF-α standard. Cytospin preparations of A431 cells were stained for EGFR on four different occasions, and expression levels were quantitated to determine the variability of the EGFR standard. The raw data from the tumor samples were analyzed as a percent of the standards (mean optical density) to control for day-to-day staining variability and to ensure that the results could be generalized for prospective data collection in other laboratories. Human skin samples were obtained from surgical pathology specimens (e.g., from limb amputations) or purchased (Carolina Biological Supply, Burlington, NC). A431 cells were obtained from the American Type Culture Collection, Rockville, MD.

Statistical Analysis

Statistical analysis was performed on the entire patient population. Survival was measured in months from the date of surgery to the date of death or to the last follow-up. Disease-free survival was defined as the time from tumor resection until the first evidence of tumor recurrence or the development of a new primary tumor of the upper aerodigestive tract. Cause-specific survival was based on death due to progression of upper aerodigestive tract cancer (i.e., recurrence of index tumor or second primary cancer) as distinct from death due to other causes. All surgical resections were considered curative rather than palliative. Patients were divided into approximately equal tertiles according to TGF-α and EGFR protein levels detected in their tumors for the purpose of generating survival curves. Survivor function curves and median survival times were calculated with the method of Kaplan and Meier *(15)*. Confidence intervals for the median values were constructed by use of Greenwood's formula on the log scale. Differences in survivor function due to prognostic factors were calculated by the logrank test *(16)*. *P* values for multiple logrank tests were adjusted with a step-down Bonferroni procedure *(17)*. All *P* values were two-sided. The joint effect of predictive variables was evaluated by Cox's regression models

(18) using continuous variables. Prognostic covariates included in the analysis were sex, age, tumor site, tumor grade, tumor stage, lymph node stage, presence of extracapsular spread, postoperative radiation therapy, postoperative chemotherapy, and the mean optical densities of TGF-α and EGFR protein levels in the tumor expressed as percents of standard. Prognostic factors were evaluated individually, and all factors having a moderate or strong impact on survival were considered jointly for Cox's regression modeling. For the assessment of the reproducibility of the mean optical density values, repeated measurements of TGF-α and EGFR immunoreactivities were obtained in a subset of samples for each marker, and the intraclass correlation was estimated.

Results

All patients underwent curative surgical resection as primary treatment for their tumors. Second primary tumors of the upper aerodigestive tract developed in seven patients during the course of the study period, and 35 patients developed local or regional recurrence or distant metastases. Four patients presented with a synchronous second primary tumor that was resected with the index tumor. Of the 91 patients entered in the study, 41 died, 32 from their head and neck cancer and nine from other causes (i.e., not as a result of carcinoma of the upper aerodigestive tract). The median follow-up for the 50 surviving patients was 49 months (range, 12–73 months). One patient was lost to follow-up at 17 months. The median survival based on all causes of death was 54 months (lower 95% confidence level = 41 months). Of the 42 patients with tumor recurrence or a new primary tumor, 32 died of their head and neck cancer, one died of other causes, and nine underwent curative resection and are currently alive without evidence of disease. The clinical, pathologic, and treatment characteristics of the patient population are summarized in Table 1. All 91 head and neck cancer patients studied had immunoreactive TGF-α and EGFR protein in their tumors, and image analysis was used to quantify the intensity of TGF-α and EGFR immunostaining. For the positive control skin samples, the average TGF-α mean optical density ± standard deviation was 25.5 ± 1.26. The average EGFR mean optical density for the positive control samples was 19.26 ± 0.99. Because of the low variability in the TGF-α and EGFR standards, the mean optical density data were analyzed as a percentage of the standard for each measurement. Median TGF-α was 145% of standard (range, 12%–521%), and median EGFR was 54% of standard (range, 5%–233%). Patients whose tumors contained high levels of immunoreactive TGF-α protein also had higher levels of EGFR (Spearman correlation = .70; $P = .0001$), and these individuals were more likely to have died of disease than patients whose tumors contained low levels of immunoreactive TGF-α and EGFR (Fig. 1).

We also determined whether TGF-α and EGFR protein levels in the tumors of the 91 patients with head and neck squamous cell carcinoma correlated with clinical, pathologic, and treatment parameters. When analyzed as continuous variables, immunoreactive TGF-α and EGFR protein levels in the primary tumor were not significantly associated with sex, age 65 years or older, tumor stage, tumor grade, or chemotherapy. Variables demonstrating some association with levels of TGF-α and/or EGFR in tumors included tumor site, lymph node stage, extracapsular spread, and radiation therapy, although the association between immunoreactive TGF-α or EGFR protein levels in tumors and survival was independent of these clinical and pathologic parameters (data not shown).

Table 1. Clinicopathologic and treatment characteristics of patients undergoing resection for head and neck squamous cell carcinoma

Characteristic	No. of patients (%)
Sex	
Male	65/91 (71)
Female	26/91 (29)
Age, y	
<65	43/91 (47)
⩾65	48/91 (53)
Tumor site	
Oral cavity	26/91 (29)
Oropharynx	12/91 (13)
Hypopharynx	17/91 (19)
Larynx	36/91 (39)
T stage*	
T1	14/91 (15)
T2	33/91 (36)
T3	23/91 (25)
T4	21/91 (23)
N stage*	
N0	49/91 (54)
N1	15/91 (16)
N2	27/91 (30)
Differentiation	
Well differentiated	25/91 (27)
Moderately differentiated	52/91 (57)
Poorly differentiated	14/91 (15)
Extracapsular spread	
Yes	29/91 (32)
No	62/91 (68)
Radiation therapy	
Yes	56/91 (62)
No	35/91 (38)
Chemotherapy	
Yes	16/91 (18)
No	75/91 (82)

*Stage was determined by pathologic analysis. T = tumor; N = lymph node.

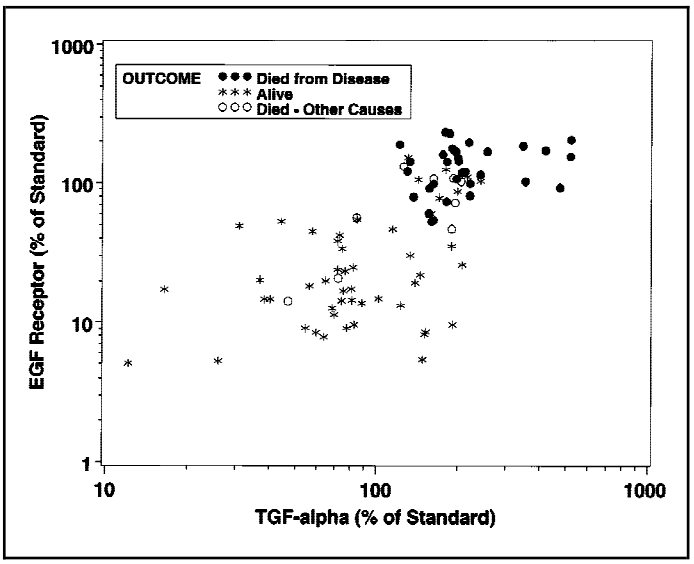

Fig. 1. Relationship between transforming growth factor-α (TGF-α) and epidermal growth factor receptor (EGFR) protein levels in 91 primary head and neck squamous cell carcinomas (Spearman correlation = .70; $P = .0001$). Patients who are alive without evidence of disease, those who died of disease, or those who died of other causes are represented according to tumor TGF-α or EGFR levels.

The association of clinical, pathologic, and treatment characteristics of the head and neck cancer patients with disease-free survival is shown in Table 2. Two-year survival was assessed because patients with head and neck cancer are generally considered to be cured of their index tumor if they demonstrate no evidence of disease 2 years after curative treatment. In the univariate analysis, the factors with no significant association with decreased disease-free survival were sex, age 65 years or older, tumor grade, tumor stage, lymph node stage, or chemotherapy. The tumor site (larynx; $P = .042$) was associated with increased disease-free survival, most likely a result of both early diagnosis and the relatively sparse lymphatic drainage associated with the vocal cords. Patients who received adjunctive external-beam radiation therapy experienced a higher incidence of disease recurrence ($P = .024$), which is likely attributed to the tendency to administer radiation therapy to patients with advanced disease. However, the level of immunoreactive TGF-α protein ($P = .0001$) or EGFR protein ($P = .0001$) in the tumor was the strongest predictor of decreased disease-free survival. Disease-free survival was more specifically examined by alternately censoring either tumor recurrences or second primary tumors. TGF-α and EGFR levels were determined to have a significant impact on the recurrence of the index tumor ($P = .001$). Although only seven patients developed second primary tumors during the course of the study, both elevated TGF-α ($P = .01$) and EGFR ($P = .001$) levels in the index tumor were associated with the occurrence of a second cancer of the upper aerodigestive tract (data not shown).

When cause-specific survival (i.e., patients did not die of their disease) was examined via univariate analysis, lymph node stage ($P = .001$), extracapsular spread ($P = .002$), and levels of TGF-α protein ($P = .0001$) or EGFR protein ($P = .0001$) showed significant association with adverse outcome (Table 3). Kaplan–Meier analysis revealed that levels of both TGF-α and EGFR in the primary tumor were highly predictive of reduced disease-free survival (Fig. 2). Similarly, levels of both TGF-α and EGFR in the primary tumor were also predictive of reduced cause-specific survival (Table 3).

A proportional hazards model for disease-free survival revealed that tumor site and TGF-α or EGFR levels were significant predictors. A test of interaction demonstrated that the increase in the risk of tumor recurrence or of a new primary tumor

Table 2. Association of potential prognostic factors and disease-free survival

Prognostic factor	Subgroup	No. of patients with recurrent disease/No. at risk	Probability of surviving 2 y	Lower 95% confidence bound, y*	Logrank P†	Adjusted P
All patients		42/91	.69	2.5	—	—
Sex	Male	27/65	.68	2.5	.301	1.0
	Female	15/26	.68	2.1		
Age	<65 y	19/43	.63	2.4	.886	1.0
	⩾65 y	23/48	.73	2.3		
Tumor site	Oral cavity	16/26	.51	1.2	.042	.248
	Oropharynx	7/12	.67	2.0		
	Hypopharynx	9/17	.62	1.7		
	Larynx	10/36	.81	4.5		
Tumor grade	Well differentiated	10/25	.70	2.1	.665	1.0
	Moderately differentiated	26/52	.69	2.4		
	Poorly differentiated	6/14	.60	1.3		
T stage‡	1	5/14	.77	2.1	.224	.988
	2	17/33	.58	1.7		
	3	9/23	.86	3.0		
	4	11/21	.58	0.6		
N stage‡	0	19/49	.74	2.5	.113	.559
	1	8/15	.80	2.3		
	2	15/27	.50	1.3		
Extracapsular spread	Yes	16/29	.54	1.7	.104	.728
	No	26/62	.74	2.5		
Radiation therapy	Yes	31/56	.63	2.1	.024	.217
	No	11/35	.78	5.2		
Chemotherapy	Yes	10/16	.50	1.3	.237	1.0
	No	32/75	.73	2.5		
TGF-α,§ % of standard	Low	5/30	.89	—	.0001	.0001
	Medium	15/31	.60	2.0		
	High	22/30	.50	1.2		
EGFR,§ % of standard	Low	5/30	.90	5.2	.0001	.0001
	Medium	11/30	.70	2.5		
	High	26/31	.49	1.2		

*This is the lower 95% confidence bound for the median survival.

†Logrank test is a test of equality of survivor function across groups.

‡Stage was determined by pathologic analysis. T = tumor; N = lymph node.

§Transforming growth factor-α (TGF-α)/epidermal growth factor receptor (EGFR) levels were determined by use of immunohistochemistry followed by computerized image analysis (*see* ''Materials and Methods'' section).

Table 3. Association of potential prognostic factors and cause-specific survival

Prognostic factor	Subgroup	Deaths from disease/No. at risk	Probability of surviving 2 y	Lower 95% confidence bound, y*	Logrank *P*†	Adjusted *P*
All patients		32/91	.88	4.5	—	—
Sex	Male	22/65	.80	4.2	.750	.750
	Female	10/26	.88	3.2		
Age	<65 y	17/43	.78	3.1	.322	.644
	⩾65 y	15/48	.86	4.2		
Tumor site	Oral cavity	10/26	.80	2.1	.093	.371
	Oropharynx	6/12	.83	2.5		
	Hypopharynx	9/17	.69	1.8		
	Larynx	7/36	.91	—		
Tumor grade	Well differentiated	5/25	.92	—	.109	.371
	Moderately differentiated	20/52	.82	3.3		
	Poorly differentiated	7/14	.69	2.0		
T stage‡	1	2/14	1.0	—	.066	.282
	2	13/33	.81	3.2		
	3	7/23	.95	4.5		
	4	10/21	.59	0.8		
N stage‡	0	11/49	.92	—	.001	.007
	1	6/15	.86	3.4		
	2	15/27	.64	1.4		
TGF-α level,§ % of standard	Low	0/30	1.0	—	.0001	.0001
	Medium	12/31	.82	2.8		
	High	20/30	.64	2.0		
EGFR level,§ % of standard	Low	0/30	1.0	—	.0001	.0001
	Medium	10/30	.81	4.5		
	High	22/31	.66	1.8		

*This is the lower 95% confidence bound for the median survival. In most cases, median survival has not been reached.

†Logrank test is a test of equality of survivor function across groups.

‡Stage was determined by pathologic analysis. T = tumor; N = lymph node.

§Transforming growth factor-α (TGF-α)/epidermal growth factor receptor (EGFR) levels determined by immunohistochemistry followed by computerized image analysis (*see* ''Materials and Methods'' section).

associated with higher levels of immunoreactive TGF-α or EGFR was independent of the primary tumor site (data not shown). A similar regression model for cause-specific survival demonstrated that the combination of increased TGF-α or EGFR levels plus lymph node stage was the strongest predictor of death from disease. The exclusion of EGFR from the model resulted in a statistically significant reduction in predictive power. However, the combination of EGFR levels and lymph node stage was as strong a predictor of outcomes as were TGF-α and EGFR levels plus lymph node stage ($P = .13$; Cox regression cause-specific survival; data not shown).

If lymph node stage (N stage) were strongly associated with steady-state levels of these two proteins, then patients with N stage 0 should tend to have low levels of immunoreactive TGF-α and EGFR, whereas patients with N stage 2 should be more likely to have elevated TGF-α and EGFR levels. To determine whether tumor levels of immunoreactive TGF-α and EGFR predicted survival independently of lymph node metastases, tests of interaction were performed. These tests revealed that the effect of TGF-α and EGFR on overall cause-specific survival was the same across N stage categories (Fig. 3). TGF-α levels were high in 12 patients with no evidence of neck metastases (N stage 0), and six of these 12 patients died of their disease during the course of the study. Similarly, 16 patients with clinicopathologic N stage 0 had high EGFR levels in their index tumors; nine of these patients subsequently died of their disease. Five patients with advanced disease in their neck (N stage 2) had low TGF-α levels in their primary tumor, and all are currently without evidence of disease. EGFR levels were low in four patients with N stage 2 clinicopathologic staging; all of these patients are currently without disease. Conversely, TGF-α tumor levels were low in 18 patients with N stage 0, none of whom died of disease, and tumor levels of EGFR were low in 21 patients with N stage 0, all of whom remain alive without evidence of disease. Fourteen patients had high levels of immunoreactive TGF-α; 11 of these patients died of disease. Moreover, nine patients had high levels of EGFR in their tumors; all of these patients subsequently died of their head and neck cancer. These results suggest that expression levels of TGF-α and EGFR in tumor predict clinical outcome independently of cervical lymph node status.

A reliability analysis was conducted by use of repeated measurements in a subset of tumor samples to determine the reproducibility of the TGF-α and EGFR assays. Twenty-two sets of repeated measurements were provided for TGF-α determinations, and nine sets were provided for EGFR determinations. Either two or four replicates were obtained. To assess reliability, we calculated the intraclass correlation coefficient. A difference was found in the reliability of quantitating the two proteins. The EGFR intraclass correlation coefficient was .9, suggesting a high degree of reliability of the measurements. In contrast, the TGF-α intraclass correlation coefficient was only .56, suggesting that quantitation of the ligand was more problematic. The concentration heterogeneity of both TGF-α (0.49 ± 0.16) and EGFR

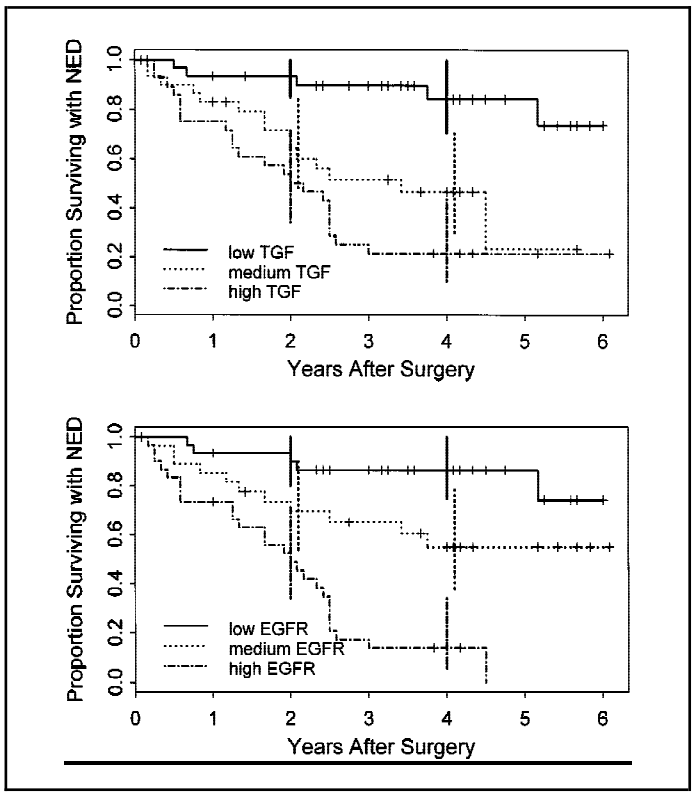

Fig. 2. Disease-free survival (i.e., survival with no evidence of disease [NED]) among 91 patients with head and neck squamous cell carcinoma according to transforming growth factor-α (TGF) level **(top panel)** or epidermal growth factor receptor (EGFR) level **(bottom panel)** in the primary tumor. Patients were grouped by lowest, middle, and highest tertiles of normalized values. Vertical lines denote 95% confidence intervals (Greenwood's formula) at 2 and 4 years following surgery. Survivor function curves and median survival times were calculated by the method of Kaplan and Meier.

(0.45 ± 0.16) staining was low, indicating low tumor heterogeneity and relatively uniform staining of the tumor specimens.

Discussion

Immunohistochemical staining of tumors from 91 patients undergoing surgical resection for head and neck squamous cell carcinoma was performed to determine if TGF-α and EGFR protein levels could serve as prognostic markers. Our analysis detected both TGF-α and EGFR immunoreactive proteins in epithelial cells from all of the tissue samples. The level of immunoreactive TGF-α and EGFR protein could be quantified with the aid of computerized image analysis. Overall, our results showed that both TGF-α and EGFR protein levels in tumors were reliable predictors of adverse outcome in head and neck cancer patients. Quantitative differences in the levels of TGF-α and EGFR were shown in this study to be superior to most of the usual clinical and pathologic prognostic factors for predicting the clinical course in patients with head and neck cancer. In our study, most of the patients (85%) had pathologic staging of their cervical lymphatics. Based on Kaplan–Meier analysis of survival data, TGF-α and EGFR protein levels were independent of lymph node stage as predictors of survival (Fig. 3).

Overexpression of members of the EGFR family has been implicated in a wide variety of human carcinomas. For example, expression of EGFR protein in breast cancer has been correlated with both a poor prognosis *(19–21)* and a lack of response to endocrine therapy *(22);* moreover, it has been reported to be a factor associated with increased metastatic potential *(23).* In patients with colon cancer, increased tumor EGFR mRNA levels have been associated with a higher rate of liver metastasis *(24).* Elevated EGFR levels have also been associated with adverse outcome in patients with cancer of the bladder *(25–28),* lung *(29),* kidney *(30),* ovary *(31),* brain *(32,33),* cervix *(34,35),* endometrium *(36,37),* esophagus *(38),* stomach *(39,40),* pancreas *(41),* or thyroid *(42).* Fewer studies have reported a correlation between enhanced TGF-α levels in the primary tumor and decreased survival *(30,38,40–43).* Elevated EGFR mRNA levels in several small series of head and neck squamous cell carcinomas have been associated with larger tumor and advanced stage and, hence, a poor prognosis *(44,45).* In laryngeal cancer, studies based on a relatively small number of patients have reported increased levels of EGFR in the tumor compared with the levels in adjacent normal mucosa as well as a higher incidence of recurrence in patients whose tumors expressed EGFR *(46,47),* although the median follow-up was relatively short (21 months). To demonstrate an inverse correlation with survival, other investigators *(48,49)* have used ligand-binding assays in a small number of patients or immunoblotting that was qualitative, rather than quantitative, to examine EGFR levels in the primary tumor. In contrast, other investigators *(50,51)* have failed to demonstrate a significant correlation between TGF-α and/or tumor levels and survival.

Levels of TGF-α and EGFR mRNA or protein in tissues can be measured by several molecular techniques, including radiolabeled ligand binding, protein blotting, RNA blotting, *in situ* hybridization, and quantitative reverse transcription–polymerase chain reaction. However, these techniques are time-consuming and require a high degree of technical expertise and meticulous processing of the tissue specimen. In addition, they are often unable to distinguish the precise cellular source of the molecule(s) under investigation (e.g., tumor cell versus normal epithelial cell versus submucosa). In contrast, immunohistochemistry using commercially available antibodies is a standard procedure in all diagnostic pathology laboratories and can be performed on paraffin-embedded specimens. While not strictly a quantitative technique, the percentage of positively staining cells as well as the intensity of the staining in the tumor cells can be measured with the aid of a computerized image analysis system, which is available in most teaching hospitals. By use of this approach, estrogen receptor levels have been accurately and reproducibly quantified in breast cancer tissue sections and have been correlated with clinical outcome in patients *(52,53).* Although the quantitative image analysis is performed only on a relatively small volume of tumor tissue, we found a remarkably low level of heterogeneity of either TGF-α or EGFR expression, suggesting that the tumor sections analyzed were truly representative of the entire tumor.

In our study, the death hazard ratio was higher in patients whose tumors had elevated TGF-α and EGFR levels. Logrank tests and Cox's regression analysis showed that TGF-α and EGFR protein levels in the head and neck squamous cell tumors were each significantly associated with both decreased disease-free and cause-specific overall survival. Our previous finding of increased TGF-α and EGFR mRNA and protein in the histo-

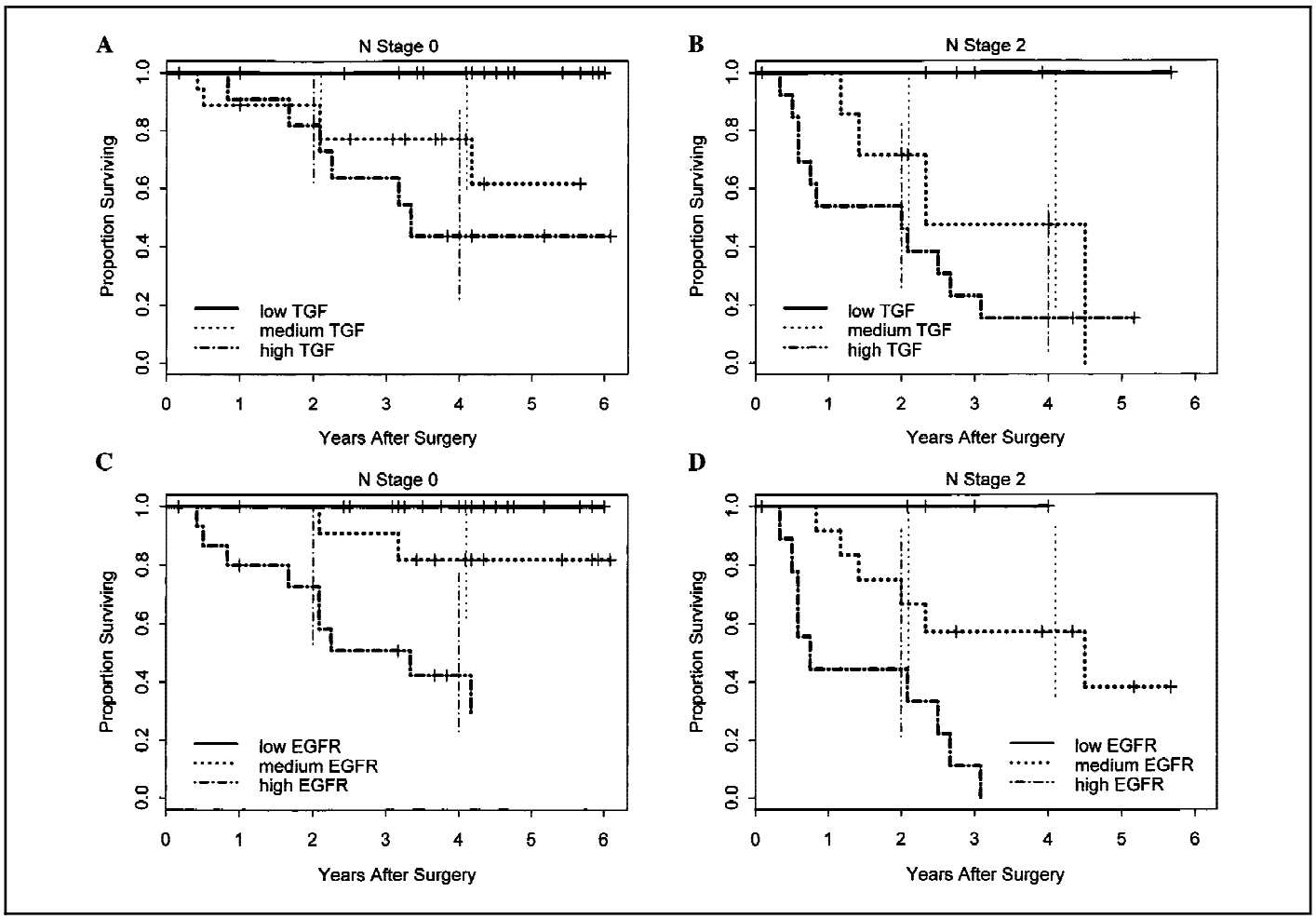

Fig. 3. Kaplan–Meier analysis showing the overall cause-specific survival among 49 head and neck cancer patients with N (i.e., lymph node) stage 0 **(panels A and C)** and 27 patients with N stage 2 **(panels B and D)** according to levels of transforming growth factor-α (TGF) **(panels A and B)** or epidermal growth factor receptor (EGFR) **(panels C and D)** in the primary tumor. Vertical lines denote 95% confidence intervals at 2 and 4 years.

logically normal mucosa distant from the tumor site in head and neck cancer patients *(8,9)* suggests that up-regulation of TGF-α and EGFR occurs throughout the upper aerodigestive tract mucosa. This diffuse alteration of the mucosa may explain the increased incidence of second primary tumors of the upper aerodigestive tract in patients whose index tumor produces high levels of TGF-α and EGFR. The presence and extent of regional lymph node metastases are reflected by the lymph node stage, which, to date, has been the most accurate clinicopathologic parameter in predicting outcome in patients with head and neck cancer. Cox's regression modeling revealed that a combination of either increased TGF-α or increased EGFR levels and greater lymph node stage provided the strongest predictors of adverse outcome. Excluding TGF-α levels from the regression analysis did not significantly reduce the predictive power. However, we think that the lower reliability of the TGF-α measurements is a more likely explanation for the superior predictive ability of quantitating EGFR levels compared with determining TGF-α levels.

In conclusion, TGF-α and EGFR levels in the primary tumor appear to be among the most important prognostic factors yet identified for patients with head and neck squamous cell carcinoma. This finding suggests that TGF-α and EGFR may serve as reliable biologic markers to identify high-risk subgroups and to guide therapy. Such therapy could theoretically include MAbs against EGFR, such as those used in patients with squamous cell carcinoma of the lung *(54,55)*, or fusion proteins or immunotoxins against TGF-α or EGFR using toxins elaborated by *Pseudomonas* or *Diphtheria* species *(56,57)*.

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Notes

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