[CANCER RESEARCH 53, 3579–3584, August 1, 1993]

Elevated Levels of Transforming Growth Factor α and Epidermal Growth Factor Receptor Messenger RNA Are Early Markers of Carcinogenesis in Head and Neck Cancer[1]

Jennifer Rubin Grandis and David J. Tweardy[2]

Departments of Otolaryngology [J. R. G.], Medicine, and Molecular Genetics and Biochemistry [D. J. T.], University of Pittsburgh School of Medicine and the Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213

ABSTRACT

The squamous mucosa of patients who develop head and neck cancer is "condemned" or predisposed to disregulated growth as reflected by the high incidence of synchronous and metachronous primary tumors. We hypothesized that transformed and nontransformed mucosa from head and neck cancer patients would produce increased levels of transforming growth factor α (TGF-α) and its cell surface receptor, the epidermal growth factor receptor (EGFR), thereby contributing to this predisposition. Using molecular biological techniques, we examined the incidence and mechanism of TGF-α and EGFR overproduction in tumors and histologically normal mucosa excised from patients with squamous cell carcinoma of the head and neck (SCCHN) to test this hypothetical mechanism of field cancerization. Northern blot hybridization was used to evaluate the frequency of increased TGF-α and EGFR mRNA production in tissue excised from 24 patients with SCCHN and 10 cell lines compared with 7 control patients without cancer or a history of alcohol and tobacco use. Southern blot hybridization was used to examine for gene amplification. In patients with SCCHN, TGF-α mRNA was elevated by a mean of 5-fold in 95% of histologically "normal" mucosa samples ($P = 0.001$) and by a mean of 5-fold in 87.5% of tumors ($P = 0.0001$) while EGFR mRNA was elevated by a mean of 29-fold in 91% of histologically normal mucosa specimens ($P = 0.0005$) and by a mean of 69-fold in 92% of tumors ($P = 0.0005$), compared with mRNA levels in control normal mucosa. In 10 SCCHN cell lines, TGF-α mRNA was increased by a mean of 16-fold and EGFR mRNA levels were increased by a mean of 77-fold. Increased production of TGF-α and EGFR mRNA in the histologically normal mucosa of patients at risk for a primary or secondary head and neck cancer may serve both as a marker for malignant transformation and as a target for preventive therapies.

INTRODUCTION

SCC[3] is the most common malignancy involving the oral cavity, oropharynx, hypopharynx, and larynx. Over the last 5 years, there has been a dramatic increase in the incidence of SCC of the oral cavity in the United States, Scandinavia, and Scotland, particularly among younger males (1–3); worldwide, the number of cases is even greater. While the management of SCCHN has improved, there is no evidence to suggest that therapeutic advances have resulted in increased survival rates. The opportunity to prevent SCCHN would be greatly enhanced by a more sophisticated understanding of the pathogenesis of these tumors.

Epidemiological studies identify alcohol and tobacco use as predisposing risk factors; however, the mechanism(s) by which the combination of these agents affects malignant transformation remains unknown. Moreover, only a minority of people who smoke and drink in any appreciable quantity subsequently develop a head and neck malignancy. No viral associations, chromosomal abnormalities, or oncogene activations have been consistently identified in patients with SCCHN.

Evidence is accumulating that TGF-α and its receptor, EGFR, may be directly involved in oncogenesis. TGF-α, when transfected into normal mouse mammary epithelial cells with a retroviral vector, results in the transformation of those cells to a malignant phenotype (4). Elevated levels of mRNA for EGFR and/or TGF-α have been detected in a variety of cultured human carcinoma cell lines including SCC of the skin, lung, esophagus, and oral cavity (5–9). This increase in mRNA is associated with increased TGF-α and EGFR protein (10). Such an elevation of EGFR protein has been documented in a number of cell lines and tumors derived from patients with SCCHN (11–13). Furthermore, patients whose tumors express increased EGFR protein appear to have a worse prognosis (12, 14–18). To date, oral cavity tumors have been reported to demonstrate evidence of mRNA expression for EGFR and TGF-α, yet data from normal mucosa in these patients are sparse and conflicting (8, 19). We hypothesized that fresh tissues and cell lines derived from patients with SCCHN would show aberrant peptide growth factor regulation. We examined fresh tumor and histologically normal mucosa samples from 24 patients with SCCHN as well as 10 cell lines to determine the frequency and cellular source of EGFR and TGF-α mRNA overexpression.

MATERIALS AND METHODS

Cell Lines. Ten cell lines (7 primary tumors and 3 metastatic lymph nodes) derived from patients with SCCHN as well as 3 control cell lines were grown in Minimal Essential Medium (GIBCO Laboratories, Grand Island, NY) with 15% fetal bovine serum (GIBCO), plus 100 units/ml of penicillin and 100 units/ml of streptomycin (GIBCO) (20). The SCCHN cell lines are part of a large collection established in the Department of Otolaryngology at this university (21). A431 is a well characterized vulvar SCC known to overproduce EGFR mRNA by 20–100-fold (5) and was used as a positive control. The negative control cell lines consisted of Detroit 551 (skin fibroblast) and IMR-90 (lung fibroblast) which are American Type Culture Collection cell lines, the latter of which is known to produce low levels of EGFR mRNA (5).

Fresh Tissue Samples. A portion of tumor and histologically normal mucosa was isolated intraoperatively from 24 patients with SCCHN undergoing surgical resection of their disease. All 24 patients were smokers and had a history of ethanol abuse. For use as controls, normal mucosa was also harvested from 7 patients without SCCHN, matched for age (within 5 years) and sex with SCCHN patients, who were undergoing unrelated head and neck procedures (*e.g.*, uvulopalatopharyngoplasty). All controls denied an antecedent history of alcohol or tobacco use.

RNA Extraction and Northern Blot Analysis of Tissues and Cell Lines. After excision, the tissues were immediately lysed in 4 M guanidine isothiocyanate (BRL, Bethesda, MD), or were snap-frozen in liquid nitrogen and processed while frozen at a later date to minimize RNA degradation. Total cellular RNA was isolated by centrifugation through a cesium chloride (BRL) cushion (22).

When the flasks containing the cell lines were 65–85% confluent, the cells were washed with phosphate-buffered saline and lysed with guanidine isothio-

Received 2/1/93; accepted 5/26/93.

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[1] Supported by Grant CH503 from the American Cancer Society (D. J. T.)

[2] To whom requests for reprints should be addressed, at Pittsburgh Cancer Institute, Biomedical Science Tower W1052, Pittsburgh, PA 15213.

[3] The abbreviations used are: SCC, squamous cell carcinoma; cDNA, complementary DNA; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor α; SCCHN, squamous cell carcinoma of the head and neck.

Table 1 *Clinical characteristics of SCCHN patients with intact RNA*

Patient	Sex/age (yr)	Primary tumor site	Clinical stage	Prior therapy
1	M/70	Larynx	$T_2N_0M_0$	Radiation
2	M/65	Larynx	$T_2N_0M_0$	None
3	M/56	Larynx	$T_3N_2M_0$	None
4	F/74	Floor of mouth	$T_2N_1M_0$	None
5	M/71	Larynx	$T_4N_0M_0$	None
6	M/77	Pyriform sinus	$T_4N_0M_0$	None
7	M/56	Larynx	$T_2N_0M_0$	Radiation
8	F/83	Buccal mucosa	$T_3N_0M_0$	None
9	M/60	Larynx	Recurrence	Radiation
10	M/63	Larynx	$T_4N_0M_0$	None
11	M/63	Retromolar trigone	$T_4N_1M_0$	None
12	F/53	Larynx	$T_3N_0M_0$	None
13	M/48	Larynx	$T_3N_0M_0$	None
14	M/64	Tongue	$T_2N_0M_0$	None
15	M/63	Hypopharynx	$T_4N_0M_0$	None
16	M/65	Pyriform sinus	$T_4M_{2b}M_0$	None
17	M/71	Larynx	$T_4N_0M_0$	None
18	M/71	Buccal mucosa	$T_4N_0M_0$	None
19	M/68	Hypopharynx	$T_4N_{2c}M_0$	None
20	M/57	Larynx	$T_4N_2M_0$	None
21	M/55	Larynx	$T_4N_2M_0$	Radiation
22	M/57	Larynx	$T_2N_0M_0$	None
23	M/63	Floor of mouth	$T_4N_{2b}M_0$	None
24	F/55	Pyriform sinus	$T_4N_1M_0$	None

cyanate. RNA was purified from crude lysate by centrifugation through a cesium chloride cushion. Twenty μg of RNA were separated on agarose-formaldehyde gels (23), blotted onto Zetabind (AMF-Cuno, Meriden, CT) according to the manufacturer's instructions, and covalently bound to the membrane by brief UV irradiation (24). Full-length cDNA clones of EGFR and TGF-α were obtained from the American Type Culture Collection collection (Rockville, MD). The EGFR was cDNA-isolated from a human cDNA library in pBR322 (25). The TGF-α cDNA was isolated frm a human kidney library in pBR327 (26). The cDNA probe for human β-tubulin was isolated from a HeLa cell in library in pUC8 (27). The cDNA probe for rat 18s rRNA was cloned in the pXC-1 derivative of pBR322 and was kindly provided by Dr. Christine Milcarek, University of Pittsburgh School of Medicine. Nucleic acid probes were radiolabeled to a high specific activity ($2–5 \times 10^8$ cpm/μg cDNA) by nick translation or random priming with [^{32}P]dCTP and used to hybridize membranes according to the manufacturer's instructions. Following hybridization overnight at 37°C, the membranes were washed exposed to Kodak XAR film with an intensifying screen at −80°C for 1–14 days.

Determination of TGF-α and EGFR mRNA Production. Two-dimensional densitometry was performed on autoradiograms following TGF-α, EGFR, and 18s ribosomal subunit cDNA hybridizations. Relative density was calculated by dividing TGF-α or EGFR signals by the 18s signal. Two to 4 controls were run on each Northern blot and the mean and SD were calculated for each set of controls. Comparisons were only made between samples run on the same blot. Several patient samples were run on 2–3 Northern blots to assess blot-to-blot variability. TGF-α values varied by as much as 15% between blots and EGFR signals by no more than 25%. When the relative densities of TGF-α or EGFR in SCCHN patient samples was greater than the mean of the controls, statistical significance was calculated using χ^2 analysis where the null hypothesis was that 50% of the patient samples would have relative densities above the controls and 50% would have values less than or equal to the controls. When the relative densities of the patients' tissues were greater than the mean plus 2 SD of the controls, significance was determined using the Fisher exact test.

DNA Extraction and Southern Blot Analysis. DNA was obtained from 10 SCCHN and 3 control cell lines by sodium dodecyl sulfate-proteinase K digestion and extraction with phenol and chloroform, followed by ethanol precipitation, as described (28). The DNA (10 μg) from cell lines and fresh tumor was digested with an excess of restriction endonuclease (*Eco*RI) and electrophoresed on a 0.8% agarose gel. The digested DNA was transferred to nylon filters using the method of Southern as described (29). Blots were then probed with [^{32}P]dCTP-labeled cDNA. The membranes were then washed and exposed to x-ray film with an intensifying screen at −80°C for 14 days.

RESULTS

TGF-α and EGFR mRNA Expression in Fresh Tumor and Normal Mucosa from Patients with SCCHN. Intact RNA was isolated from samples of tumor and histologically normal mucosa from 24 patients undergoing surgical resection at the Eye and Ear Pavilion of the Montefiore University Hospital, University of Pittsburgh, from July 1990 through November 1991 (Table 1). Total RNA from the normal mucosa of 7 patients matched for age and sex with cancer patients with neither SCCHN nor a significant history of alcohol and tobacco exposure who underwent unrelated head and neck surgery was also extracted for use as controls.

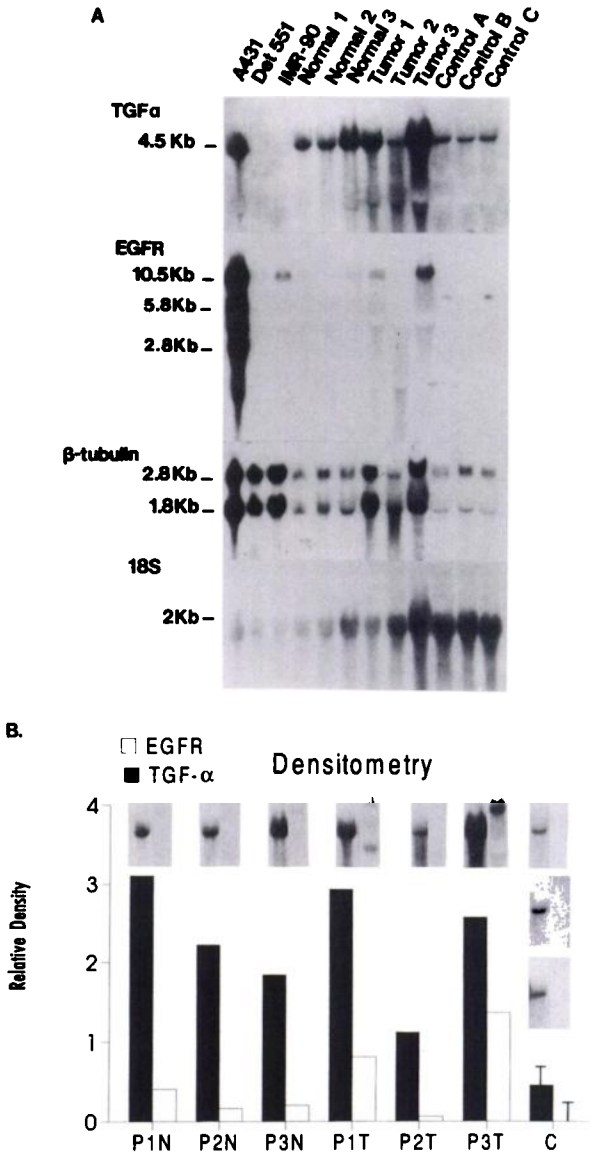

Fig. 1. Sample Northern blot of the tissues of SCCHN patients showing increased TGF-α and EGFR mRNA production. In *A*, total RNA (20 μg) from tumor tissue and histologically normal mucosa from three SCCHN patients and three control cell lines and mucosa from three control patients without cancer were fractionated by gel electrophoresis, blotted onto nylon membranes, hybridized sequentially with TGF-α, EGFR, β-tubulin, and the cDNA for the 18s ribosomal subunit, and stripped between hybridizations. Exposure time was 14 days for TGF-α, 72 h for EGFR, 48 h for β-tubulin, and 2 h for 18s. Hybridization with β-tubulin, a "housekeeping" gene, and the probe for 18s ribosomal RNA, a structural RNA, was performed to control for RNA loading and integrity. In *B*, the autoradiograms were analyzed by densitometry and the TGF-α and EGFR values were corrected by dividing by the 18s value. The corrected scanning ratio was then plotted for each individual sample; the control data are presented as mean ± SD of the three controls. *Kb*, kilobases; *P*, patient; *N*, histolgically normal mucosa; *T*, tumor; *C*, control.

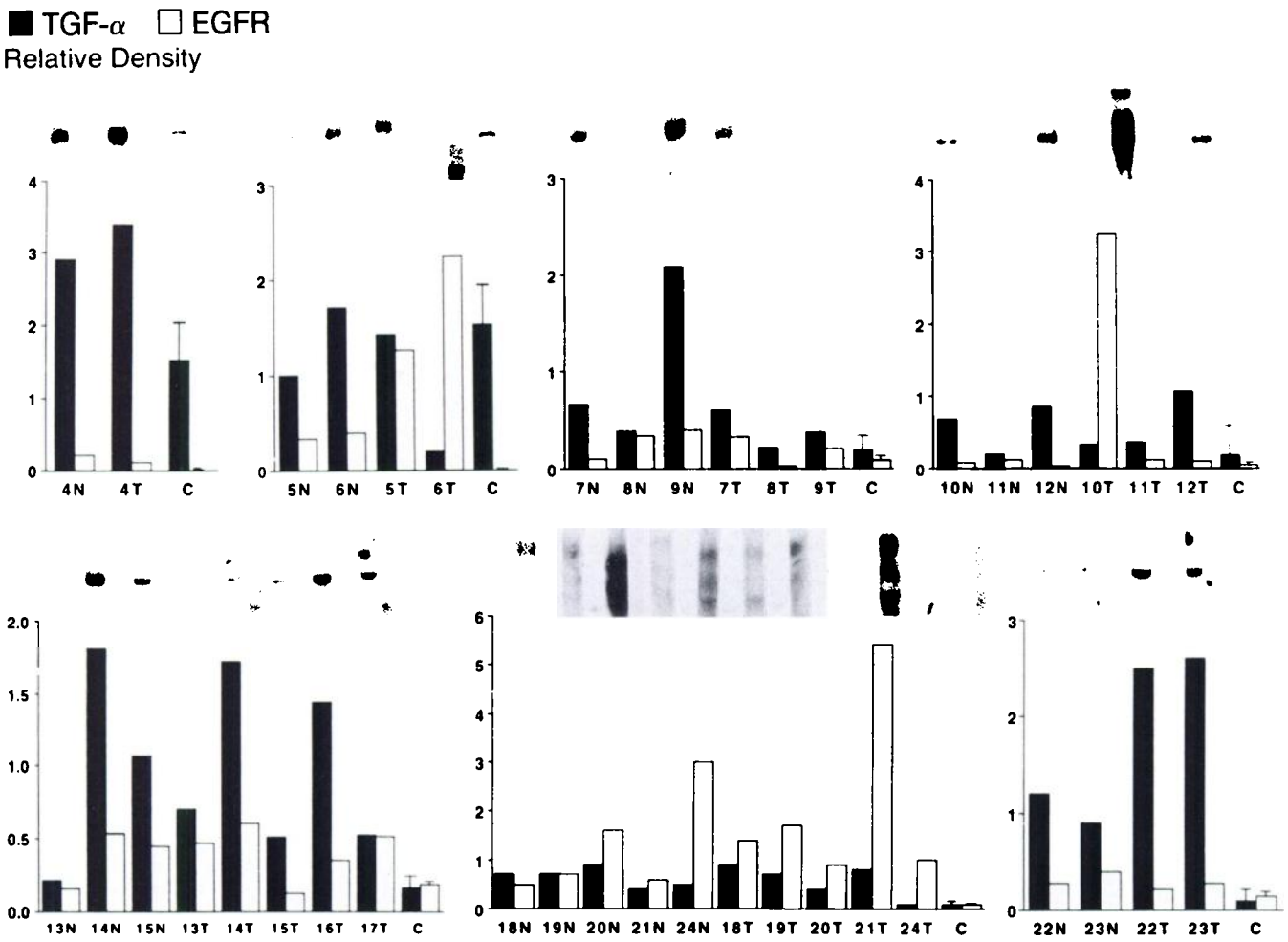

Fig. 2. Up-regulation of TGF-α and EGFR mRNA in patient samples. Densitometric analysis of 24 SCCHN patients and 7 normal controls analyzed on 9 Northern blots. Relative density was calculated by dividing the density of the TGF-α or EGFR hybridization by the 18s ribosomal subunit hybridization. The region of the autoradiogram corresponding to the TGF-α and EGFR bands is shown above each sample. *N*, histologically normal mucosa; *T*, tumor; *C*, control.

Table 2 *Summary of TGF-α and EGFR mRNA levels in SCCHN cell lines and patient tissues*

Cell/tissue sample	TGF-α mRNA levels		EGFR mRNA levels	
	Level in sample > mean level in controls[a]	Level in sample > mean level in controls + 2 SD	Level in sample > mean level in controls[a]	Level in sample > mean level in controls + 2 SD
SCCHN cell lines	10/10 (100%) $P = 1.1 \times 10^{-5}$	10/10 (100%) $P = 1.1 \times 10^{-5}$	10/10 (100%) $P = 1.1 \times 10^{-5}$	10/10 (100%) $P = 1.1 \times 10^{-5}$
SCCHN tumors	21/24 (87.5%) $P = 0.001$	17/24 (71%) $P = 2.9 \times 10^{-7}$	22/24 (92%) $P = 0.0005$	20/24 (83.3%) $P = 2.3 \times 10^{-8}$
Histologically "normal" mucosa[b]	21/22 (95%) $P = 0.0001$	16/22 (73%) $P = 2.5 \times 10^{-6}$	20/22 (91%) $P = 0.0005$	18/22 (82%) $P = 1.1 \times 10^{-6}$

[a] Control, mRNA levels excised from patients without cancer.
[b] "Normal" mucosa, histologically normal mucosa excised from patients with SCCHN.

The RNA isolated from patient tissues was analyzed on Northern blots. Up-regulation of EGFR and TGF-α mRNA levels was determined by comparing EGFR and TGF-α levels in tissues (tumor and histologically normal mucosa) from patients with SCCHN with EGFR and TGF-α mRNA levels in normal mucosa excised from control patients; only comparisons made between samples run on the same Northern blot were considered valid. Levels of mRNA were quantified by two-dimensional densitometry; an 18s ribosomal cDNA probe was used to control for loading. To assess the reproducibility of results, initially 5 patient samples were analyzed on 2–3 Northern blots. The blot-to-blot variability of each specimen proved negligible. Specifically, EGFR mRNA levels varied by 3–25% while the levels of TGF mRNA varied by 2–15%.

Normal mucosa from non-SCCHN patients consistently demonstrated low levels of TGF-α mRNA and little or no detectable EGFR mRNA. The overwhelming majority of tumor specimens as well as histologically normal mucosa samples harvested from patients with SCCHN were found to demonstrate increased levels of TGF-α and EGFR mRNA compared with mRNA levels produced by normal mucosa from non-cancer patients (Figs. 1 and 2). Overall, TGF-α mRNA was up-regulated in 95% of histologically normal mucosa samples ($P = 0.0001$) and 87.5% of tumor samples compared with

Table 3 *Degree of Up-regulation of TGF-α and EGFR in patient tissues*

Patient	TGF-α in tumor[a]	TGF-α in normal mucosa[a]	EGFR in tumor[a]	EGFR in normal mucosa[a]
1	2.26	2.52	118.6	139
2	1.39	2.75	23.5	123.8
3	5.64	4	690	100
4	2.23	1.92	19.3	35.3
5	0.94	0.66	210	55.5
6	0.13	1.13	373.3	64.7
7	3.1	3.4	3.95	1.14
8	1.12	2	0.32	4.1
9	1.9	10.8	2.5	4.9
10	1.9	3.84	64.8	1.48
11	2	1.13	2.4	2.4
12	5.95	4.8	1.8	0.6
13	4.22	1.3	2.42	0.8
14	10.4	10.9	3.1	2.73
15	3.1	NA[b]	0.64	NA
16	8.67	NA	1.8	NA
17	3.14	NA	2.67	NA
18	9.6	7.2	16.1	6.12
19	7.5	6.8	20.5	8.23
20	4.1	10.4	10.1	19
21	8.6	4.25	64.2	6.9
22	19.6	9.61	1.21	2
23	19.9	7	1.8	1.15
24	0.8	5.66	13.6	36.9
Mean	5.34	4.86	68.7	29.4
SD	5.35	3.38	157.5	4.3

[a] Level in sample/mean level in control.
[b] NA, not available (due to RNA degradation).

TGF-α mRNA in control mucosa (P = 0.001). EGFR mRNA was minimally expressed in 1 control sample and not expressed at all in the remaining 6 control normal mucosa samples; however, EGFR mRNA was detected and greater than normal control mucosa in 92% of tumors and in 91% of histologically normal mucosa samples resected from patients with SCCHN (P = 0.0005) (Table 2). TGF-α mRNA was elevated approximately 5-fold in both tumor and histologically normal mucosa samples from SCCHN patients compared with controls. EGFR mRNA was increased 69-fold in tumor samples and 29-fold in histologically normal mucosa samples from patients with SCCHN compared with levels in controls (Table 3).

TGF-α and EGFR mRNA Expression in SCCHN Cell Lines. When Northern blot hybridization is used to analyze mRNA species in homogenized fresh tissue samples, one cannot precisely identify which cells (*e.g.*, tumor, connective tissue, etc.) are responsible for the message detected. Using *in situ* RNA hybridization, a recent study demonstrated TGF-α expression by the infiltrating eosinophils in oral cavity tumors (19). To determine if the increased TGF-α as well as EGFR mRNA expression was of epithelial cell origin, we examined 10 cell lines derived from patients with SCCHN (21). All 10 SCCHN cell lines (both primary and metastatic) show increased levels of both TGF-α and EGFR mRNA compared with levels in normal mucosa from control patients ($P = 1.1 \times 10^{-5}$) (Fig. 3; Table 2). TGF-α mRNA was elevated by a mean of 10-fold (range, 1.7–36.3) and EGFR mRNA was elevated by a mean of 77-fold (range, 9.3–36.2) compared with control.

Mechanism of TGF-α and EGFR mRNA Up-regulation. Increased steady-state mRNA levels may result from a variety of mechanisms including increase in gene copy number (DNA), enhanced message stability, or an elevated message transcription rate. To determine if overexpression of EGFR and TGF-α mRNA is due to multiple gene copies in the cell lines, genomic DNA was purified and quantitated and 10 μg were linearized with an excess of restriction enzyme (*Eco*RI) and then subjected to Southern blot hybridization. Compared with A431 which is known to amplify EGFR DNA sequences 15–30-fold (30, 31), the 10 SCCHN cell lines derived from patients did not show a significant increase in EGFR or TGF-α gene dosage or any evidence of rearrangements of these genes (data not shown).

DISCUSSION

Our examination of TGF-α and EGFR mRNA production by fresh head and neck tumors and samples of histologically normal mucosa excised from a large number of patients with SCCHN revealed markedly increased levels of both TGF-α and EGFR mRNA in the overwhelming majority of specimens compared with levels detected in normal mucosa samples from control patients without cancer. The apparent up-regulation of growth factor and receptor mRNA expression has implications for the pathogenesis of head and neck cancer.

Alterations in growth factor signal transduction may contribute to disordered cell growth via a variety of mechanisms, including altered growth factor production, quantitative or qualitative changes in growth factor receptors, and disturbances of intracellular signalling events following receptor binding. When a cell develops the ability to produce a growth factor that stimulates its own proliferation, an autocrine growth pathway may develop, resulting in uncontrolled growth. The acquisition *in vitro* of such an autocrine pathway, involving the growth factor bombesin and its receptor, may contribute to the development of small cell carcinoma of the lung (32). Our discovery of increased mRNA levels of TGF-α, and its receptor, EGFR, in fresh

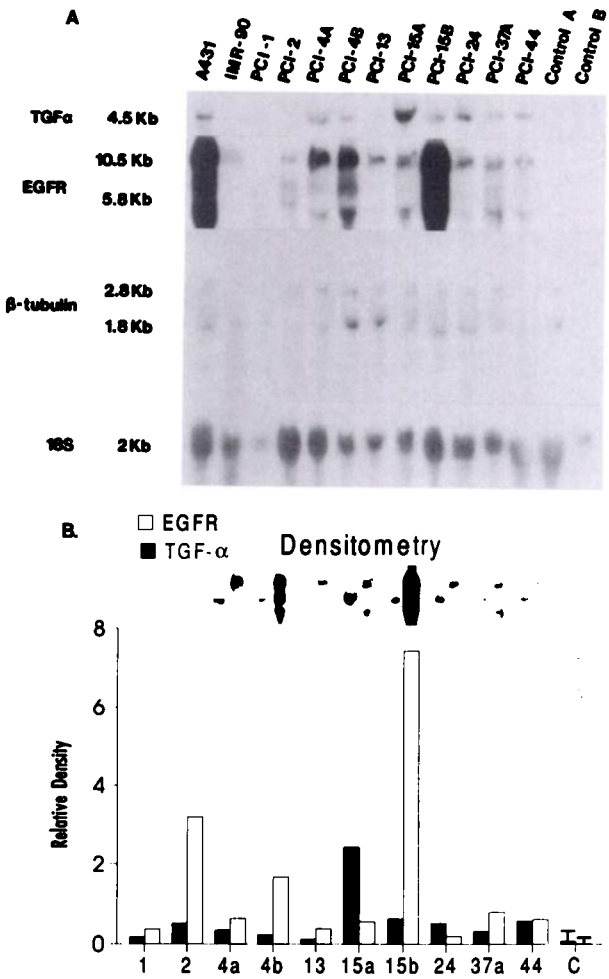

Fig. 3. Northern blot of SCCHN cell lines demonstrating increased TGF-α and EGFR mRNA production compared with control. *A*, total RNA (20 μg) from 10 SCCHN cell lines, two control cell lines, and mucosa from two control patients without cancer, hybridized sequentially with TGF-α, EGFR, β-tubulin, and the cDNA from the 18s ribosomal subunit, and stripped between hybridizations. Exposure time was 14 days for the TGF-α and EGFR, 24 h for β-tubulin, and 2 h for 18s. In *B*, the autoradiograms were analyzed by two-dimensional densitometry and the TGF-α and EGFR values were corrected by dividing by the 18s value. The corrected scanning ratio was then plotted for each individual sample; the control data are presented as the mean ± SD of the 2 controls. *Kb*, kilobases. *C*, control.

tumors and cell lines derived from patients with SCCHN suggests that an autocrine growth pathway may be operating in this tumor system.

TGF-α production was originally thought to be restricted to transformed and fetal or placental cells (33), but synthesis has now been detected in normal colonic mucosa (34) as well as gastric epithelium (35). The elevation of both TGF-α and EGFR mRNA in histologically normal mucosa extracted from a large number of patients with SCCHN was striking. The concept of "field cancerization" or "condemned mucosa," whereby multiple foci of premalignant or malignant changes occur as a result of exposure of the entire epithelium to initiating agents (*e.g.*, tobacco smoke and alcohol), has been invoked to explain why patients with SCCHN are susceptible to the significant development of synchronous and metachronous tumors (36, 37). The detection of elevated levels of mRNA for EGFR and its ligand, TGF-α, in the histologically normal mucosa of patients with SCCHN suggests that this is one mechanism whereby exposed mucosa becomes "condemned." This finding also indicates that the development of elevated TGF-α and EGFR mRNA levels may signal an early event in carcinogenesis in SCCHN.

The precise contribution of epithelial toxins, ethanol, and tobacco to the genetic disregulation which subsequently leads to the head and neck cancer phenotype is uncertain. Specifically, experiments analyzing the effects of alcohol and tobacco products on growth factor and receptor mRNA levels have yet to be reported. It is possible that in the genetically susceptible individual, prolonged exposure to the combination of alcohol and tobacco results in transcriptional activation of the TGF-α and EGFR genes resulting in an autocrine regulatory pathway, with subsequent malignant transformation. Future studies incorporating controls with a history of alcohol and tobacco use but without head and neck cancer are necessary to address this issue.

The identification of aberrant epithelial growth factor regulation in histologically normal mucosa of patients at risk for SCCHN could be useful as a true molecular marker of premalignancy as well as serve as a target for chemomodulation or prevention. Retinoids are differentiation agents the effects of which on cells are mediated by regulating gene transcription. In head and neck mucosa, retinoids have been shown to both eliminate premalignant lesions such as leukoplakia (38) and reduce the incidence of second primary tumors in patients with SCCHN (39). Studies in other systems reveal that retinoic acid down-regulates both EGFR (40) and TGF-α (41) mRNA expression. Preliminary data from our laboratory and others confirm that SCCHN cell lines, when treated with retinoic acid, produce less TGF-α and EGFR mRNA (42). An enticing explanation for the effects of retinoic acid on mucosal head and neck neoplasms is therefore provided by our findings. The overproduction of TGF-α and EGFR mRNA by tumors and mucosa at risk in patients with SCCHN may be interrupted by treatment with retinoic acid which down-regulates TGF-α and EGFR mRNA production at the level of gene transcription.

An understanding of growth factor disregulation has led to new approaches to therapy in other types of cancer, such as the use of anti-IL-2 receptor antibodies to treat T-cell lymphomas (43). Numerous animal studies have demonstrated an anti-tumor effect of anti-EGFR monoclonal antibodies against human squamous cell carcinomas (A431) xenografted into nude mice (44). A recent Phase I trial at the National Cancer Institute confirmed the feasibility of administering anti-EGFR monoclonal antibodies to patients with squamous cell carcinoma of the lung (45). Our discovery of elevated TGF-α and EGFR production by squamous epithelial cells from patients with SCCHN suggests that therapies targeting these genes may be effective in the prevention of both recurrent disease and the development of a second primary malignancy of the upper aerodigestive tract.

ACKNOWLEDGMENTS

We would like to thank Dr. Eugene N. Myers and Dr. Jonas T. Johnson for providing patient tissue specimens; Dr. Theresa L. Whiteside for supplying the SCCHN cell lines; and Dr. Victor L. Yu for reviewing the manuscript.

REFERENCES

1. Davis, S., and Severson, R. K. Increasing incidence of cancer of the tongue in the US among young adults. Lancet, *2:* 910–911, 1987.
2. Hakulinen, T., Anderson, A. A., Malker, B., Pukkala, B., Schou, G., and Tulinius, H. Trends in cancer incidence in the Nordic countries. A collaborative study of the five nordic cancer center registries. Acta Pathol. Microbiol. Immunol. Scand., *288* (Suppl.): 1–151, 1986.
3. McFarlane, G. J., Boyle, P., and Scully, C. Rising mortality from cancer of the tongue in young Scottish males. Lancet, *2:* 912, 1987.
4. McGeady, M. L., Kerby, S., Shankar, V., Ciardiello, F., Salomon, D., and Seidman, M. Infection with a TGF-α retroviral vector transforms normal mouse mammary epithelial cells but not normal rat fibroblasts. Oncogene, *4:* 1375–1382, 1989.
5. Derynk, R., Goeddel, D. V., Ullrich, A., *et al.* Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. Cancer Res., *47:* 707–712, 1987.
6. Partridge, M., Green, M. R., Langdon, J. D., and Feldman, M. Production of TGF-α and TGF-β by altered keratinocytes, skin and oral squamous cell carcinomas-potential autocrine regulation of normal and malignant epithelial cell production. Br. J. Cancer, *60:* 542–548, 1989.
7. Yoshida, K., Kyo, E., Tsuda, T., *et al.* EGF and TGF-α, the ligands of hyperproduced EGFR in human esophageal carcinoma cells, act as autocrine growth factors. Int. J. Cancer, *45:* 131–135, 1990.
8. Todd, R., Donoff, B. R., Gertz, R. *et al.* TGF-α and EGF-receptor mRNAs in human oral cancers. Carcinogenesis (Lond.), *10:* 1553–1556, 1989.
9. Weichselbaum, R. R., Dunphy, E. J., Beckett, M. A., *et al.* Epidermal growth factor gene amplification and expression in head and neck cancer cell lines. Head Neck, *11:* 437–442, 1989.
10. Mydlo, J. H., Michaeli, J., Crodon-Cardo, C., Goldenberg, A. S., Heston, W. D. W., and Fair, W. R. Expression of transforming growth factor α and epidermal growth factor receptor messenger RNA in neoplastic and nonneoplastic human kidney tissue. Cancer Res., *49:* 3407–3411, 1989.
11. Shirasuna, K., Hayashido, Y., Sugiyama, M., Yoshioka, H., and Matsuya, T. Immunohistochemical localization of epidermal growth factor (EGF) and EGF receptor in human oral mucosa and its malignancy. Virchows Arch. Pathol. Anat., *418:* 349–353, 1991.
12. Kawamoto, T., Takahashi, K., Nishi, M., Kimura, T., Matsumura, T., and Taniguchi, S. Quantitative assay of epidermal growth factor receptor in human squamous cell carcinomas of the oral region by an avidin-biotin method. Jpn. J. Cancer. Res., *82:* 403–410, 1991.
13. Maxwell, S. A., Sacks, P. G., Gutterman, J. U., and Gallick, G. E. Epidermal growth factor receptor protein-tyrosine kinase activity in human cell lines established from squamous cell carcinomas of the head and neck. Cancer Res., *49:* 1130–1137, 1989.
14. Mukaida, H., Toi, M., Hirai, T., Yamashita, Y., and Toge, T. Clinical significance of the expression of epidermal growth factor and its receptor in esophageal cancer. Cancer (Phila.), *68:* 142–148, 1991.
15. Yasui, W., Hata, J., Yokozaki, H., *et al.* Interaction between epidermal growth factor and its receptor in progression of human gastric carcinoma. Int. J. Cancer, *41:* 211–217, 1988.
16. Lacroix, H., Igelhart, J. D., Skinner, M. A., and Kraus, M. H. Overexpression of erbB-2 or EGF receptor proteins present in early stage mammary carcinoma is detected simultaneously in matched primary tumors and regional metastases. Oncogene, *4:* 145–151, 1989.
17. Ishitoya, J., Toriyama, M., Oguchi, N., *et al.* Gene amplification and overexpression of EGF receptor in squamous cell carcinoma of the head and neck. Br. J. Cancer, *59:* 559–562, 1989.
18. Ozawa, S., Masakazu, U., Ando, N., Abe, O., Hirai, M., and Shimizu, N. Stimulation by EGF of the growth of EGF receptor-hyperproducing tumor cells in athymic mice. Int. J. Cancer, *40:* 706–710, 1987.
19. Todd, R., Chou, M. Y., Matossian, K., Gallagher, G. T., Donoff, R. B., and Wong, D. T. W. Cellular sources of transforming growth factor alpha in human oral cancer. J. Dent. Res., *70:* 917–923, 1991.
20. Sacchi, M., Snyderman, C. H., Heo, D. S., *et al.* Local adoptive immunotherapy of human head and neck cancer xenografts in nude mice with lymphokine-activated killer cells and interleukin 2. Cancer Res., *50:* 3113–3118, 1990.
21. Heo, D. S., Snyderman, C. H., Gollin, S. M., *et al.* Biology, cytogenetics, and sensitivity to immunological effector cells of new head and neck squamous cell carcinoma lines. Cancer Res., *49:* 5167–5175, 1989.
22. Chirgwin, J. M., Praybyla, A. E., McDonald, R. J., and Rutter, J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, *18:* 5294–5299, 1979.
23. Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry, *16:* 4743–4751, 1977.
24. Church, G. M., and Gilbert, W. Genomic sequencing. Proc. Natl. Acad. Sci. USA, *81:* 1991–1995, 1984.
25. Ullrich, A., Coussens, J. S., Hayflick, J. S., *et al.* Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature (Lond.), *309:* 806–810, 1984.

26. Derynck, R., Roberts, A. B., Winkler, M. E., *et al.* Human transforming growth factor α: precursor structure and expression in *E. coli*. Cell, *38:* 287–297, 1984.
27. Hall, J. L., Dudley, L., Dobner, P. R., Lewis, S. A., and Cowan, N. J. Identification of two human β-tubulin isotypes. Mol. Cell. Biol., *3:* 854–862, 1983.
28. Maniatis, T., Fritsch, E. F., and Sambrook, J. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1982.
29. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., *98:* 503–517, 1975.
30. Merlino, G. T., Xu, Y., Ishi, S., and Clark, A. J. L. Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. Science (Washington DC), *224:* 417–419, 1984.
31. Ullrich, A., Coussens, L., Hayflick, J. S., *et al.* Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature (Lond.), *309:* 418–425, 1984.
32. Minna, J. D. Genetic events in the pathogenesis of lung cancer. Chest, *96* (Suppl.): 17s–23s, 1989.
33. Derynck, R. Transforming growth factor α. Cell, *54:* 593–595, 1988.
34. Malden, L. T., Novak, U., and Burgess, A. W. Expression of transforming growth factor α messenger RNA in the normal and neoplastic gastro-intestinal tract. Int. J. Cancer, *43:* 380–384, 1989.
35. Beauchamp, R. D., Barnard, J. A., McCutchen, C. M., *et al.* Localization of transforming growth factor α and its receptor in gastric mucosal cells: implications for a regulatory role in acid secretion and mucosal renewal. J. Clin. Invest., *84:* 1017–1023, 1989.
36. Slaughter, D. P., Southwick, H. W., and Smejkal, W. "Field cancerization" in oral stratified squamous epithelium, clinical implications of multicentric origin. Cancer (Phila.), *5:* 963–968, 1953.
37. Strong, M. S., Inceze, J., and Vaughan, C. W. Field cancerization in the aerodigestive tract—its etiology, manifestation, and significance. J. Otolaryngol., *13:* 131–136, 1984.
38. Hong, W. K., Endicott, J., Itri, L. M., *et al.* 13 *cis*-retinoic acid in the treatment of oral leukoplakia. N. Engl. J. Med., *315:* 1501–1505, 1986.
39. Hong, W. K., Lippman, S. M., Itri, L. M., *et al.* Prevention of second primary tumors with isotretinoin in squamous cell carcinoma of the head and neck. N. Engl. J. Med., *323:* 795–801, 1990.
40. Hudson, L. G., Santon, J. B., Glass, C. K., and Gill, G. N. Ligand-activated thyroid hormone and retinoic acid receptors inhibit growth factor receptor promotor expression. Cell, *62:* 1165–1175, 1990.
41. Dmitrovsky, E., Moy, D., Miller, W. H., Jr., Li, A., and Masui, H. Retinoic acid causes a decline in TGF-α expression, cloning efficiency, and tumorigenicity in a human embryonal cancer cell line. Oncogene Res., *5:* 233–239, 1990.
42. Kim, J. S., Steck, P. A., Gallick, G. E., *et al.* Suppression by retinoic acid of epidermal growth factor receptor autophosphorylation and glycosylation in cultured human head and neck squamous carcinoma cells. J. Natl. Cancer Inst. Monogr., *13:* 101–110, 1992.
43. Waldmann, T. A., Goldman, C. K., Bongiovanni, K. F., *et al.* Therapy of patients with human T-cell lymphotropic virus I-induced adult leukemia with anti-tac, a monoclonal antibody to the receptor for interleukin-2. Blood, *72:* 1805–1816, 1988.
44. Masui, H., Moroyama, T., and Mendelson, J. Mechanism of antitumor activity in mice for anti-epidermal growth factor receptor monoclonal antibodies with different isotypes. Cancer Res., *46:* 5592–5598, 1986.
45. Divgi, C. R., Welt, S., Kris, M., *et al.* Phase I and imaging trial of indium III-labeled anti-epidermal growth factor receptor monoclonal antibody 225 in patients with squamous cell lung carcinoma. J. Natl. Cancer Inst., *83:* 97–104, 1991.