

# Differential Expression of the Epidermal Growth Factor Receptor and Its Ligands in Primary Non-Small Cell Lung Cancers and Adjacent Benign Lung<sup>1</sup>

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## ABSTRACT

The epidermal growth factor receptor (EGFR) and one of its ligands, transforming growth factor  $\alpha$  (TGF- $\alpha$ ), are thought to function as a potential autocrine loop in non-small cell lung cancer (NSCLC). However, the expression pattern of EGFR and the TGF- $\alpha$ -related ligands have not been fully characterized in primary NSCLC and adjacent benign lung tissue. For this reason, we comprehensively examined the coexpression and differential expression of EGFR and its ligands, TGF- $\alpha$ , epidermal growth factor (EGF), and amphiregulin (AR), by Northern analysis, in paired samples of primary tumors and uninvolved lung. For those RNA species overexpressed in malignant lung, single cell expression patterns were studied by immunohistochemistry. Specimens were obtained from 57 consecutive patients who underwent resection of carefully staged resectable NSCLC and were followed prospectively. Most (112 of 114) tissue samples yielded high-quality RNA. EGFR was expressed in 82 of 88 (93%) tissue samples, while TGF- $\alpha$  was expressed in 62 of 72 (86%) samples, and AR was expressed in 64 of 70 (92%) samples. EGF was unexpressed in total cellular RNA in both tumor and uninvolved lung. In a comparison of RNA expression patterns in tumors and uninvolved lung, overexpression of EGFR was found in 45% (22 of 44) of tumors, while overexpression of TGF- $\alpha$  was seen in 61% (22 of 36) of tumors, and decreased expression of AR was seen in 63% (22 of 35) of tumors. Cell type and stage did not influence differential expression, indicating that this is a frequent event in primary NSCLC. Simultaneous overexpression of EGFR and TGF- $\alpha$  was seen in only 38% of tumors. Simultaneous overexpression of EGFR and decreased expression of AR were seen in only 21% of tumors. Thus far, the differential expression of EGFR, TGF- $\alpha$ , and AR does not correlate with either disease-free or overall survival. These findings indicate that histologically dissimilar tumors can express similar components of autocrine or paracrine growth factor loops. Differential expression of EGFR and its ligands in tumor specimens compared to uninvolved lung is a common event in NSCLC and may participate in tumor growth without necessarily influencing tumor progression or histology.

## INTRODUCTION

Autocrine loops involving specific growth factors and their receptors are thought to play an important role in the development and progression of lung cancers. One well-characterized autocrine loop is that formed by gastrin-releasing peptide and its receptor in SCLC<sup>3</sup> (1). In NSCLC, the EGFR and one of its ligands, TGF- $\alpha$ , are hypothesized to function in a similar autocrine manner (2). However, this has not been clearly documented in primary tumors for several reasons. Growth factor expression has usually been examined by immunohistochemistry due to a lack of large numbers of primary tumor specimens from which intact RNA could be isolated. Expression of EGFR

at the RNA level has been investigated primarily in cell lines which may not accurately reflect expression patterns *in vivo*. Immunohistochemistry cannot discriminate between autocrine or paracrine growth factor expression and may be inaccurate if the antibody used cross-reacts with related antigens (3). To date, there have been only selected attempts to contrast growth factor expression in primary tumors and in paired samples of uninvolved lung, making it difficult to assess differential expression within tumors. In addition, the coexpression of EGFR and its ligands, TGF- $\alpha$ , EGF, and AR, has not been fully characterized in carefully staged NSCLC. Efforts to correlate the coexpression pattern of EGFR and its ligands with clinical findings have been limited.

This study was undertaken to characterize in detail the expression of EGFR and its ligands in primary NSCLC at the RNA level; to identify differential expression in primary tumors compared to paired benign lung tissue; to extend the analysis of differentially overexpressed RNA species within tumors to the single cell level of immunohistochemical expression; and to correlate these findings with histology, stage, and outcome in a carefully staged group of patients with completely resectable NSCLC who have been followed prospectively.

## MATERIALS AND METHODS

**Tumor Bank.** Tissue specimens were obtained from consecutive patients who were undergoing potentially curative operations for primary NSCLC. Pulmonary resection was accompanied by careful intraoperative staging with complete mediastinal lymph node dissection as described by Martini (4). Lymph nodes were removed and separately labeled for the pathologist according to the American Thoracic Society Lymph Node Map (5).

Within 10 min of the pulmonary resection being completed, a specimen of the primary tumor trimmed of the surrounding lung tissue and of any grossly necrotic material was snap frozen in liquid nitrogen. A separate specimen of uninvolved lung tissue was harvested from an area distant from the primary tumor and frozen in liquid nitrogen. Immediately adjacent specimens of tumor and uninvolved lung were submitted to Pathology as frozen and fixed samples, to correlate gene expression results with immunohistochemical findings. Two pathologists reviewed all of the submitted specimens in order to confirm the histology and tumor stage, determine the amount of viable tumor present, and exclude concurrent lung pathology. The tumor-node-metastasis stage of the primary tumor was determined according to the new International Staging System for NSCLC (6).

**Isolation of Total Cellular RNA.** Stored tissues were then removed from liquid nitrogen, minced with a sterile razor blade, placed in ice-cold guanidine isothiocyanate solution, sonicated before being placed on a cesium chloride cushion, and ultracentrifuged to purify RNA as previously described (7). To prevent RNA degradation, all solutions and instruments which came in contact with the specimens were sterilized and washed with diethylpyrocarbonate-treated H<sub>2</sub>O. Following ultracentrifugation, RNA pellets were resuspended in H<sub>2</sub>O, ethanol precipitated with 0.3 M sodium acetate on dry ice, washed once with dry ice-cold 70% ethanol, lyophilized, and resuspended in H<sub>2</sub>O as a 1  $\mu$ g/ $\mu$ l stock stored at -70°C.

**Nucleic Acid Probes.** Probes were obtained as inserts contained within plasmid vectors. Inserts were freed from vectors as specified by the unique flanking restriction sites for each probe. The fragments were gel purified, electroluted, and further purified through passage over an elutip-D (Schleicher and Schuell) column. The probes included: a 2.4-kilobase *Cla*I-cut human EGFR cDNA (a gift of Glenn Merlino, National Cancer Institute); a 1.35-

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<sup>3</sup> The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor; TGF- $\alpha$ , transforming growth factor  $\alpha$ ; AR, amphiregulin; cDNA, complementary DNA; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mAb, monoclonal antibody; DAB, diaminobenzidine.

kilobase *EcoRI*-cut human TGF- $\alpha$  cDNA (8); a 3.8-kilobase *XhoI*-cut human EGF cDNA (a gift of Dr. Graeme Bell, Howard Hughes Medical Institute Research Laboratories, University of Chicago, Chicago, IL); a *EcoRI*-cut human AR cDNA (a gift of Dr. Greg Plowman, Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA); and a 1.9-kilobase *BamHI*-cut human  $\beta$ -actin cDNA (9). Probes for hybridization were radiolabeled with [<sup>32</sup>P]dCTP using random-priming techniques.

**Northern Analysis.** Ten  $\mu$ g of total cellular RNA from each of the paired samples of primary tumor and normal lung were used for Northern analysis. This was performed by size fractionation on a 1% agarose-formaldehyde gel in a 0.2 M 3-*N*-morpholino-propanesulfonic acid/0.05 M sodium acetate/0.01 M EDTA buffer. Transfer to nitrocellulose filters was performed as previously described, as were hybridizations, washings, and autoradiography (7, 10). The stringency of the washings was optimized for each probe. Autoradiography was accomplished with Kodak XAR film and exposure to an intensifying screen at -70°C. The appropriate exposure time was determined for each probe.

**Immunohistochemistry.** To correlate the RNA findings for those species overexpressed in the NSCLCs with a single cell expression assay, established immunoperoxidase staining methods were utilized to examine the fresh frozen tissue specimens for EGFR and TGF- $\alpha$  expression (11, 12). To ensure that tissue sections adhered, slides were cleaned in 95% alcohol and subbed in 0.3% gelatin solution containing 0.05% chromium potassium sulfate in distilled water. Cryostat-cut sections (4–8  $\mu$ m thick) were taken from the fresh tumor and normal lung tissues that had been stored in liquid nitrogen at -70°C and were placed for 10 min in 1% formalin in PBS (pH 7.4), cold acetone, or 95% ethanol. Endogenous peroxidase activity was quenched by incubation with 0.1% hydrogen peroxide in distilled H<sub>2</sub>O for 10 min. Tissue sections were washed three times in PBS for 5 min each and then incubated with blocking serum (10% normal horse serum in 2% PBS/BSA) for 20 min. All incubations were performed at room temperature in wet chambers. Blocking serum was drained off, and the primary antibody was incubated for 1 h, appropriate dilution having been previously determined. Sections were washed with PBS and incubated for 45 min with secondary horse anti-mouse antibodies (also previously titrated for optimal dilutions), followed by avidin-biotin peroxidase complexes (1:100 in PBS) and DAB solution as chromogen. The DAB solution was filtered and incubated with the tissue sections for 6 to 12 min. After treatment, the sections were washed with distilled H<sub>2</sub>O counterstained with hematoxylin and mounted with permount.

Mouse mAb 528 detecting EGFR (Hybritech, Inc., San Diego, CA) was used at 20  $\mu$ g/ml in PBS containing 2% BSA. A mouse monoclonal antibody to an epitope of TGF- $\alpha$  (mAb 4.4; Oncogene Science, Inc., Manhasset, NY) was utilized at 20  $\mu$ g/ml in PBS/BSA. As a negative control, purified mouse mAb IgG<sub>1</sub> class directed against the cell surface antigen anthranilate synthase of *E. coli* (bcr-25; Oncogene Science, Inc.) was used at the same working dilutions (20  $\mu$ g/ml in PBS/BSA). As a positive control, purified mAb against cytokeratins and other intermediate filaments (Cambridge Research Laboratories, Cambridge, MA) were also used at the same concentrations (20  $\mu$ g/ml). The secondary antibodies used were biotinylated horse anti-mouse IgG (heavy and light chain specific) affinity-purified antibodies (1:100 dilution in PBS) followed by avidin-biotin peroxidase complexes (1:100 dilution in PBS) (Vector Laboratories, Inc., Burlingame, CA). DAB was used as a chromogen (5 mg of DAB tetrahydrochloride in 100 ml of PBS with 100  $\mu$ l of 0.3% hydrogen peroxide).

**Scoring of Assays.** Scoring of the results of hybridizations performed on the paired samples of primary tumor and normal lung was done independently by two individuals, one of whom was aware of the clinical data and one of whom was not. The hybridization to  $\beta$ -actin was used as a control for the integrity and amount of RNA loaded for Northern analysis. Results of the hybridizations performed on each of the primary tumors were compared to the paired samples of normal lung and were recorded as expressed or unexpressed; and equal, increased, or decreased expression in the primary tumor compared to the paired normal lung sample.

Scoring of the immunohistochemical results was performed by two individuals who were unaware of the RNA and clinical data. The immunohistochemical staining was scored as negative if staining was absent and as + or ++, depending on the intensity of the staining when present. The percentage of cells that stained in each sample was estimated.

**Clinical Database.** After pulmonary resection, patients were seen in follow-up by a single surgeon every 3 months for the first 2 postoperative years, then every 6 months thereafter. Follow-up included a history and physical exam, chest X-ray, complete blood count, and chemistry profile. Computerized tomography and radionuclide bone scans were used selectively to investigate new signs or symptoms or to provide more accurate follow-up in patients whose potential area of recurrence could not be adequately evaluated by standard clinical means. Suspected areas of recurrent disease (e.g., adrenal metastases) were confirmed by biopsy whenever technically possible. The clinical and pathologic parameters recorded included patient age and gender, tumor cell type and stage, the estimated percentage of viable tumor in the specimen, and the length of disease-free and overall survival as calculated from the date of the operation. The clinical information, RNA, and immunohistochemical data were entered prospectively into a computerized database.

The proportion of tumors with differential expression of EGFR or its ligands was compared across the levels of prognostic factors using Pearson's  $\chi^2$  test. Overall and disease-free survival were calculated from the date of thoracotomy and described using the product limit method of Kaplan and Meier (13). Overall and disease-free survival were compared across the levels of prognostic factors using the log-rank test (14). All statistical tests were conducted at the two-sided 0.05 level.

**RESULTS**

Tissue specimens were harvested from 57 patients. There were 27 women and 30 men with a mean age of 64 years (range, 40 to 77 years). The stage and histopathology of these tumors are shown in Table 1. Thirty of the tumors were Stage I, 6 were Stage II, 20 were Stage III, and one was Stage IV (solitary brain metastasis resected by craniotomy prior to thoracotomy). There were 31 adenocarcinomas, 19 squamous cell cancers, 3 large cell cancers, and 4 tumors that had a mixture of squamous cell and adenocarcinoma components. Six patients had received cisplatin-based chemotherapy, and one patient both chemotherapy and radiotherapy prior to thoracotomy because of positive mediastinal nodes found at initial mediastinoscopy (N2 disease). One other patient had received preoperative radiotherapy because of a tumor initially thought to be too locally advanced (T4 by virtue of involvement of the main carina) to allow resection. With a median follow-up of 14.8 months for all 57 patients, the median disease-free and overall survival have not yet been reached. The overall 2-year survival (Fig. 1) for the Stage I and II tumors was 85%, while it was 45% for the Stage III and IV tumors ( $P = 0.01$ ). As yet, there is no significant difference in the survival of patients with

Table 1 Stage and histopathology of the 57 tumors harvested

Tumor stage	Tumor cell type			
	Squamous cell cancer	Adeno-carcinoma	Large cell cancer	Mixed Adeno and squamous cancer
Stage I				
T <sub>1</sub> N <sub>0</sub> M <sub>0</sub>	4	4		
T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	8	9	2	3
Stage II (n = 6)				
T <sub>2</sub> N <sub>1</sub> M <sub>0</sub>	1	4		1
Stage IIIA (n = 19)				
T <sub>3</sub> N <sub>0</sub> M <sub>0</sub>	4	4		
T <sub>1</sub> N <sub>2</sub> M <sub>0</sub>		3	1	
T <sub>2</sub> N <sub>2</sub> M <sub>0</sub>	1	4		
T <sub>3</sub> N <sub>2</sub> M <sub>0</sub>	1	1		
Stage IIIB (n = 1)				
T <sub>4</sub> N <sub>0</sub> M <sub>0</sub>		1		
Stage IV (n = 1)				
T <sub>2</sub> N <sub>2</sub> M <sub>1</sub> <sup>a</sup>		1		
Total: 57	19	31	3	4

<sup>a</sup> Simultaneous solitary brain metastasis resected prior to thoracotomy.

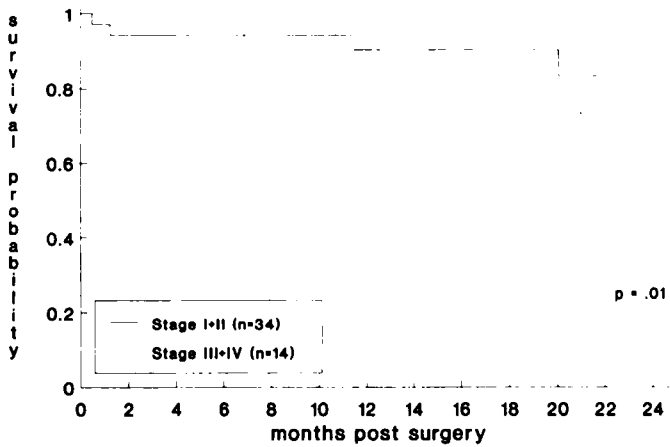


Fig. 1. Overall survival by stage of the 48 patients whose specimens contained at least 50% viable tumor.



Fig. 2. Northern analysis of growth factor expression in total cellular RNA isolated from a primary tumor (T) and its adjacent benign lung (N). The RNA isolated from the tumor specimen from this patient exhibited overexpression of EGFR and TGF- $\alpha$  and decreased expression of AR relative to the RNA isolated from its adjacent benign lung. As a control for the amount and integrity of loaded RNA, hybridization to  $\beta$ -actin was performed. Hybridization of the indicated radiolabeled probes and autoradiography were performed as indicated in "Materials and Methods."

squamous cell cancers compared to those with nonsquamous cell cancers ( $P = 0.91$ ).

Northern analysis revealed that 112 of the 114 tissue samples (57 tumor and 57 paired uninvolved lung samples) yielded high-integrity RNA. On histological review, 9 of the tumor specimens contained less than 50% viable tumor. Even though these samples yielded intact quality RNA, they were omitted from the analysis of growth factor expression because it was felt that the findings might not accurately

reflect growth factor expression within the malignant tumors. Four of these tumors were very small (T1N0) adenocarcinomas with extensive fibrosis. The other 3 tumors contained a large amount of necrotic tissue perhaps due to the preoperative radiation or chemotherapy given to these patients with Stage III disease. The results reported here are based on hybridizations that allowed unequivocal comparison of the expression of EGFR and its ligands in the paired specimens of uninvolved lung to the tumor. Northern analysis revealed that EGFR, TGF- $\alpha$ , and AR RNAs of the expected sizes (15–17) were widely expressed in both tumor and uninvolved lung. Eighty-two of 88 samples (93%) examined were found to express EGFR, 62 of 72 (86%) samples examined expressed TGF- $\alpha$ , and 64 of 70 (92%) samples examined expressed AR. In contrast, none of the 90 samples (45 tumors and 45 paired samples of uninvolved lung) examined were found to express EGF at the level of total cellular RNA (data not shown).

Differential expression in the tumor compared to the paired uninvolved lung was frequent. An example of a tumor that demonstrated overexpression of EGFR and TGF- $\alpha$  and decreased expression of AR relative to the paired sample of uninvolved lung is shown in Fig. 2. Twenty of 44 (45%) tumor samples showed overexpression of EGFR, while 22 of 36 (61%) tumor samples showed overexpression of TGF- $\alpha$ . In contrast, 22 of 35 (63%) tumor samples showed decreased expression of AR, and 4 of 35 (11%) showed increased expression of AR compared to the paired samples of uninvolved lung (Fig. 3). Immunohistochemical staining of individual cells for EGFR and TGF- $\alpha$  demonstrated that overexpression at the RNA level tightly correlated with an increase in protein expression which was localized to the tumor cells and not the stroma (Fig. 4, a–c). However, all tumors that demonstrated increased expression at the RNA level were associated with immunohistochemical staining that was rated as ++ in 100% of cells. Less intense staining, or specific staining in few cells, did not correlate as highly with overexpression at the total cellular RNA level of detection. The intensity of RNA expression and of immunohistochemical staining was less for TGF- $\alpha$  than for EGFR (Figs. 2 and 4), suggesting that this is a less abundantly expressed protein.

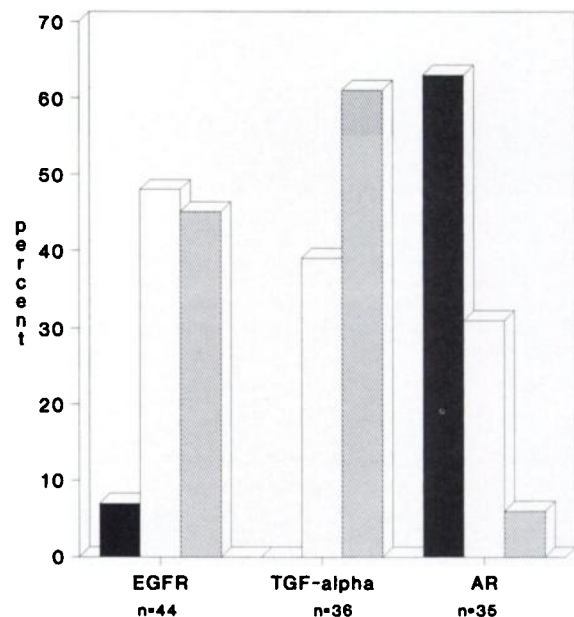


Fig. 3. Proportion of tumors that demonstrated differential expression of EGFR, TGF- $\alpha$ , and AR in the primary tumor compared to the paired sample of uninvolved lung. ■, decreased; □, equal; ▨, increased.

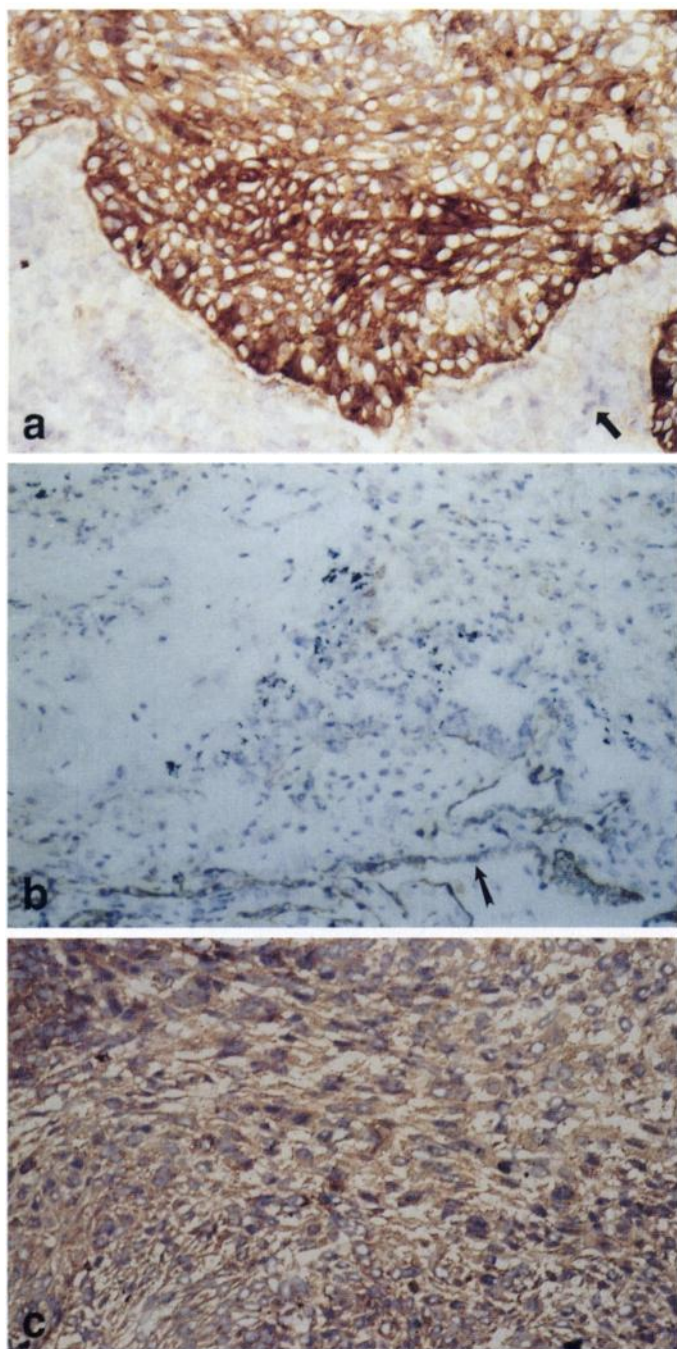


Fig. 4. Representative example of immunohistochemical staining of a primary tumor specimen that showed overexpression of EGFR and TGF- $\alpha$  relative to adjacent uninvolved lung. The tumor shows intense staining for EGFR, while nonepithelial cells surrounding the tumor (arrow) do not stain for EGFR (a). A sample of benign lung tissue from the same patient shows staining for EGFR in the bronchial epithelium (arrow) but not in the remainder of the lung tissue (b). Tumor from the same patient also showed diffuse intense staining for TGF- $\alpha$  (c).

As illustrated in Fig. 5, differential expression of EGFR, TGF- $\alpha$ , and AR in tumor compared to paired uninvolved lung was seen in all tumor stages. As shown in Fig. 6, cell type also did not influence differential expression. Nine of 25 nonsquamous tumors (36%) and 11 of 19 squamous cell cancers (58%) overexpressed EGFR ( $P = .25$ ). Fifteen of 22 nonsquamous tumors (68%) and 7 of 14 (50%) squamous cell cancers overexpressed TGF- $\alpha$  ( $P = 0.46$ ). Fifteen of 23 nonsquamous tumors (65%) and 7 of 12 (58%) squamous cell tumors showed decreased expression of AR ( $P = 0.97$ ).

Far fewer tumors demonstrated simultaneous overexpression of EGFR and TGF- $\alpha$  than overexpression of either of these alone. Only 13 of 34 tumors (38%) showed overexpression of both EGFR and TGF- $\alpha$ . Likewise, only 7 of 34 (21%) tumors showed simultaneous overexpression of EGFR and decreased expression of AR. Thus far, differential expression of EGFR, TGF- $\alpha$ , or of AR in tumor versus uninvolved lung has not been associated with a significant difference in either disease-free or overall survival. However, the survival curves suggest a trend toward worse overall survival for the patients whose tumors had overexpression of either EGFR or TGF- $\alpha$  (Figs. 7-9). The number of patients whose tumors have simultaneous differential expression of EGFR and TGF- $\alpha$  and of EGFR and AR is small, but they also do not have a significantly worse disease-free or overall survival. The number of tumors that had simultaneous overexpression of EGFR

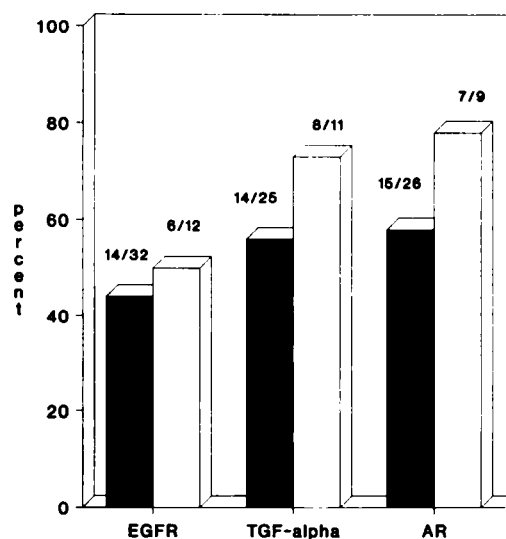


Fig. 5. Differential expression of EGFR, TGF- $\alpha$ , and AR by tumor stage. The y-axis shows the percentage of tumors with expression different from that of normal lung. Differential expression is seen in all stages, and there is no significant difference in Stages I and II, compared to III and IV. ■, Stages I and II; □, Stages III and IV.

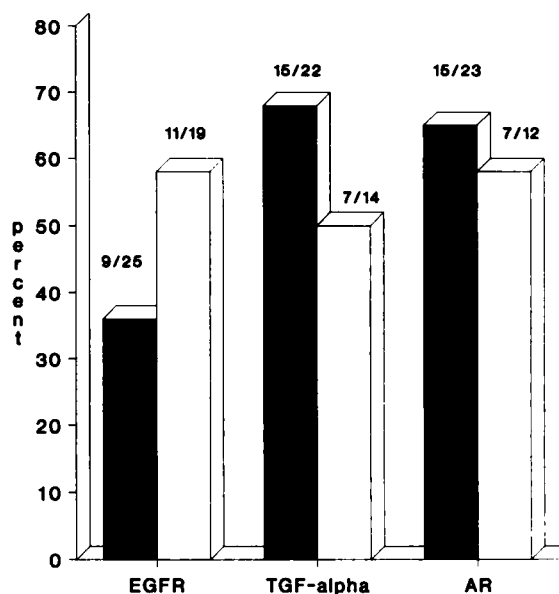


Fig. 6. Differential expression of EGFR, TGF- $\alpha$ , and AR by cell type. The y-axis shows the percentage of tumors with expression different from that of normal lung. There is no significant difference between squamous cell and non-squamous cell tumors. ■, non-small cell cancer; □, small cell cancer.

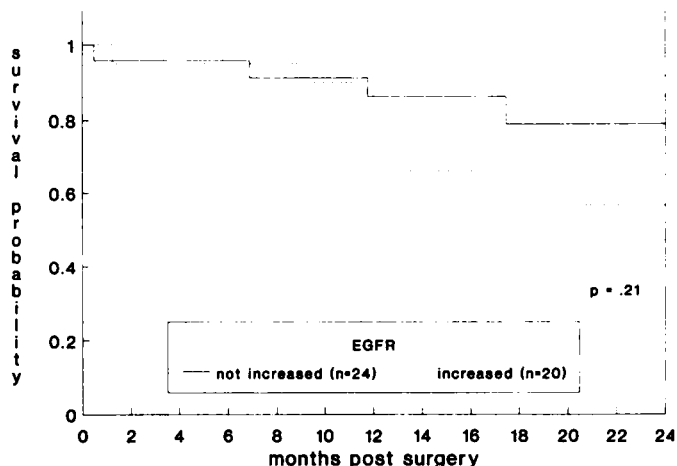


Fig. 7. Overall survival of patients who had overexpression of EGFR in the primary tumor compared to those who did not.

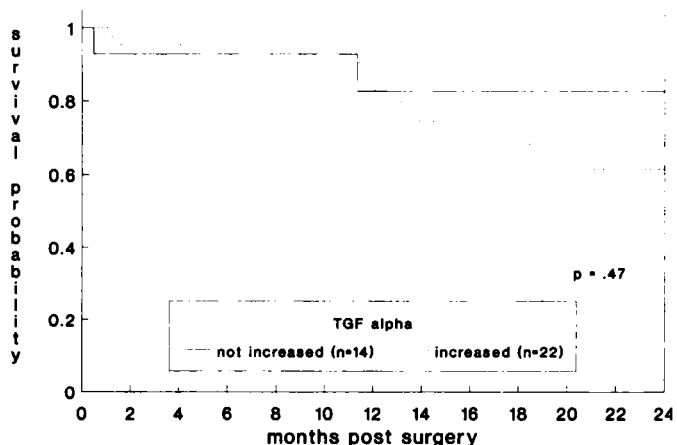


Fig. 8. Overall survival of patients who had overexpression of TGF- $\alpha$  in the primary tumor compared to those who did not.

and TGF- $\alpha$  and decreased expression of AR was too small to allow a statistically valid correlation with survival.

**DISCUSSION**

The identification of steps involved in lung carcinogenesis is a subject under intensive study. Activation of oncogenes and inactivation of tumor suppressor genes have been linked to lung tumorigenesis (18). Growth factors and their receptors are also hypothesized to play an important role in lung tumor formation. Examples include gastrin releasing peptide and its receptor, which act as an autocrine growth loop in SCLC cell lines (1). NSCLC cell lines are known to express high levels of EGFR (3), and it has been hypothesized that EGFR and TGF- $\alpha$  function in a manner analogous to that of an autocrine loop in NSCLC.

To date, studies have largely focused on the role of EGFR in lung tumorigenesis. Immunohistochemical studies on primary tumors have confirmed the finding in cell lines that expression of EGFR is a frequent event in NSCLC and is rare in SCLC (19). Additional immunohistochemical studies on primary tumors have suggested that EGFR expression is higher in tumors than in uninvolved lung, is more common in squamous cell cancers than in adenocarcinomas, and may be related to tumor stage or differentiation state (20–23). Other studies have found no clear relationship between EGFR expression, cell type, or tumor cell differentiation but have confirmed the differential expression of EGFR in tumors and uninvolved lung (24–26). An in-

crease in EGFR binding has been reported and is thought to be due to an increase in the number of receptors, rather than to a change in receptor affinity (27, 28). DNA studies on primary NSCLC suggest that amplification and rearrangements of the EGFR gene are infrequent (29–32). Augmented EGFR RNA and protein levels are therefore unlikely to be secondary to gene amplification (32). Attempts to correlate EGFR overexpression by immunohistochemistry with patient survival have led to conflicting results, with some studies reporting no impact on survival (26), some a worse survival (33), and others an improved survival (3). The present study simultaneously analyzed RNA and immunohistochemical growth factor expression and correlated these findings with clinical outcome.

The expression pattern of TGF- $\alpha$  in NSCLC has not been as extensively explored. A study of human lung cancer cell lines found TGF- $\alpha$  mRNA expression in one squamous cell line, 2 adenocarcinoma cell lines, and one of 3 large cell lines (15). Another study of RNA expression levels in various solid tumors and normal tissues found high levels of expression in one squamous cell and one large cell cancer of the lung, and no expression in 3 lung adenocarcinomas and 2 normal lungs (16). A subsequent report found TGF- $\alpha$  expression, as assessed by radioimmunoassay and by mRNA, was present in normal lung as well as primary lung tumors (34). There did not appear to be any correlation between TGF- $\alpha$  expression and cell type or tumor stage. In contrast, an immunohistochemical study of TGF- $\alpha$  expression in 138 lung adenocarcinomas suggested that the intensity of staining correlated with 5-year survival (35).

AR is a recently identified ligand for EGFR and has been studied primarily in breast cancer cell lines. AR is thought to be a bifunctional growth factor, interacting with EGFR to promote the growth of normal epithelial cells but inhibiting the growth of carcinoma cell lines (17, 36). To our knowledge, AR expression has not yet been compared in lung cancers and paired uninvolved lung tissues. The observed overexpression of AR in benign compared to malignant lung might exert a growth-inhibiting effect on these malignant tissues. In contrast, total cellular RNA expression of the related ligand EGF was not observed in either benign or malignant lung tissues.

The potential clinical importance of the differential expression of EGFR and its ligands in NSCLC compared to uninvolved lung has been previously recognized. Radiolabeled monoclonal antibodies to EGFR have already been used to image NSCLC in preliminary clinical trials (37). Immunotoxins based on murine monoclonal antibodies to EGFR have been developed and are known to be cytotoxic to cell lines that overexpress EGFR (38–41). Clinical trials utilizing this

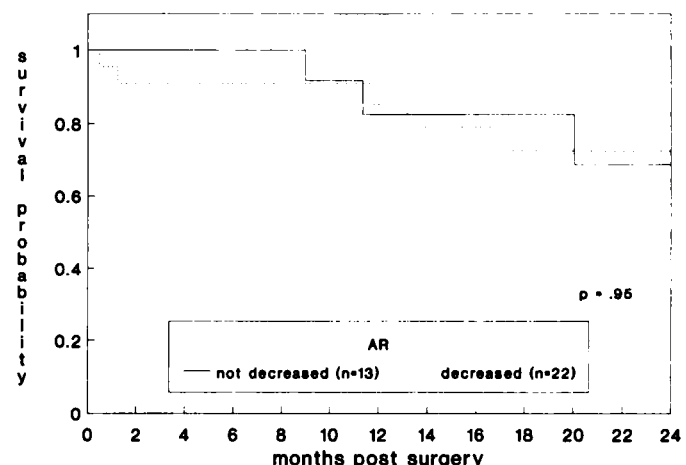


Fig. 9. Overall survival of patients who had decreased expression of AR in the primary tumor, compared to those who did not. Thus far, there is no significant difference in survival between groups compared in Figs. 7, 8, or 9.

growth factor receptor or its ligands as targets for therapy are planned. However, the applications of such therapeutic strategies would be aided by an improved understanding of the expression pattern of EGFR and its ligands in lung cancer compared to normal lung. The reported relationship of EGFR and its ligands to histology, tumor stage, and survival has been inconsistent. Most studies have utilized immunohistochemistry and suffer from the inherently qualitative nature of this approach. Comparison of expression pattern within tumors and uninvolved lung has not been extensively studied. RNA studies have been performed primarily on cell lines, perhaps attesting to the prior difficulty encountered in developing tumor banks containing large numbers of fresh tumor and lung specimens that yield intact RNA of high integrity.

This study provides a comprehensive analysis of EGFR and its ligands in a large, well-staged cohort of patients with resectable NSCLC. The correlation of the analysis at the RNA level with a single cell assay by immunohistochemistry within the same clinical specimens is an unusual feature of this study. This study demonstrates that intact RNA can be reliably obtained from both primary lung cancers and paired uninvolved lung, if the samples are harvested and snap frozen in liquid nitrogen in the operating room immediately after the lung tissue is removed. This approach permits the precise isolation of tissue useful for the identification of molecular genetic abnormalities in primary NSCLC.

Our findings confirm that expression of EGFR and TGF- $\alpha$  is frequent in both primary NSCLC and uninvolved lung. Expression of EGF was not detectable at the level of total cellular RNA, but expression of AR was widespread in tumor and in uninvolved lung. Of note is the observation that only uniform and intense levels of immunohistochemical staining in the primary tumor compared to the uninvolved lung correlated with overexpression at the RNA level. This finding underscores the importance of examining the expression pattern in tissues at both the RNA and immunohistochemical levels.

Differential expression of EGFR, TGF- $\alpha$ , and AR is also common and is seen in approximately one-half of all NSCLC. Both expression and differential expression are seen in all cell types. The decreased expression of AR in many primary tumors compared to uninvolved lung is consistent with the hypothesis that this protein product exerts an inhibitory growth effect on tumors. Hence, decreased expression of AR in lung tumors could promote their growth. Simultaneous differential expression of EGFR and either of its ligands, TGF- $\alpha$  or AR, occurs in a small subset of tumors that had no distinguishing clinical or histological features. The fact that differential expression of individual growth factors occurs more frequently than differential coexpression suggests that expression of EGFR and its ligands may be responsive to independent regulatory mechanisms.

Both expression and differential expression of EGFR, TGF- $\alpha$ , and AR are seen in NSCLC of all stages as compared to adjacent benign lung tissues. It is not yet known whether overexpression of these protein products participates in tumor initiation via the transformation of preneoplastic lesions. However, since overexpression occurs in all stages of overt lung cancer, it may represent a common event. Overexpression of EGFR and its ligands does not yet appear to correlate with either disease-free or overall survival in this study. Although longer follow-up is needed to confirm this initial clinical correlation, it is conceivable that these growth factors act to promote local tumor growth without having an impact on tumor progression or metastasis.

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