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Activated Akt Expression has Significant Correlation with EGFR and TGF-α Expressions in Stage I NSCLC

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Abstract. *Background: In vitro studies have indicated that epidermal growth factor receptor (EGFR) may intensify signaling output by the receptor's overexpression, heterodimerization with HER-2, or autocrine expression of ligands. The purpose of the present study was to evaluate the correlation between EGFR and its related proteins and to explore the prognostic value of the proteins. Materials and Methods: Immunohistochemical staining of transforming growth factor alpha (TGF-α), EGFR, HER-2, and phosphorylated (p-)Akt was performed in specimens surgically excised from 91 consecutive patients with p-stage I non-small cell lung cancer (NSCLC). Expression or coexpression of TGF-α and the receptors were related to expression of p-Akt. The prognostic impact of these peptides was also tested. Results: TGF-α, EGFR and HER-2 overexpressions were detected in 32%, 79% and 13% of tumors, respectively. Coexpressions of TGF-α & EGFR and EGFR & HER-2 were observed in 29% and 11% of tumors, respectively. P-Akt expression was found in 73% of tumors. Significant correlations between EGFR, TGF-α or coexpression of TGF-α & EGFR and p-Akt expression were found (p=0.006, 0.008 and 0.010, respectively). No proteins examined had an impact on relapse-free survival. Conclusion: The Akt pathway is frequently involved in NSCLC and overexpression of EGFR and autocrine expression of TGF-α may increase the potency of Akt activation.*

The members of the epidermal growth factor receptor (EGFR) family, including c-erbB-1 (EGFR), c-erbB-2 (also known as HER-2/neu), c-erbB-3 and c-erbB-4 are transmembrane glycoproteins with an intracellular domain possessing intrinsic tyrosine-kinase activity (1). EGFR is the family member most widely studied in relation to non-small cell lung cancer (NSCLC). Once EGFR is bound by its ligands, which include transforming growth factor alpha (TGF-α), it is autophosphorylated by forming a homodimer or heterodimer with another receptor family member and triggers downstream signal transduction cascades (1). Of the downstream proteins, Akt is one of the best understood pathways triggered by growth factor receptors including EGFR and HER-2. Akt pathways are generally thought to result in more aggressive tumor phenotypes such as decreased apoptosis or cell growth (2). Several *in vitro* studies have demonstrated that receptor overexpression, autocrine expression of receptor ligands, heterodimerization with HER-2 and EGFR mutations could function as mechanisms of increased signaling output from EGFR (3).

Overexpression of EGFR has been reported to be present in 51–88% of NSCLC tumors (4-8). Several studies have reported that EGFR expression correlates with reduced survival (9), lymph node metastasis (8) and poor chemosensitivity in NSCLC (4), although other studies have yielded opposite findings (5-6). There are also reports demonstrating relationships between HER-2 overexpression and poor survival or chemoresistance in lung cancer (10). Furthermore, some reports have indicated that EGFR and HER-2 coexpression may correlate with poor prognosis in lung cancer (7, 11). An immunohistochemical study using 131 primary lung adenocarcinomas indicated that coexistence of EGFR and TGF-α appears to correlate with shorter survival (12), but this finding was not confirmed in a subsequent RNA study of 36 specimens that included those of other NSCLC histologic types (13)

Recently, positive correlations between levels of TGF-α, EGFR, or HER-2 and phosphorylated (p-)ERK in head and neck tumor (14), TGF-α or EGFR and p-ERK in gastric tumor (15) and HER-2 and p-Akt in breast tumor have been demonstrated (16). We also found frequent participation of p-ERK, p-Akt and p-STAT3 in NSCLC

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tumors (28%, 53% and 58% of 60 specimens, respectively) and positive correlations of level of EGFR expression with p-ERK and p-Akt expressions but not with that of STAT3 (17). These findings appeared to suggest that receptor overexpression or autocrine ligand expression play a role in increased downstream signaling in tumors, as suggested by *in vitro* studies. However, while there is plenty of data regarding EGFR in NSCLC tumors, it still remains to be clarified how coexpression of TGF-α as its ligand or HER-2 as its heterodimer cognate influences expression of downstream activated protein. Additionally, the prognostic value of downstream activated proteins has not yet been evaluated in NSCLC. These issues are of great interest, considering that anti-EGFR drugs have already begun to be applied to the treatment of NSCLC.

Table I. *Clinicopathological characters.*

Total number of patients	91
Gender	
Male	56
Female	35
Histology	
Squamous cell carcinoma	33
Adenocarcinoma	50
Bronchioloalveolar carcinoma	6
Large cell carcinoma	2
Tumor differentiation (BAC and La. excluded)	
Poorly	25
Moderately	24
Well	34
Pathological T status	
T1	46
T2	45

BAC, bronchioloalveolar carcinoma; La.; Large cell carcinoma

Patients and Methods

Patients and tumor tissues. Ninety-one consecutive patients with pathologically stage I NSCLC who had undergone curative resection between December 16, 1992 and December 27, 1999 were studied. Patients who underwent either neoadjuvant chemotherapy or radiation therapy before surgery were excluded. Tumor specimens excised at surgery were formalin-fixed and paraffin-embedded for histological and immunohistochemical processing. Tumors were classified using the World Health Organization (WHO) histological classification (18) and Union Internationale Contre le Cancer (UICC) criteria (19).

Immunohistochemistry. For immunohistochemical staining of all proteins except HER-2, the usual avidin biotin complex (ABC) method was employed. Briefly, after formalin-fixed, paraffin-embedded tissue sections (3 μm) were deparaffinized, the slides were washed with PBS. For staining of TGF-α and p-Akt, the sections were microwaved at 400 W for 25 min in a citrate buffer solution (pH 6.0) for antigen unmasking. The slides were treated for 30 min at room temperature with methanol containing 0.1% hydrogen peroxide. The sections were then blocked for 30 min with either 10% goat or 10% horse serum, washed with PBS and incubated overnight with the appropriate primary antibody at 4 ˚C. The Vectastain ABC kit (1:200 dilutions, Vector Laboratories, Burlingame, CA, USA) was used to detect the resulting immune complexes following the manufacturer's instruction. The peroxidase activity was visualized by incubating the sections for 1-2 min in 0.05% diaminobenzidine. The slides were then counterstained with hematoxylin and mounted. The primary antibodies used included mouse monoclonal antibody recognizing TGF-α (Oncogene Research Product, Boston, MA, USA) at a dilution of 1:500, 31G7 recognizing EGFR (Zymed Laboratory Institute, South San Francisco, CA, USA) at a dilution of 1:100 and phospho-Akt [Ser473] antibody (IHC specific, Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:50.

For HER-2 staining, the method described by Herceptest (DAKO, Carpinteria, CA, USA) was used as follows. After deparaffinization, the sections were placed in a citrate buffer and heated in a water bath to 95˚C for 40 min. The sections were cooled in the solution for 20 min at room temperature and rinsed in distilled water. The slides were then treated for 10 min at room temperature with 3% hydrogen peroxide and transferred to a bath of TBS. Subsequently, they were incubated for 30 min with HER2/neu rabbit polyclonal antibody (DAKO) at a dilution of 1:100 including 1% bovine serum albumin at room temperature. Sections were washed three times with TBS and treated with a secondary antibody (ENVISION+, DAKO) for 30 min. Following several washes with TBS, they were incubated for 10 min in diaminobenzidine substrate including hydrogen peroxide (3,3'-Diaminobenzidine Tetrahydrochloride Liquid System, DAKO). The slides were then rinsed in distilled water, counterstained with hematoxylin and mounted.

For positive controls for each of the antibodies, sections previously validated by us to be strongly positive were used. Negative controls were performed for each of the antibodies using nonimmune serum instead of the primary antibodies to exclude nonspecific staining.

Evaluation of immunoperoxidase staining. A pathologist without any clinical information regarding the specimens performed the scoring. For EGFR and TGF-α, microscopic examination of the cell membrane for EGFR and cytoplasm for TGF-α reaction product were performed and scored as follows: negative= <5% of cells stained; += 5-29% of cells stained; 2+= 30-69% of cells stained; 3+= 70-100% of cells stained. For HER-2, evaluation standardized by DAKO was performed as follows. No staining was negative (-). 1+, 2+ and 3+ indicated faint-partial, weak to moderate-complete and moderate to strong-complete membrane staining in more than 10% of the tumor cells, respectively. For EGFR and HER-2, positive staining of 2+ or greater was evaluated as overexpression. When both TGF-α and EGFR or EGFR and HER-2 were expressed at 2+ or greater, they were treated as coexpression. P-Akt was evaluated subjectively as follows: negative= <5% of cells staining; += diffuse but weak nuclear staining of cells with more than 5% of cells staining; 2+= diffuse and strong nuclear staining of cells with more than 5% of cells staining.

Statistical analysis. Statistical analysis was performed with StatView 5.0 software. Levels of TGF-α, EGFR and HER-2 expression (- ~ 3+), p-Akt expression (- ~ 2+) and tumor differentiation (poorly

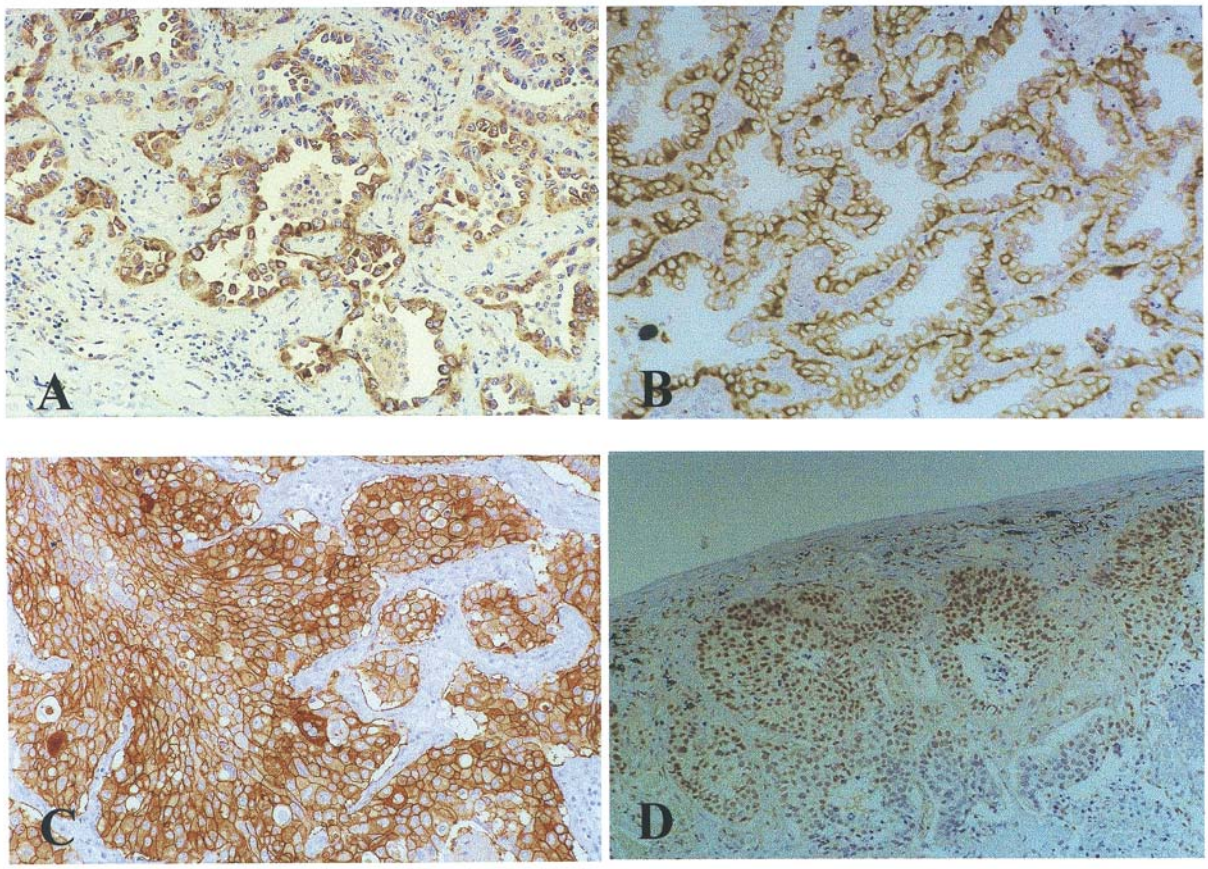

Figure 1. *Characteristic patterns of immunohistochemical staining for TGF-α, EGFR, HER-2 and p-Akt. An adenocarcinoma exhibiting 3+ staining for TGF-α (A) demonstrates diffuse cytoplasmic staining. An adenocarcinoma exhibiting 3+ staining for EGFR (B) and a squamous cell carcinoma exhibiting 3+ staining for HER-2 (C) demonstrate strong cytoplasmic membrane staining. A squamous cell carcinoma exhibiting 2+ staining for p-Akt (D) demonstrates diffuse and strong nuclear staining and slight cytoplasmic staining.*

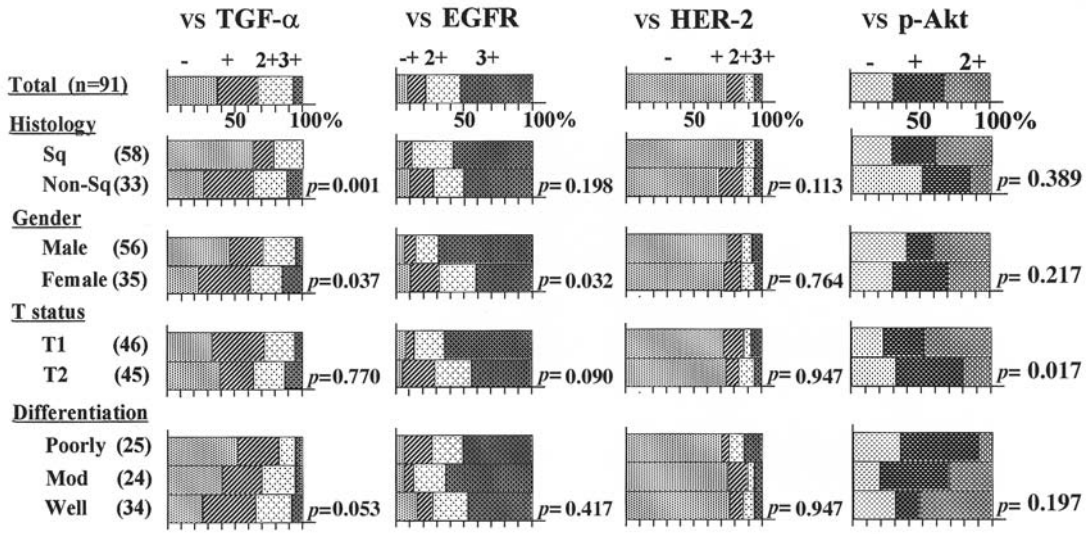

Figure 2. *Relationships between TGF-α, EGFR, HER-2 or p-Akt expression and clinicopathological factors. The level of TGF-α expression appeared to correlate with non-squamous tumor and female sex (Mann-Whitney U-test). The level of EGFR appeared to correlate with male sex (Mann-Whitney U-test). Staining for p-Akt appeared to correlate with T1 tumor (Mann-Whitney U-test). The abbreviations are: Sq, squamous cell carcinoma; Mod, moderately-differentiated.*

Table II. *Relationships between TGF-α, EGFR, or HER-2 and p-Akt.*

		TGF-α				EGFR				HER-2			
	(N)	-	+	2+	3+	-	+	2+	3+	-	+	2+	3+
p-Akt													
-	(24)	12	8	3	1	3	5	7	9	18	1	4	1
+	(37)	15	12	8	2	2	6	12	17	26	5	3	3
2+	(30)	7	8	12	3	2	1	5	22	23	6	0	1
				* p=0.008				* p=0.006				* p=0.596	

* Spearman rank correlation coefficient

~ well) were converted to integral values of 0-3, 0-2 and 1-3, respectively and treated as continuous variables. Bronchiolo-alveolar carcinomas and large cell carcinomas were excluded from the analysis related to tumor differentiation. The Mann-Whitney *U*-test or Spearman rank correlation coefficient determination was employed to relate pairs of these variables. Relapse-free survival (without censoring for death) was calculated from the date of surgery using the Kaplan-Meier method (20); differences between curves were assessed using the log-rank test (21). Statistical significance was set at $p<0.05$.

Results

Tissue specimens were collected from 91 consecutive patients with NSCLC who underwent curative resection in our hospital between December 16, 1992 and December 27, 1999. Clinicopathological characteristics are reviewed in Table I. The characteristic patterns of immunohistochemical staining for TGF-α, EGFR, HER-2 and p-Akt are demonstrated in Figure 1. P-Akt was found mainly in the margins of tumors, which appeared to be invading normal tissue.

The relationships between TGF-α, EGFR, HER-2, or p-Akt expression and clinicopathological factors are demonstrated in Figure 2. Twenty-eight (31%), 23 (25%) and 6 (7%) specimens exhibited 1+, 2+ and 3+ TGF-α staining, respectively. TGF-α expression appeared to correlate with non-squamous tumor and female sex (p=0.001 and 0.037, respectively). However, since a significantly greater number of patients with non-squamous tumor were included in the female population than in the male (data not shown), it could not be determined which variable or whether both variables were significantly related to TGF-α expression. Twelve (13%), 24 (26%) and 48 (53%) specimens exhibited 1+, 2+ and 3+ EGFR staining, respectively. Although EGFR appeared to be correlated with male sex (p=0.032), this relationship might have been due in part to the fact that more male patients had squamous tumors, as described above, in addition to the finding that squamous cell carcinoma tended to exhibit greater EGFR expression. Twelve (13%) and 7 (8%) and 5 (5%) specimens exhibited +, 2+ and 3+ HER-2 staining, respectively. No significant relationship was found between HER-2 and any of the clinicopathological factors examined. For p-Akt, 37 (36%) and 30 (33%) specimens exhibited + and 2+ staining, respectively and the level of p-Akt expression appeared to correlate with T1 tumor (p=0.017).

The relationships between the level of expression of TGF-α, EGFR, or HER-2 and p-Akt are shown in Table II. While levels of EGFR and TGF-α expression appeared to be associated with p-Akt expression (p=0.006 and 0.008, respectively), this relationship was not found between HER-2 and p-Akt.

Coexpression of TGF-α & EGFR and EGFR & HER-2 was observed in 29% and 11% of tumors, respectively. As exhibited in Table III, coexpression of TGF-α & EGFR, while representing autocrine secretion of the ligand, appeared to be associated with expression of p-Akt (p=0.010). On the other hand, coexpression of EGFR & HER-2 did not appear to correlate with p-Akt expression.

For determination of relapse-free survival, the median follow-up period was 8.1 years (range: 4.1-12.2). At the time of analysis, of 91 patients, 32 had died and 19 patients had relapsed. No subgroup related to peptides reached median relapse-free survival time. No peptides examined had exhibited significant impact on relapse-free survival (TGF-α ≤1+ *versus* [*vs.*] ≥2+, p=0.391; EGFR ≤1+ *vs.* ≥2+, p=0.500; HER-2 ≤1+ *vs.* ≥2+, p=0.439; p-Akt negative *vs.* ≥1+, p=0.809).

Discussion

In the present study, 73% of specimens exhibited staining for p-Akt. While some reports have indicated overexpression of EGFR, HER-2 and TGF-α in NSCLC, in 51-88% (4-8), 3.4-60% (5, 7, 10, 22) and 33-61% of patients, respectively (12, 13), there have been few reports on p-Akt in tumors. Perez-Tenorio's group found that p-Akt was exhibited in 54% of 93 breast cancer specimens (23). The present study also indicated the frequent participation of Akt pathway in NSCLC.

In the analyses relating each peptide to clinicopathological factors, p-Akt expression appeared to be correlated with T1

Table III. *Relationships between coexpressions of EGFR & HER-2 or TGF-α & EGFR and p-Akt.*

		Co-exp. of EGFR & HER-2		Co-exp. of TGF-α & EGFR	
	(N)	No (81)	Yes (10)	No (65)	Yes (26)
p-Akt					
-	(24)	20	4	20	4
+	(37)	32	5	29	8
3+	(30)	29	1	16	14
			* p=0.109		* p=0.010

Co-exp., coexpression; * Mann-Whitney *U*-test

tumor. Roy's group found immunohistochemically that Akt overexpression occurs frequently during human colon carcinogenesis but is less common in colon cancers with microsatellite instability (24). Thus, it may be worth determining how p-Akt participates in dysplasia or carcinoma *in situ*, in examining possible roles of Akt in early steps of tumorigenesis.

We selected p-Akt as a marker for downstream activation because, in a previous study, we found the most promising correlation between the level of EGFR and p-Akt (17). Consistent with the previous study, we reproduced a significant correlation between EGFR and p-Akt levels. Furthermore, in the present study, we found a positive correlation between TGF-α and p-Akt and coexpression of TGF-α & EGFR and p-Akt. EGFR overexpression and coexpression of receptor ligands are considered mechanisms of increased receptor signaling output from the receptor (3). Our findings suggest that this may be true in tumors at least between EGFR and Akt. Akt is reported to mediate many biological effects such as up-regulation of cyclin D1, activation of S6 kinase and down-regulation of p27 promoting cell cycle progression and inhibition of Bad, caspase-9 and Forkhead resulting in anti-apoptosis (25-27). Thus, the potential connection between the level of EGFR expression and p-Akt expression may be an important finding from the aspect of understanding the roles of EGFR and TGF-α overexpression in NSCLC tumors. Albanell and colleagues demonstrated immunohistochemically that ZD1839, a selective tyrosine kinase inhibitor of EGFR, decreases p-ERK in interafollicular epidermis and hair follicles of patients treated with this drug (28). It is of great interest to see if the phosphorylated proteins, p-ERK and p-Akt, can be used as biological markers in actual tumor.

Focusing on the roles of coexpression of HER-2 with EGFR, HER-2 is thought to potentiate EGFR function by increasing EGF binding affinity, stabilizing and recycling EGF-HER-2 heterodimers and expanding the repertoire of receptor-associated substrates and signaling responses (3). In fact, positive relationships between the level of p-ERK and those of EGFR and HER-2 were demonstrated in head and neck cancer tumors (14). Furthermore, Brabender's group demonstrated an additive effect of EGFR and HER-2 coexpression in worsening of stage I-III NSCLC patient survival compared with high expression of only one of the other receptors (11). In the present study, however, we did not find significant relationships of coexpression of EGFR and HER-2 with either downstream activated proteins or patient outcome. This failure might be due to the patient selection being limited to p-stage I, potentially causing a very low rate of HER-2 overexpression. In fact, some groups indicated that HER-2 overexpression is more common in higher-stage NSCLC and two different studies including only early-stage NSCLC failed to find any significant prognostic value for HER-2 overexpression (22, 29-31). Thus, a study using specimens collected from higher-stage tumors may be needed and is ongoing in our laboratory.

There have been several reports suggesting the deleterious effects of EGFR, HER-2 and TGF-α on patient survival in NSCLC (3-5, 9). However, we did not find an impact on patient outcome due to expression of p-Akt, an activated downstream protein. This result was unexpected, because there are increasing numbers of reports that predict tumor character or patient prognosis based on these downstream proteins (32-34). Although a definitive conclusion could not be drawn from our present study, the absence of prognostic significance in p-Akt might reflect the possible contribution of various intra-cellular signaling pathways to advancement of NSCLC.

It should be recalled here that EGFR and HER-2 are only two of the many types of growth factor receptors sharing the downstream pathways. Inter-growth factor receptor crosstalk, such as that between EGFR and insulin-like growth factor receptor (IGFR) or platelet-derived growth factor receptor (PDGFR), has also been suggested by results of studies of cell lines (35). Furthermore, many factors other than growth factor receptors, such as hormonal-receptors, cytokine receptors, or non-receptor tyrosine kinases, can trigger the common downstream cascade. Additionally, immunohistochemistry is not strictly quantitative and is prone to inter-observer scoring error.

Our findings thus need to be supported by other objective and quantitative approaches.

In summary, the results of the present study suggested frequent participation of the Akt pathway in NSCLC and showed that expression of EGFR or TGF-α and coexpression of TGF-α and EGFR are correlated with p-Akt expression. The latter findings suggest that EGFR overexpression and autocrine secretion of TGF-α may increase the potency of Akt activation in NSCLC tumors. We could not demonstrate the usefulness of these peptides as prognostic factors.

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