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Lung Carcinoid Cell Lines Have Bombesin-Like Peptides and EGF Receptors

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The biochemical properties of lung cancer cell lines were investigated. Bombesin-like peptides were present in three small cell lung cancer (SCLC) cell lines examined and three of four lung carcinoids but not in five non-small cell lung cancer (NSCLC) cell lines. Therefore SCLC and some lung carcinoids, but not NSCLC, are enriched in neuroendocrine properties. In contrast, ^{125}I-EGF bound with high affinity to all five NSCLC cell lines and three of four lung carcinoids but not to the three SCLC cell lines examined. For lung carcinoid cell line NCI-H727, ^{125}I-EGF bound with high affinity (Kd = 6 nM) to a single class of sites (Bmax = 110,000/cell). The ^{125}I-EGF bound was rapidly internalized at 37°C but not 4°C. Using Western blot techniques and antiphosphotyrosine antibodies, EGF induced phosphorylation of a major 170 Kd protein. Using immunoprecipitation techniques and anti-EGF receptor antibodies a major 170 Kd protein was labeled. These data indicate that biologically active EGF receptors are present on NSCLC and lung carcinoid cell lines.

Key words: lung cancer, lung carcinoids, EGF receptors, BN-like peptides

The main types of lung cancer are small cell lung cancer (SCLC) and non-SCLC (NSCLC). SCLC is a neuroendocrine tumor that utilizes bombesin(BN)-like peptides as autocrine growth factors [1]. BN-like peptides are synthesized in [2] and secreted from SCLC cell lines [3]. The peptides bind to cell surface receptors [4] and stimulate phosphatidyl inositol turnover [5], elevate cytosolic Ca^{2+} levels [6], and stimulate clonal growth [7]. This autocrine growth cycle is disrupted by a monoclonal antibody, which binds to and neutralizes BN-like peptides [1], and by BN receptor antagonists [8].

In contrast, NSCLC may use TGFα as an autocrine growth factor [9,10]. Immunoreactive TGFα has been detected in some NSCLC cell lines and the clonal growth of NSCLC is inhibited by a monoclonal antibody against TGFα [10]. Also,

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approximately 100,000 high affinity EGF receptors per cell have been detected in all NSCLC cell lines examined [11,12]. The EGF receptors may mediate the growth of NSCLC, and addition of anti-EGF receptor antibodies inhibit the growth of some NSCLC tumors [13].

Recently, lung carcinoid cell lines were established [14]. Here, the biochemical properties of the lung carcinoid cell lines were investigated and compared to those of SCLC and NSCLC cells.

METHODS

Human lung cancer cell lines were cultured in serum supplemented medium (RPMI-1640 containing 10% heat-inactivated fetal bovine serum). The SCLC cell lines grew as floating aggregates whereas the NSCLC cell lines were adherent [15]. The lung carcinoid cell lines were nonadherent with the exception of NCI-H727 [14]. Routinely, the cells had greater than 90% viability and were mycoplasma free. The cells were used when they were in exponential growth phase.

The cell lines were assayed for BN-like peptides. Cells were washed three times in PBS, then extracted in boiling 2 N acetic acid. Lyophilized extracts were quantitated for BN-like peptides by radioimmunoassay as described previously [9]. Protein was determined using the Lowry method [16]. For receptor studies, SCLC cell lines were harvested and washed two times in serum free SIT medium (RPMI-1640 containing 3×10^{-8} M Na_2SeO_3, 5 μg/ml insulin, and 10 μg/ml transferrin). The cells (2×10^6) were added to ^{125}I-EGF (500 Ci/mmol) in the presence or absence of competitor (1 μg/ml EGF) in receptor binding buffer (SIT medium containing 0.1% BSA). After a 30 min incubation at 37°C, bound peptide was separated from free using the centrifugation techniques described previously [4]. NSCLC cells were harvested after treatment with trypsin and 5×10^4 cells were added to 24 well plates, which were pretreated with human fibronectin (10 μg). After a monolayer of cells had formed (3 days) the cell number was determined and receptor binding studies were conducted. The cells were washed 4×with receptor binding buffer and incubated with ^{125}I-EGF in receptor binding buffer. Free radiolabeled peptide was removed and the cells that contained bound peptide dissolved in 0.2 N NaOH and counted in a gamma counter. The adherent lung carcinoid (NCI-H727) was assayed like NSCLC cells, whereas the nonadherent lung carcinoids were assayed like SCLC cells.

The tyrosine phosphorylation induced by EGF was investigated. NSCLC cells and NCI-H727 were cultured in 10 cm diameter dishes and after a monolayer of cells had formed, they were rinsed with RPMI-1640 and cultured at 37°C in the presence or absence of EGF (100 ng/ml). After 20 min the medium was removed and the cells solubilized in Laemmli buffer. The phosphotyrosine-containing proteins were analyzed using Western blot techniques [17]. Samples (200 μg protein) were electrophoresed onto an 8% polyacrylamide slab gel and transferred to nitrocellulose sheets using an American Bionetics polyblot transfer system. Blots were probed with 12 μg/ml of polyclonal antiphosphotyrosine antibodies, followed by ^{125}I-protein A (Amersham Corp.). Autoradiograms were exposed with an intensifying screen for 1–2 days and the Mr of the labeled protein compared to the electrophoretic mobility of protein standards (Sigma Chemical Co.).

The ability to immunoprecipitate EGF receptors was investigated. NSCLC cells and NCI-H727 were cultured in 10 cm dishes and the cells (1×10^7) rinsed twice with methionine-free medium and incubated overnight in methionine-free medium containing

50 μCi/ml [^{35}S]methionine plus 10% fetal bovine serum. The labeled cells were washed twice with PBS and solubilized with 20 mM HEPES · NaOH (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 1 mM PMSF, and 10 μg/ml aprotinin. The solubilized cells were centrifuged in a Beckman Airfuge for 30 min at 4°C and incubated for 30 min at 4°C with Sepharose-protein A to which monoclonal antibody 108 against the EGF receptor was bound [18]. The immunoprecipitates were washed three times with 50 mM HEPES . NaOH (pH 8.0), 500 mM NaCl, 0.1% SDS, 0.2% Triton X-100, and 5 mM EGTA. Then the immunoprecipitates were washed three times with 50 mM HEPES . NaOH (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, and 5 mM EGTA. Lastly the immunoprecipitates were washed three times with 10 mM Tris · HCl (pH 8.0) and 0.1% Triton X-100. The washed beads were heated at 95°C for 3 min after the addition of sample buffer and analyzed by polyacrylamide gel electrophoresis. The gels were treated with AMPLIFY for 30 min, dried and autoradiographed on Kodak XAR2 film.

RESULTS

The neuroendocrine properties of the lung cancer cell lines were investigated. Table I shows that the three SCLC cell lines examined had high levels of BN/GRP. In particular, cell line NCI-H209 had the highest levels of BN/GRP (18.3 pmol/mg protein). Three of the four lung carcinoids examined had moderate levels of BN/GRP, ranging from 0.9–2.2 pmol/mg. In contrast, BN/GRP was not detectable in one lung carcinoid and the five NSCLC cell lines examined. These data indicate that SCLC and some lung carcinoids, but not NSCLC, are enriched in their neuroendocrine properties.

Specific ^{125}I-EGF binding was absent in the three SCLC cell lines examined (Table I). Specific ^{125}I-EGF binding was present in three of the four lung carcinoids examined and in all five of the NSCLC cell lines examined. In particular, the density of EGF receptor sites in lung carcinoid cell line NCI-H727 was similar to that observed in

TABLE 1. Concentration of BN-Like Peptides and EGF Receptors in Lung Cancer Cell Lines*

	BN/GRP	^{125}I-EGF
SCLC		
NCI-H209	18.3	<0.2
NCI-H345	4.7	<0.2
NCI-N592	0.2	<0.2
Lung carcinoid		
NCI-H679	2.2	1.8
NCI-H720	0.9	0.4
NCI-H727	<0.1	7.1
NCI-H835	1.5	<0.2
Adenocarcinoma		
ADLC-5M2	<0.1	8.4
Large cell carcinoma		
NCI-H157	<0.1	7.0
Squamous cell carcinoma		
NCI-H520	<0.1	6.1
EPLC-32M1	<0.1	4.2
EPLC-65H	<0.1	8.5

*The concentration of BN-like peptides (pmol/mg protein) and the density of ^{125}I-EGF binding sites (fmol/10^6cells) using 1 nM ^{125}I-EGF was determined. The mean value of three determinations is indicated.

the NSCLC cell lines. The EGF receptors in cell line NCI-H727 were characterized further and compared to NSCLC EGF receptors.

The amount of ^{125}I-EGF bound was determined as a function of unlabeled EGF concentration. Figure 1 shows that total binding to squamous cell carcinoma cell line EPLC-32M was approximately 9,000 cpm, whereas nonspecific binding in the presence of 0.5 μM unlabeled EGF was 400 cpm. Little specific binding was inhibited by 0.5 nM EGF, whereas ^{125}I-EGF binding was half maximally inhibited (IC_{50}) using 5 nM EGF. For adenocarcinoma cell line ADLC-5M2, the IC_{50} was 3 nM (Fig. 1). Similarly, for cell lines EPLC-65H, H-157, H-727, and H-520 the IC_{50} values ranged from 3–5 nM.

The concentration dependence of ^{125}I-EGF binding to lung carcinoid cell line NCI-H727 was determined. Figure 2 (left) shows that specific ^{125}I-EGF binding was saturable. A Scatchard replot of the specific binding data indicated that ^{125}I-EGF bound with high affinity (Kd = 6 nM) to a single class of sites (Bmax = 110,000/cell). Previously, it was demonstrated that the EGF-receptor complex was rapidly internalized at 37°C [19]. Figure 3 shows that at 4°C, 73% of the bound ^{125}I-I-EGF was at the cell surface. In contrast, at 37°C, only 25% of the bound ^{125}I-EGF was found on the cell surface. Therefore, at 37°C, but not 4°C, the receptor-^{125}I-EGF complex is internalized.

The biological activity of the EGF receptors was investigated. When EGF was added to adenocarcinoma cell line ADLC-5M2, a major 170 Kd band was phosphorylated on tyrosine amino acid residues (Fig. 4, lane b) relative to controls (Fig. 4, lane a). In addition, minor bands whose phosphorylation was increased after addition of EGF and contained phosphotyrosine were present at 140, 115, and 55 kd. Similar results were obtained for squamous cell carcinoma cell line EPLC-65H (Fig. 4, lane e). In contrast, less phosphotyrosine was present using cell line NCI-H727 (Fig. 4, lane c); however, minor bands at 170, 130, 115, and 55 kd were stimulated by EGF (Fig. 4, lane d).

The ability to immunoprecipitate the EGF receptor was investigated. Cells were incubated with [^{35}S]methionine and the EGF receptors immunoprecipitated with monoclonal antibody 108. A major 170 Kd protein was detected using cell lines EPLC-65H and NCI-H727 (Fig. 5). Similar results were obtained for cell lines ADLC-5M2 and EPLC-32M.

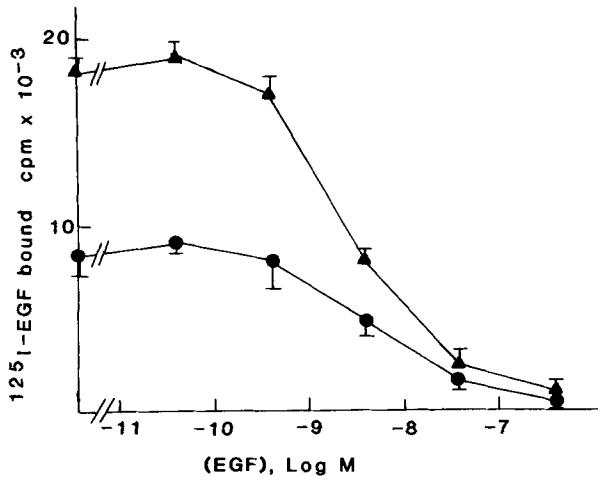

Fig. 1. EGF binding to NSCLC cells. ^{125}I-EGF was incubated with squamous cell carcinoma cell line EPLC-32M (●) and adenocarcinoma cell line ADLC-5M2 (▲) in the presence of increasing concentrations of unlabeled EGF. The mean value ± S.E. of four determinations is indicated.

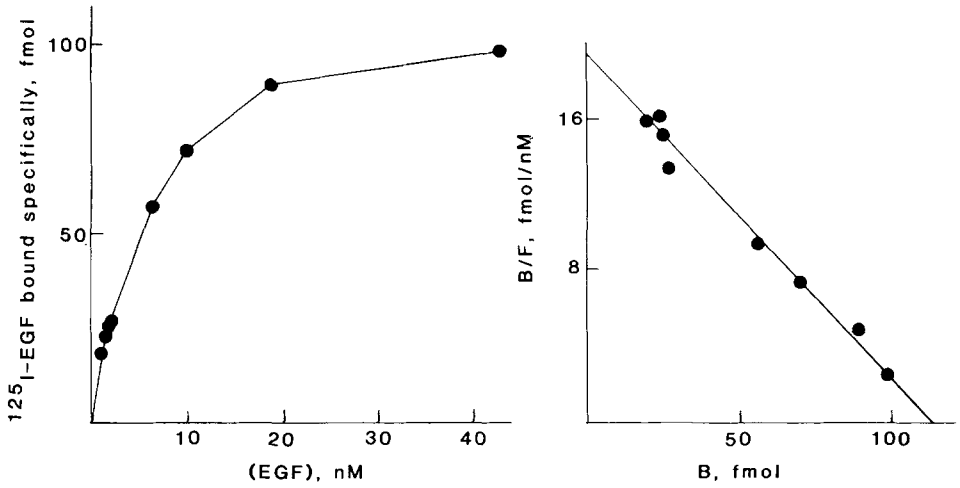

Fig. 2. Dose response curve of EGF binding to lung carcinoid cell line NCI-H727 at equilibrium. **Left:** The amount of ^{125}I-EGF bound specifically was determined as a function of ^{125}I-EGF concentration; each well contained 6 $\times 10^5$ viable NCI-H727 cells. The mean value of four determinations is indicated. **Right:** Scatchard replot of the specific binding data.

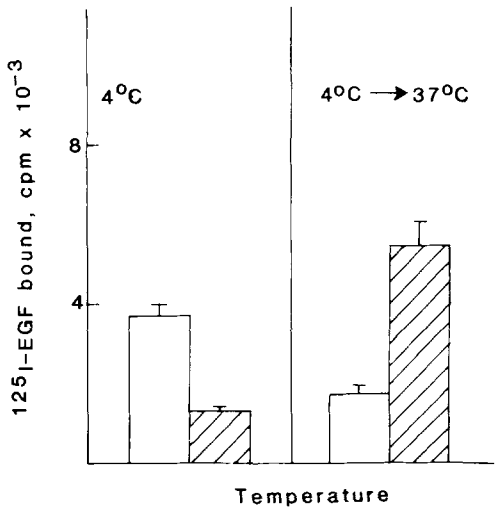

Fig. 3. Internalization of bound EGF. ^{125}I-EGF was incubated with cell line NCI-H727 for 2 h at 4°C and the cells washed to remove free peptide. **Left:** The cells were incubated for an additional 30 min at 4°C and (right) the cells were incubated at 37°C for 5 min. The cells containing bound ^{125}I-EGF were treated with 0.5 ml of 0.5 M acetic acid, 0.15 M NaCl (pH 2.5) at 4°C. After 5 min the supernatant was removed and counted (open bar) and the pellet that contained internalized peptide (stripped bar) solubilized in 0.2 N NaOH and counted in a gamma counter.

DISCUSSION

It is generally accepted that tumor cells may produce autocrine growth factors that stimulate cancer proliferation [20]. For SCLC, BN-like peptides function as autocrine growth factors [1]. For certain types of cancer such as NSCLC, TGFa, which binds with high affinity to EGF receptors, may function as an autocrine growth factor [21]. The

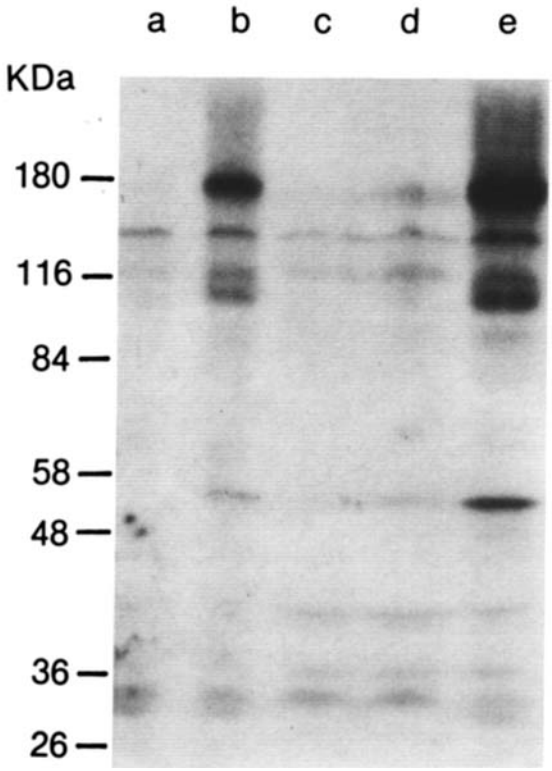

Fig. 4. In vitro phosphorylation of lung cancer cell lines. Adenocarcinoma ADLC-5M2 (**a,b**), lung carcinoid NCI-H727 (**c,d**), and squamous cell carcinoma EPLC-65H (**e**) cell lines were incubated in the absence (**a,c**) and presence (**b,d,e**) of 100 ng/ml EGF. A western blot analysis was performed and the samples treated with an antiphosphotyrosine antibody followed by incubation with ^{125}I-protein A and autoradiography.

EGF receptor, which is a glycoprotein composed of 1,186 amino acids and has protein kinase activity, is present on glioblastoma and NSCLC cells [11,22]. Also, the EGF receptor gene is amplified in glioblastomas [22] and NSCLC [23]. Here we investigated the biochemical properties of lung carcinoids.

High densities of EGF receptors were found in lung carcinoid cell line NCI-H727, whereas lower concentrations were detected in cell lines H679 and H720. Lung carcinoid cell line NCI-H835 did not have significant ^{125}I-EGF binding activity nor did the three SCLC cell lines examined. The five NSCLC cell lines examined all had high densities of EGF receptors (60,000–130,000 per cell). Similarly, cell line NCI-H727 had 110,000 EGF receptors/cell. It remains to be determined if the EGF receptor gene is amplified in this cell line.

^{125}I-EGF bound with high affinity to lung carcinoid cell line NCI-H727 (Kd = 6 nM). Similarly, EGF bound with high affinity (IC_{50} = 3–5 nM) to the five NSCLC cell lines examined. Previously, Haedner et al. [11] found that all NSCLC cell lines examined bound ^{125}I-EGF with high affinity (Kd = 0.5–4.5 nM) to a large number of sites (480–1,000 fmol/mg protein). These data indicate that EGF binds with high affinity to NSCLC cell lines and lung carcinoids. Previously, it was found that high levels of EGF receptors were present in squamous carcinoma but not carcinoid tumors [24,25].

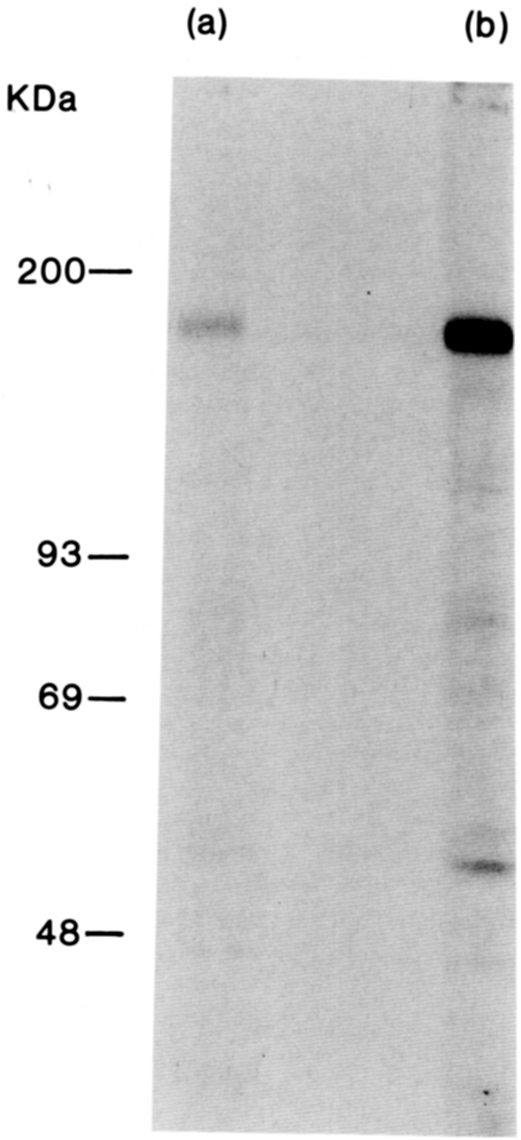

Fig. 5. Immunoprecipitation of the EGF receptor. Cell lines NCI-H727 (**a**) and EPLC-65H (**b**) were labeled with [^{35}S]methionine, solubilized and immunoprecipitated with anti-EGF receptor monoclonal antibodies. After elution of the EGF receptor, the samples were analyzed by SDS gel electrophoresis followed by autoradiography.

In addition, some SCLC cell lines bind EGF with high affinity, but the density of EGF receptors is 1–2 orders of magnitude lower than that in NSCLC cells [11,12].

In human epidermoid carcinoma (A431) cells, which have 2,000,000 EGF receptors per cell, EGF receptors cluster on both coated pits and noncoated membrane areas [26]. The EGF-receptor complexes undergo endocytosis and after internalization into lysosomes, the EGF-receptor complex is degraded by enzymes. Similarly, in lung carcinoid cell line NCI-H727, the EGF-receptor complex is localized to the cell surface at 4°C but is rapidly internalized at 37°C.

In A431 cells, when EGF binds to its receptors, the tyrosine-specific phosphorylation of numerous membrane proteins occurs [27]. Subsequent studies indicated that the EGF receptor is a tyrosine kinase that undergoes autophosphorylation [28]. Here, EGF stimulated the phosphorylation of a major 170 Kd protein and minor 130, 115, and 55 Kd proteins using NSCLC cell lines. It is likely that the major 170 Kd band is the EGF receptor, whereas the minor bands may represent protein substrates or EGF receptor degradation products. The pattern of tyrosine phophorylation using lung carcinoid cell line NCI-H727 was similar to that using the NSCLC cells, although the degree of EGF-stimulated phosphorylation was lower. The decreased phosphorylation in cell line NCI-H727 may result because of decreased tyrosine kinase activity, increased tyrosine phosphate phosphatase activity, and/or deletion of some tyrosine amino acid residues. Because the anti-EGF receptor antibody (108) immunoprecipitated a major 170 Kd protein, however, it is unlikely that substantial portions of the cytoplasmic domain of the EGF receptor are truncated in NCI-H727. In contrast, the EGF receptor is truncated in human gliomas [22].

EGF and TGFα stimulates the proliferation of a wide variety of cells [29]. Recently, we found that EGF stimulates the clonal growth of lung carcinoid NCI-H727 and NSCLC cells [30]. Because immunoreactive TGFα was identified in NSCLC cell line extracts [10], TGFα may function as an autocrine growth factor in NSCLC. The 108 antibody, which inhibits binding of EGF to its receptor, may disrupt this autocrine growth cycle. Xenograft formation and experimental lung metastasis of human oral epidermoid carcinoma cell line KB is inhibited by 108 [31]. Also, 108 inhibits the binding of ^{125}I-EGF, inhibits the clonal growth, and immunoprecipitates a major 170 Kd protein using cell line NCI-H727 [30]. Because similar data were obtained using NSCLC cell lines, EGF receptor monoclonal antibodies may serve as therapeutic agents in the treatment of lung cancer. Currently, clinical trials are being conducted on squamous cell carcinoma patients using anti-EGF receptor monoclonal antibodies [13].

In addition to having cell surface receptors for EGF, some lung carcinoids produce BN-like peptides. Recent data (Moody et al., unpublished) indicate that cell line NCI-H720 also has high affinity binding sites for BN-like peptides. Currently, we are investigating the effects of BN on the growth of this lung carcinoid cell line.

In summary, one type of lung cancer, SCLC, produces high levels of BN-like peptides and has cell surface receptors for BN. Another type of lung cancer, NSCLC, produces TGFα and has high concentrations of EGF receptors. Lung carcinoids are of an intermediate type whereby some cell lines produce BN-like peptides and/or have EGF receptors.

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