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Elevation of Epidermal Growth Factor Receptor Expression in a Brain-Metastasized Human Squamous Lung Cancer Cell Line

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ABSTRACT

The metastatic variant of human squamous cell cell line NCI-H226 (H226)was established by selection *in vivo* and its brain metastatic variant cell line H226B was examined. TGF- α increased cell proliferation of parental cell line H226 at an optimal concentration of 5 ng/ml, whereas H226B was less responsive to exogenous TGF- α (0.2 ng/ml - 100 ng/ml) in the assay. I^{125}-EGF binding assays showed that H226B has two types of EGF receptors, whereas H226 has only one type of EGF receptor. H226B cells have twice as many EGF receptors as that of H226. The kinase activity of H226B EGF receptors is down-regulated as shown by the immune complex assay. The brain metastaic variant H226 has an altered autocrine growth machanism compared to the parental cell line H226. This work provides a model for understanding the molecular events during tumor metastasis progression.

Keywords: Epidermal Growth Factor Receptors, Transforming Growth Factor- α , Metastasis.

INTRODUCTION

The human epidermal growth factor (EGF) receptor is a single-chain transmembrane glycoprotein that has an intrinsic tyrosine-protein kinase (Carpenter, 1987; Carpenter *et al.*, 1979; Hunter *et al.*, 1981) which is stimulated by EGF or EGF-like factors (Ullrich and Schlessinger, 1990; Fitzpatrick *et al.*, 1984). Elevated expression or activity of EGF receptor has been reported in neoplasms of the bladder (Smith *et al.*, 1989), breast (Ro *et al.*, 1988), and brain (Liberman *et al.*, 1984) and in renal (Petrides *et al.*, 1990) and squamous cell carcinomas (Kamata *et al.*, 1986; Cowley *et al.*, 1984). The increased EGF receptor activity in tumorigenesis can be attributed to autocrine stimulation by transforming growth factor- α (TGF- α). Increasing EGF receptor levels have also been induced by gene amplification (King *et al.* 1985), en-

hanced transcription (Downward *et al.*, 1984), translation and decreased metabolic turnover rate (Gamou *et al.*, 1987).

The human squamous cell carcinoma cell line has been reported to be metastatic(Schackert *et al.*, 1989). The cell line used in this report, H226B, was established from the brain metastatic progeny in BALB/c mice after internal carotid artery injection of the human non-small cell lung squamous cell line NCI-H226 (H226). The EGF receptor expression of H226B was studied. The TGF - α - mediated mitogenic responsiveness does not correlate with the increasing EGF-binding capacity.

MATERIALS AND METHODS

Cell lines and conditions

Human lung squamous cell carcinoma cell line H226 was obtained from Drs. J. Minna and A. Gazdar (NCI-Navy Medical Oncology Branch, National Navy Research Center, Bethesda, MD). The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, sodium pyruvate, and L-glutamine. The cell line was examined periodically and was free of mycoplasma, retrovirus type 3, mouse pneumonia virus, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus, extromella virus, and lactate dehydrogenase virus.

Intracarotid artery injection and cell line isolation

The technique used was described previously (Schackert *et al.*, 1989). Briefly, each mouse was anesthetized by intraperitoneal injection of sodium pentobarbital and fixed on its back on a paper board under a dissecting microscope. The animal's head was stabilized with a rubber band held between the teeth of the upper jaw. A mediolateral incision was made by a No.10 scalpel to serve as a landmark to separate the right common carotid artery from the vagal nerve. The nerve and the artery were prepared for an injection distal to the point of division. The artery was nicked with a pair of microscissors and a 30-gauge glass cannula was inserted into the lumen. H226 cells (1×10^5) suspended in 50 μl of Hank's Balanced Salt Solultion were injected slowly into the artery. The mice were killed when they became moribund or after 45 days. All tumors found in their brain were separated from the normal tissue; one was dissected with a sterile scalpel, minced with scissors, and cultured in 6-well plates in fetal calf serum-supplemented medium. Once tumor cells outgrew fibroblast cells, the culture was transferred to a new

culture flask; it continued to be transferred to increasingly larger culture flasks. Thus, the cell line H226B, with morphology similar to that of parent cell line H226, was established; the cells were maintained in RPMI-1640 medium with 5% fetal calf serum and free of mycoplasma contamination.

Growth Factor Responses

TGF-α wse added to the cells in various concentrations from 2 to 1×10^2 ng per ml. Cells (2×10^4 per well) were first cultured in 96-well microtiter plates in RPMI-1640 medium with 5% fetal bovine serum overnight. The medium was changed to 200 μl of serum-free RPMI containing insulin (5 μg/ml), transferrin(10 μg / ml) and sodium selenite (30nM) and various concentrations of TGF - α . After 48 hrs. at 37℃, the mixture were added 20 μl of PBS - dissolved MTT (3-(4,5-dimethylthiazol - 2 - yl) - 2 , 5 - diphenyl tetrazolium bromide, Sigma) for 4 hrs. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added and mixed thoroughly to dissolve the formazan crystals. The plate was read on a microplate reader, using 570 nm as the wavelength while 630 nm was set as a reference wavelength.

^{125}I-labeled EGF binding assay

Receptor grade EGF (Collaborative Research, Boston, MA) was labeled with carrier-free Na^{125}I (Amersham Corp.) using chloramine-T method (Carpenter and Cohen, 1976). Cells (5×10^5 per well) were cultured into 6-well plates and allowed to attach overnight. The medium was then aspirated and replaced with serum-free medium for an additional 3 h. incubation at 37℃. After determining of cell numbers per well, the cells were washed twice with ice-cold Dulbecco phosphate-buffered saline (DPBS) medium supplemented with 0.2% bovine serum albumin. Different concentrations of ^{125}I-labeled EGF in 1 ml DPBS-BSA were added to each well. After a 2h incubation at 4 ℃ , the cells were washed three times in ice-cold DPBS-BSA, and the bound radioactivity was determined after the cells were lysated in a 50 mM NaOH and 10% SDS mixture. The nonspecific binding was determined in assays containing a 100-fold molar excess of native EGF. Quantitation of binding sites per well, and the dissociation constant, K_d, were determined by Scatchard analysis (Scatchard, 1949). The A431 human epidermoid cell line was used as a positive control.

Immune complex kinase assay for EGF receptor

Kinase assays were performed as described with modification (Maxwell *et al.*,1989). Cells from 75% confluent

flasks were lysated and Dounce-homogenized in RIPA lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (ED TA), 1% aprotinin, 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml leupeptin, and 20mM sodium phosphate, (pH 7.0)). Five hundred micrograms of clarified cell lysates were incubated for 1 h with 5 μ l of EGF receptor extracellular domain specific monoclonal antibody, R_1 (Amersham Corp., Arlington Heights, IL). Immune complexes were harvested by addition of *Staphylococcus aureus* (Cowan strain) (Calbiochem, La Jolla, CA) for 30 min. A buffer containing 10 μ l [γ ^{32}P] adenosine-5'-triphosphate (ATP), 6 mM $MnCl_2$, 20 mM sodium N-2-hydroxyethylpiperazine- N' - 2 - ethanesulfonate (HEPES) (pH 7.0), and 10 μ M sodium orthophosphate was added. Phosphorylated proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% resolving gels). The gels were washed in 1N NaOH at 80°C for 1 hr. and dried. The dried gels were exposed to Kodak X-Omat film before development.

RESULTS

The levels of EGF receptor expression by cell lines H226 and H226B were determined by ^{125}I-labeled EGF binding. The maximum cell-bound radioactivity in both cell lines indicated that brain variant H226B has more EGF receptor binding capacity than the parental cell line (Fig. 1). Scatchard analysis of ^{125}I-labeled EGF binding indicated that the parental cell line, H226, has one type of binding site (4.5 $\times$ 10^4 binding sites per cell) with a dissociation constant, K_d, of 2.5×10^{-12} M; whereas EGF receptors of the brain variant H226B are degenerated into two types of binding sites: 6.9×10^4 binding sites per cell, with K_d of 2.5×10^{-12} M, for low-affinity receptors and 2.26×10^4 binding sites per cell, with K_d of 1.6×10^{-13} M, for high-affinity receptors.

The cells were incubated in serum-free and growth factor-free medium before TGF-α was added. Viability of the cells was measured by MTT colorimetric assay after incubation with growth factors for 48 h. The parental cell line, H226, showed maximum response to TGF-α at a concentration of 5 ng/ml (Fig. 2). In the presence of TGF-α at concentrations above 10 ng/ml, the proliferation rate of H226 was reduced. However, the brain variant cell line H226B was not as responsive toTGF-α concentrations in the range of 0.2 to 100 ng/ml as H226.

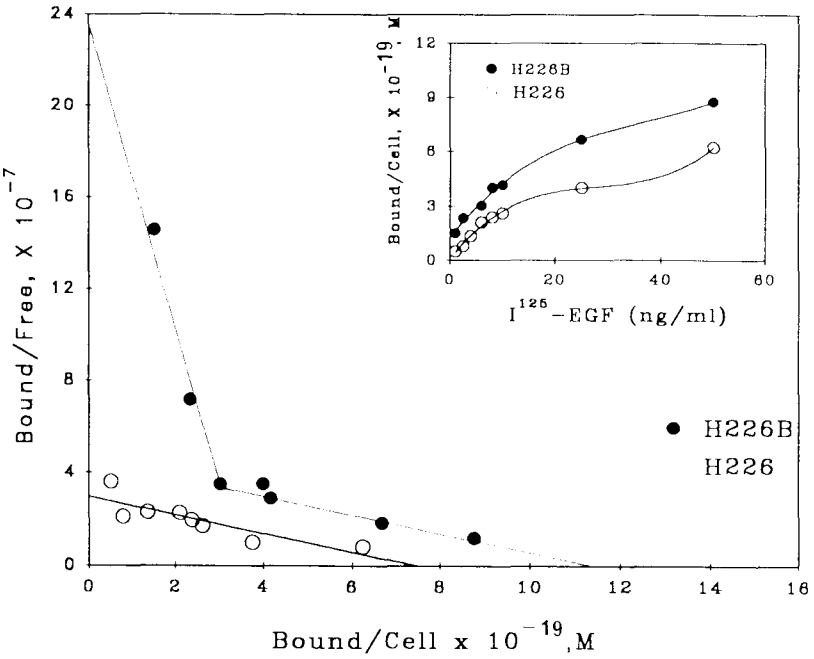

Fig. 1. ^{125}I-labeled EGF binding analysis. ^{125}I-EGF (0-50 ng/ml) in PBS was added to 5×10^5 cells in 60mm petri dishes. Bound reactivity was determined after a 2 hrs. incubation at 4°C. Non-specific binding was determined after addition of a 100-fold excess of unlabeled EGF and was always less than 3% of total binding. Scatchard analysis was used to determine binding sites and dissociation constants, K_d, using regression analysis. The binding of ^{125}I-labeled EGF to A431 cells was performed at the same time with binding sites of 1×10^6 receptors per cell and exhibited two types of binding sites.

The kinase activity of the EGF receptors of each cell line was determined by immunoprecipitation with an extracellular domain-specific monoclonal antibody, R_1, followed by kinase assay of the immune complex by incubation with [γ ^{32}P]ATP. Normal EGF receptor auto-phosphorylation was observed for H226 (Fig. 3, lane 3 and 5), but the EGF receptor auto-phosphorylation of the brain variant cell line H226B, was down-regulated significantly (Fig. 3, lane 4). A trace of activity could barely be detected in the X-ray film after 4 h exposure (Fig. 3, lane 6); the kinase activity is disproportional to the numbers of EGF receptors present on the cell surface (as determined by the ^{125}I-labeled EGF binding analysis).

DISCUSSION

The squamous cell carcinoma cell lines have been reported to express high levels of EGF receptors (Cowley *et al.*, 1984; Kamata *et al.*, 1986). EGF receptors in primary human glioblastoma and xenografted glioblastomas were reported to be amplified and

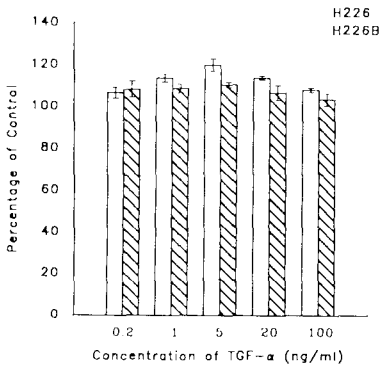

Fig.2: Dose-titration of TGF-α on H226 and H226B. Cells (1×10^4/well) were cultured in 96-well microtiter plates in RPMI-1640 medium with 5% fetal calf serum overnight. The medium was changed to insulin, transferrin and sodium selenite-supplemented RPMI-1640 overnight. Growth Factors (0.2-100 ng/ml) in RPMI was added. After 18 hrs. incubation at 37 °C, the cells were stained with MTT for 4 hrs., followed by solublization in isopropanol-acid mixture. The colored assay was read at 570 nm with 630 nm reading as reference. The cells incubated in TGF-α was compared to cells in medium alone (100%). All concentrations were tested in quadruplicate, and the errors represent standard errors.

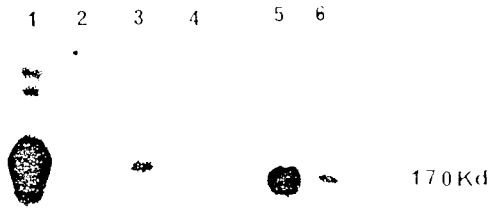

Fig.3: Immune complex kinase assay for EGF receptors. Cell lysate in RIPA buffer was incubated in EGF-receptor antibody R1. Immune complexes were harvested by addition of staphylococcus aureus and incubated with [γ ^{32}P]ATP. Phosphorylated protein was separated by SDS-PAGE gel (7.5%). The gels were washed in 1 N NaOH at 80 °C and dried before exposure. A431 cells (lane 1 and 2) were incubated with R1 antibody (lane 1) and a non-specific antibody (lane 2). Lane 3 and 4 were exposed for 1 hr.; whereas lane 5 and 6 were exposed for 4 hrs.. Lane 3 and 5 are H226. Lane 4 and 6 are H226B.

expressed in altered forms (Sugawa *et al.*, 1990) The squamous lung cancer cell line H226 was introduced into athymic BALB/c nude mice through the interior carotid artery to form brain tumors. One of the tumors was excised from brain tissues and used to develop a variant cell line free of fibroblasts. Unlike the parental cell line H226, the brain metastatic variant is tumorigenic in mice. We have shown before that H226 growth is modulated by a TGF-α - mediated autocrine loop (Putnam *et al.*, 1992). The results of this study indicated, however, that TGF-α does not play a significant role in naintaining H226B immortality. By histochemical staining and RNA dot blot hybridization, TGF-α was found expressed in the cytoplasm of H226B, albeit at a smaller level than the parental cell line (data not shown)

This suggests other growth factor(s) may emerge as the predominant

regulator. Only one type of EGF receptor was found on the cell surface of H226. However, in H226B, the EGF receptors are degenerated into two types. H226B has more EGF receptors than the parental cell, as shown by the dose-saturation binding curve, and its low-affinity receptor has a dissociation constant identical to that of the parental cell line. To examine the receptor functions, the protein kinase activity of EGF receptor immunoprecipitates was determined. The result showed that H226B EGF receptor kinase activity is significantly down-regulated. Since we were unable to detect the kinase activity of EGF receptors with the available antibody, we anticipate that EGF receptors on the cell surface of H226B may have adopted a different protein conformation from the parental cell line. It has been reported that the conformational changes of EGF receptors altered the intracellular tyrosine kinase activity and the stimulation of DNA synthesis (Greenfield *et al.*, 1989). Alternatively, the molecular structure for protein kinase activity of the receptors may have been altered. Both epidermoid cells A431 and breast cancer cells MDA-468 contain amplified EGF receptors that did not enhance their sensitivity on TGF- α - mediated autocrine proliferation (Downward *et al.*, 1984).

The principal origin of human brain metastases are lung carcinomas, which metastasize to the brain in 27% of patients (Gamache *et al.*, 1982). Schackert *et al.* (1989) developed an *in vivo* model for studying the growth pattern and biological behavior of brain metastases of different human carcinomas. The intracaratoid inoculated cells invaded through the blood-brain barrier and produced multiple lesions through the brain. This work underscores that, in contrast to H226, the brain metastatic variant H226B elicited the autocrine growth pathway despite expression of TGF- α and an excessive expression of EGF receptors on the cell surface. The results suggest, at late tumor progression event, TGF- α may have triggered a different growth regulation mechanism. It has been also shown that cell lines expressing defective kinase-negative mutant EGF receptors have a suppressive mitogenic response and undertake different endocystic pathways (Honegger *et al.*, 1990), thus retaining their immortality. Previous studies indicated that EGF receptor gene is amplified in most malignant variants of human glioblastomas and xenografted glioblastomas and is associated with the rearrangements that caused truncation of the extracellular domain (Hung *et al.*, 1986) or expression of larger

mass EGF receptor (Henderlee *et al.*, 1984, Steck *et al.*, 1986). Our results indicate that the parental and brain metastatic variant cell line showed two distinct EGF receptors phenotypes. Further elucida-tion of the molecular structure of H226B EGF receptors should assist us in understanding their variation during tumor cell metastasis progression.

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REFERENCES

Carpenter, G. 1987. Receptors for epidermal growth factor and other polypeptide mitogens. *Ann. Rev. Biochem.* **6**:881-919.

Carpenter, G., L. King, and S. Cohen. 1979. Rapid enrichment of protein phosphorylation in A431 cell membrane preparations by epidermal growth factor. *J. Biol. Chem.* **254**:4884-4891.

Carpenter, G., and S. Cohen. 1976. ^{125}I-labeled human epidermal growth factor, binding, internalization and degradation in human fibroids. *J. Cell Biol.* **71**:159-171.

Cowley, G., J. A. Smith, B. Gusterson, F. Hendler, and B. Ozanne. 1984. The amount of EGF receptor is elevated on squamous cell carcinomas. *Cancer Cells* **1**:5-10.

Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Tatty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield. 1984. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature(London)* **307**:521-527.

Fitzpatrick, S., J. Brightwell, J. L. Wittliff, G. H. Barrows, and G. S. Schultz. 1984. Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen receptor and progestin receptor levels. *Cancer Res.* **44**:3448-3453.

Gamache F. W., J. B. Posner, and R. Patterson. 1982. Metastatic brain tumors. **In**: Youman GR, ed. Neurological Surgery, 2nd edition, Saunders, Philadelphia, 5, 2872-2878.

Gamou, S., and N. Shimiyu. 1987. Change in metabolic turnover is an alternative mechanism increasing cell surface epidermal growth factor receptor levels in tumor cells. *J. Biol. Chem.* **262**:6708-6713

Greenfield C., I. Hiles, M. D. Waterfield, W. Federwisch, A. Wollmer, T. L. Blundell, and N. McDonald. 1989. Epidermal growth factor binding induces a conformational change in the extra domain of its receptor. *The EMBO J.* **8**:4115-4123.

Henderlee, F. J., and B. W. Ozanne. 1984. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J. Clin. Inves.* **74**:647-651.

Honegger, A. M., A. Schmidt, A. Ullrich, and J. Schlessinger. 1990. Evidence for epidermal growth factor (EGF) induced intermolecular autophophorylation of EGF receptors in living cell. *Mol. Cell. Biol.* **10**:4035-4040.

Hung, M. C., K. L. Thompson, I. M. Chiu, and W. Rosner. 1986. Characterization of rodent epidermal growth factor receptor transcripts using a mouse genome probe. *Biochem. Biophy. Commun.* **141**:1109-1115.

Hunter, T., and J. A. Cooper. 1981. Epidermal growth factor induces rapid tyrosine phosphorylation of protein in A431 human tumor cells. *Cell* **24**:741-752.

Kamata, N., K. Chida, K. Rikimaru, M Horikoshi, S. Enomoto, and T. Kuroki. 1986. Growth inhibitory effects of epidermal growth factor and overexpression of its receptors on human squamous cell carcinomas in culture. *Cancer Res.* **46**: 1648-1653.

King, C.R., M. H. Kraus, L.T. Williams, G. T. Merlino, I. Pastan, and S. Aaronson. 1985. Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant-sized mRNAs. *Nuc. Acid Res.* **13**:8477-8486.

Liberman, T. N., Razon, A. Bartal, Y.Yarden, J. Schlessinger, and H. Soreq. 1984. Expression of epidermal growth factor receptors in human brain tumors. *Cancer Res.* **44**:753-760.

Maxwell, S. A., P. G. Sacks, J. U. Gutterman, and G. Gallick. 1989. Epidermal growth factor receptor protein-tyrosine kinase activity in human cell lines established from squamous carcinoma of head and neck. *Cancer Res.* **49**:1130-1137.

Petrides, P.E., S. Bock, J. Bovens, R. Hofman, and G. Jaske. 1990. Modulation of pro-epidermal growth factor, pro-transforming growth factor and epidermal growth factor receptor gene expression in human renal carcinoma. *Cancer Res.* **50**:3934-3938.

Putnam E. A., N. Yen, G. E. Gallick, P. A. Steck, K. Fang, B. Akpakip, A. F. Gazdar, and J. A. Roth. 1992. Autocrine growth stimulation by transforming growth factor-α in human non-small lung cancer. *Surg. Oncol.* **1**:49-60.

Ro, J., S. M North, G.E. Gallick, G. N. Hortobagyi, J. U. Gutterman, M. Blick. 1988. Amplified and overexpressed epidermal growth factor receptor gene in uncultured primary human breast carcinoma. *Cancer Res.* **48**:161-164.

Scatchard, G. 1949. The alteration of protein for small molecules and ions. *Annals of N.Y. Acad. Sci.* **51**:660-672.

Schackert, G., J. E. Price, C. D. Bucana, and I. J. Fidler. 1989. Unique patterns of brain metastasis produced by different human carcinomas in athymic nude mice. *Intl. J. of Cancer* **44**:892-897.

Smith, K., J. A. Fennely, D. E. Neal, R. R. Hall, and A. L. Harris. 1989. Characterization and quantitation of epidermal growth factor receptor in invasive and superficial bladder tumor. *Cancer Res.* **49**:5810-5815.

Steck, P.A., G. E. Gallick, S. A. Maxwell, W. S. Kloetzer, R. B. Arlinghaus, R. P. Moser, J. U. Gutterman, and W. K. A. Yung. 1986. Expression of epidermal growth factor receptor and associated glycoprotein on cultured human brain tumor cells. *J. Cell. Biochem.* **32**:1-10.

Sugawa, N., A. J. Ekstrand, C. D. James, and V. P. Collins. 1990. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc. Natl. Acad. Sci.* **87**:8602-8606.

Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**:203-212.

人類肺癌扁平細胞在腦移轉後表皮生長因子受體的異常變化

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摘　要

人類肺癌細胞中以扁平細胞最具移轉能力。本文在以肺癌扁平細胞為模式，探討表皮生長因子受體(EGF receptor)在移轉前後的表型變化。將非小細胞肺癌細胞株(NSCLC) NCI-H226(H226)由裸鼠頸動脈注入，使之產生腦移轉腫瘤後加以培養成細胞株。實驗結果顯示EGF receptor於腦移轉後在細胞表面有增加的現象，但EGF receptor本身所應具之磷酸化反應及對轉形生長因子-α(TGF-α)刺激反應卻有降低的趨勢，這種現象可能說明了人類肺癌扁平細胞在腦移轉之後不再侷限於早期細胞生長所需由TGF-α所引導之自身促激反應(autocrine)，而可能由其它反應機制所取代。

關鍵詞：表皮生長因子受體、轉形生長因子-α、癌細胞移轉