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Quantitative Assay of Epidermal Growth Factor Receptor in Human Squamous Cell Carcinomas of the Oral Region by an Avidin-Biotin Method

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A quantitative assay method for epidermal growth factor receptors (EGFRs) of human tumor tissues was established, based on enzyme-labeled avidin-biotin (LAB) interaction with anti-human EGFR monoclonal antibody 528IgG. A standard calibration curve for EGFR estimation in human tumor tissues was obtained with A431#8 cells cloned from A431 human epidermoid carcinoma cell line. The coefficient of variance for the standard curve was below 35% in the application to tumor tissues from nude mice implanted with human tumor cell lines. The minimum tissue amount required for the quantitative assay was around 0.1 g (wet weight). Using the LAB method, the correlation between the level of EGFR number and tumor malignancy was examined for 14 human squamous cell carcinomas (SCCs) from the oral region. Seven of the SCCs showed a more than two-fold higher EGFR number compared to normal gingival tissues. Three highly aggressive carcinomas with poor prognosis possessed five to ten times higher levels of EGFR number than normal tissues. The elevated EGFR level in the SCCs seems to correlate to increasing tumor size and the stage of SCCs as clinically classified according to the 1987 UICC TNM system.

Key words: Epidermal growth factor receptor — Avidin-biotin method — Monoclonal antibody — Squamous cell carcinoma — Oral cancer

Epidermal growth factor (EGF) is a potent mitogen that plays important roles in growth control by binding to cell surface receptors. The EGF receptor (EGFR) shares significant homology with the v-*erb*-B oncogene product,[1] and the over expression of the amplified EGFR gene (c-*erb*-B) has also been reported for A431 cells[2–5] and several other epithelial carcinomas.[6–14] Thus, the aberrant expression of EGFRs is considered to be involved in the pathogenesis of certain epithelial neoplasms, and to represent a potential prognostic marker in some of them.[15–22]

To examine the frequency of abnormal EGFR expression in human neoplasms, it is obviously essential to develop a simple and reliable quantitative assay method for human EGFR. Though several methods with radioisotope-labeled reagents have been proposed for EGFRs expressed in cultured cells or tissues,[23–27] they are still not satisfactory for the purpose of clinical investigation of human cancers. The use of the avidin-biotin interaction[28] should provide a simple, rapid and sensitive solid-phase assay method for detecting the EGFRs without using radioisotopes. In this report, we present a quantitative assay method for EGFRs in human tissues, based on the enzyme-labeled avidin-biotin (LAB) interaction with anti-human EGFR monoclonal antibody (MoAb). The relationship between the EGFR number thus determined and the clinical malignancy of squamous cell carcinomas (SCCs) in the oral region was also investigated.

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MATERIALS AND METHODS

Materials Anti-human EGFR MoAb (528IgG) and anti-hapten MoAb (ARB229IgG: incapable of binding to EGFR) were prepared from hybridoma conditioned media as previously described.[29,30] Mouse EGF was purchased from collaborative Research, Inc. (USA). Avidin D, biotinylated horse anti-mouse IgG antisera and horseradish peroxidase-conjugated avidin were from Vector Laboratories, Inc. (USA), and 4-methoxy-1-naphthol for color development was from Aldrich Chemical Co. Inc. (USA). Other reagents were all of analytical grade.

Cell culture A431#8 cells cloned from A431 human epidermoid carcinoma cell line[31] were maintained in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DME) (Nissui, Japan) and Ham's F12 nutrient medium (F12) (Nissui) containing 15 m*M* Hepes, 2.2 g/liter $NaHCO_3$, pH 7.4, (DME/F12), supplemented with 5% fetal calf serum (FCS) (Gibco, USA). Human foreskin fibroblast (HFF) cell line[30] and six human tumor cell lines of different origins, HeLa-J (cervical), HeLa-S (cervical), Li-7A (liver), T-84 (colon), T24-9 (brain) and A431-derived variant cell line A431#18,[31]

were maintained in DME/F12 medium supplemented with 10% FCS. Cells were grown under humidified air containing 5% CO_2 at 37°C. Fairly confluent cells were washed three times with Ca^{2+},Mg^{2+}-free phosphate-buffered saline (PBS(−): pH 7.4), detached with a rubber policeman, and harvested by low-speed (500–1000*g*) centrifugation. Cell pellets were stored at −20°C until use.

Tumor formation in nude mice Approximately 1×10^7 cells of each of the human tumor cell lines were inoculated subcutaneously into KSN nude mice (Shizuoka Lab. Animal Center, Japan). After two to four weeks, the tumors were carefully removed from the nude mice, rinsed with PBS (−), weighed and stored at −20°C until use.

Collection and storage of tissues from human oral region SCC tissue samples of around 0.1–0.5 g (wet weight) were obtained from tumors removed by surgical operation. Small pieces of normal gingival tissues were provided from subjects with no evidence of tumor at the time of tooth extraction. These tissues were rinsed with PBS(−) and stored at −20°C until use. Clinical staging of tumors was done according to the 1987 UICC TNM system.[32]

Preparation of membrane fractions Frozen stored tissues or cultured cells were homogenized in 2 ml of ice-cold extraction solution (20 m*M* boric acid, 0.2 m*M* EDTA, 1 m*M* iodoacetic acid, 125 m*M* NaCl, pH 10.2) according to the method of Thom *et al.*[33] The homogenate volume was then adjusted to 40 ml with the extraction solution and stirred for 10 min. Five ml of 500 m*M* borate buffer (pH 10.2) was then added to the homogenate solution and the suspension was further stirred for 10 min. Five ml of the tissue suspension was used for DNA analysis according to Burton.[34] The remaining 40 ml of the tissue suspension was subjected to EGFR assay. After removal of the tissue debris by low-speed centrifugation (450*g*, 20 min), the cell membrane was collected by centrifugation at 12,000*g* for 30 min, then resuspended in 1 ml of Tris-buffered saline (TBS: 20 m*M* Tris, 500 m*M* NaCl, pH 7.4) and stored at −20°C until use. All procedures for the membrane preparation were performed at 4°C. The membrane fractions could be stored for at least 3 months without loss of 528IgG binding activity of EGFR.

EGFR assay EGFR assay was performed by the LAB method using a Bio-Dot microfiltration apparatus (Japan Bio-Rad)[35] as summarized in Table I. The total EGFR number of the membrane fractions was assayed with 528-IgG and ARB229IgG by subtracting the non-specific binding as determined with the latter, and measured with a refractance scanning densitometer (Bio-Rad model 1650-2).

The EGFR assay was also performed by radioimmunoassay (RIA) with ^{125}I-labeled 528IgG MoAb as previously described[29] to confirm the reproducibility and accuracy of the present LAB method.

Table I. EGFR Assay Procedures with LAB Method[a]

1. Protein binding
   (a) 100 μl of each membrane fraction prepared from tissue suspension (0.1–1 μg of DNA per well) is placed onto each well of a nitrocellulose membrane (5 μm pore size: S & S) properly wetted with TBS[b] and placed in a microfiltration apparatus.
   (b) after gravity drainage for 30 min, each well is washed with 100 μl of TBS (×1).
2. Blocking of non-specific binding
   (a) 300 μl of 100 n*M* avidin D (VEC. A-2000)/1% BSA/TBS is added to each well.
   (b) after gravity drainage for 20 min, each well is washed with TBS (×5).
   (c) 100 μl of 1 n*M* biotin (BIOCHEM. RES. 2031)/1% BSA/TBS is added to each well.
   (d) after gravity drainage for 20 min, each well is washed with TBS (×5).
3. Antibody binding
   (a) 100 μl of 50 n*M* 528IgG/1% BSA/TBS or ARB229IgG/1% BSA/TBS is added to each well.
   (b) after standing for 10 min at 37°C, each well is washed with TBS (×5).
   (c) 100 μl of 50 n*M* biotinylated anti-mouse IgG antisera/1% BSA/TBS is added to each well.
   (d) after standing for 10 min at 37°C, each well is washed with TBS (×5).
4. Color development and counting
   (a) 200 μl of 2 μg/ml horseradish peroxidase-conjugated avidin in TBS is added to each well. After standing for 30 min at 37°C followed by gravity drainage for 30 min, each well is washed with TBS (×5).
   (b) the nitrocellulose membrane is transferred into a dish and rinsed with TBS (×3).
   (c) 12 ml of color development solution[c] is added to the dish, then the dish is gently agitated for a few min until blue dots appear.
   (d) the reaction is stopped by washing the nitrocellulose membrane with distilled water, then the membrane is dried.
   (e) each blue dot is scanned with a refractance densitometer.

a) All procedures were performed at room temperarure unless otherwise stated.
b) Tris-buffered saline (see the text).
c) Mixture with one part of 3 mg/ml 4-methoxy-1-naphthol in methanol and five parts of 0.018% H_2O_2 in TBS prepared immediately before use.

RESULTS

Estimation of EGFR number by the LAB method 528-IgG used for the LAB method in the present work binds specifically to the external domain of human EGFR with

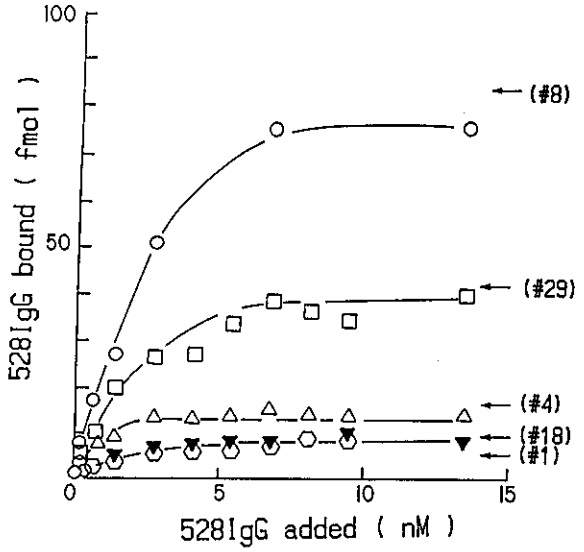

Fig. 1. Maximum level of 528IgG binding for variant A431 cells. Cells used were A431 clonal variants: A431#8, #29, #4, #18 and #1. Variant cells 2×10^4 per well were plated in a 96-well microtiter plate and 528IgG binding assay was performed as previously described.[29] Arrows show the maximum binding levels of EGF to each cell line.

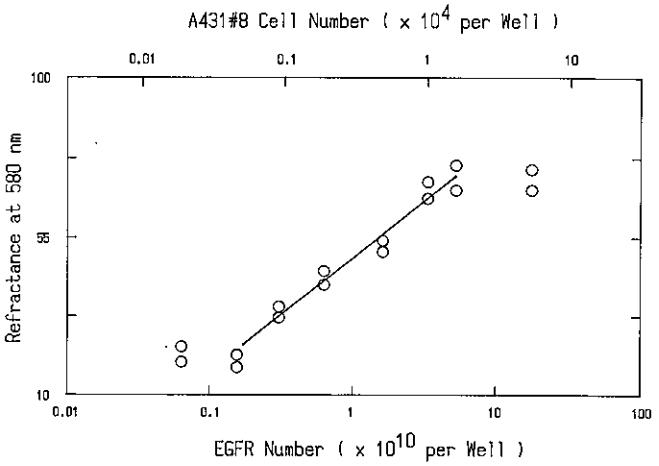

Fig. 2. Calibration curve of EGFR number determined by the LAB method using A431#8 cells as a standard. As described in "Materials and Methods," membrane fractions prepared from A431#8 cells were used, and cell number was estimated from the DNA content. EGFR number (3×10^6) per cell of A431#8 was estimated by Scatchard plot analysis with a direct ^{125}I-528IgG RIA method as described previously.[29]

a high affinity ($K_d=1.5$–2.5×10^{-9} M).[30] The present quantitative LAB method was based on measuring the maximal binding level of MoAbs to EGFRs. As shown in Fig. 1, 10 nM 528IgG was enough to obtain the maximum binding level in variant A431 cells with various receptor numbers. This antibody level was found to be applicable to other cell types examined: HFF, HeLa-J, HeLa-S, Li-7A, T-84 and T24-9 (data not shown).

A431 clonal variant, A431#8 cell, has a high and steady EGFR number (3×10^6 per cell) and is therefore suitable as a standard to obtain a calibration curve for the quantitative analysis of EGFRs in cells and tissues. Figure 2 presents the calibration curve between the refractance measured and EGFR number with A431#8 by the LAB method. The standard curve was linear over the range of 6×10^2–2×10^4 cells per well, corresponding to the EGFR number of 2×10^9–6×10^{10} per well. The restriction of the analytical range was found to depend not on the receptor number but rather on the cell number applied per well, probably due to steric obstruction among layered cells on the nitrocellulose membrane, because linearity was also obtained over the same range of cell number as above with A431#18 or HFF cells possessing 5–10% as many EGFRs as A431#8 cells (data not shown).

Application of the LAB method to estimation of EGFR number in tumor tissue and cultured cells Cell numbers in tumor tissues and cultured cells were estimated from the DNA level based upon the DNA contents (around 10 pg per cell) predetermined with A431#8 and other variant cells. The above-mentioned analytical range of cell number corresponded to approximately 6–200 ng of DNA per well.

Table II. Estimation of EGFR Number in Tumor Tissues and Cultured Cells by the LAB and RIA Methods

Cell line	EGFR number ($\times10^5$/cell)		
	LAB method		RIA method[b]
	tumor tissue[a]	cells	cells
A431#8	25.3±3.09	30.0	29.5
Li-7A	27.4±9.55	28.5	31.2
T-84	1.37±0.38	1.28	0.95
T24-9	0.54±0.075	0.38	0.32
HeLa-J	0.14±0.026	0.18	0.18

a) Mean±SD of three separate sets of experiments for tumor tissues formed in nude mice as described in "Materials and Methods."
b) EGFR number determined by Scatchard plot analysis with ^{125}I-528IgG as described previously.[30]

The EGFR numbers of five distinct human carcinoma cell lines (A431#8, Li-7A, T-84, T-24-9, HeLa-J) were estimated by the LAB method. Table II indicates that there is an accordance of EGFR number per cell between *in vitro* (cultured cells) and *in vivo* (human tumor tissues

Table III. EGFR Number and Clinical Classification of SCCs of the Oral Region and EGFR Number of Normal Gingival Tissues

No.	Age	Sex	Clinical diagnosis	Differentiation[a]	TNM	Stage	Recurrence[b] (months)	EGFR number[c] ($\times 10^5$/cell)
1	48	M	upper alveolus and gingival Ca	p	$T_4N_0M_0$	4	(+6)	10.9±3.38
2	63	F	buccal mucosa (gingival Ca)	m	$T_2N_1M_0$	3	(+4)	1.04±0.14
3	75	F	lower alveolus and gingival Ca	w	$T_4N_0M_0$	4	(+5)	5.91±2.34
4	57	M	upper alveolus and gingival Ca	w	$T_4N_2M_0$	4	(+1)	10.7±2.17
5	73	M	lower alveolus and gingival Ca	m	$T_1N_0M_0$	1	(−)	1.93±0.52
6	73	M	lower alveolus and gingival Ca	p	$T_4N_0M_0$	2	(−)	1.68±0.12
7	79	F	recurrence of tongue Ca	m	$T_2N_0M_0$	2	(−)	0.76±0.21
8	66	M	retromolar buccal mucosa Ca	m	$T_3N_{2b}M_0$	4	(−)	1.69±0.19
9	74	M	mouth floor lower alveolus and gingival Ca	m	$T_4N_0M_0$	4	(−)	3.88±1.34
10	54	M	lower alveolus and gingival Ca	m	$T_4N_{2a}M_0$	4	(−)	4.94±1.05
11	74	F	tongue Ca	m	$T_2N_{2a}M_0$	4	(−)	2.24±0.12
12	76	F	lower alveolus and gingival Ca	w	$T_4N_1M_0$	4	(−)	1.61±0.18
13	73	M	lower alveolus and gingival Ca	w	$T_4N_0M_0$	4	(−)	3.98±0.99
14	55	M	recurrence of tongue Ca	w	$T_4N_{2b}M_0$	4	(−)	1.22±0.19
15	44	F	NMT					1.40±0.34
16	44	F	NMT					0.61±0.12
17	54	M	NMT					0.30±0.06
18	39	M	NMT					0.37±0.07
19	54	M	NMT					0.44±0.10
20	61	M	NMT					0.40±0.15
21	44	M	NMT					1.19±0.08
22	39	F	NMT					1.10±0.10

a) p=poorly, m=moderately, w=well differentiated.
b) presence (+) or absence (−) of recurrence after surgical operation.
c) Mean±SD of 2–6 separate sets of EGFR assay.
Ca: carcinoma. NMT: non malignant tissues [normal gingival tissues from subject with no evidence of tumor].

inoculated in nude mice) conditions. The EGFR numbers of these cell lines confirmed by Scatchard plot analysis with the RIA method using ^{125}I-labeled 528IgG are also recorded in Table II. The EGFR numbers of each tumor tissue obtained from a set of three nude mice were assayed separately by the LAB method, and the coefficient of variance (CV) in these assays was always below 35%. When a known amount of A431#8 cells was added as an inner standard of EGFR to the original sample, the recovery of the EGFRs during the membrane preparation step was estimated to be more than 80% for both tumor tissues and cultured cells.

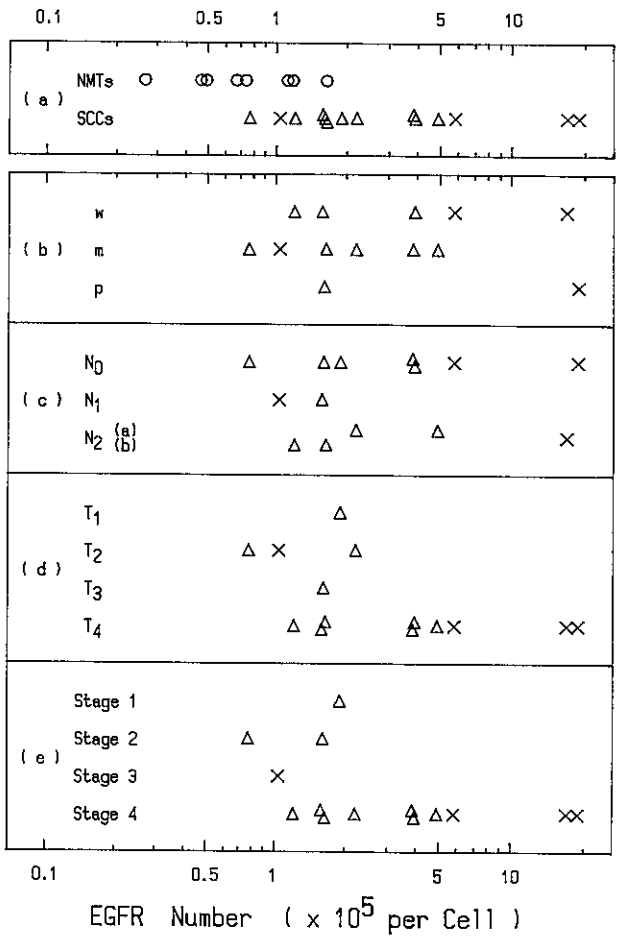

Fig. 3. Relationship between EGFR number and prognosis of SCCs of the oral region. (○) non malignant tissues (NMTs) from subjects with no evidence of tumor, (△) SCCs and (×) SCCs of poor prognosis (early recurrence). (a) Comparison of EGFR number of NMTs and SCCs of the oral region. (b) Relationship between EGFR number and histological grade of SCCs. w=well, m=moderately, p=poorly differentiated. (c) Relationship between EGFR number and potential of regional lymph node metastasis (category N) of SCCs. (d) Relationship between EGFR number and increasing size and/or local extent of primary tumor (category T) of SCCs. (e) Relationship between EGFR number and clinical stage of SCCs.

Correlation between EGFR number and prognosis of human oral cancer SCCs at the oral region from 14 patients and normal human gingival tissues from 8 subjects with no evidence of tumor were subjected to the EGFR assay as described in "Materials and Methods." The results are summarized in Table III and Fig. 3. With 7 SCCs out of 14 patients, the EGFR numbers per cell of tumor tissues were over two-fold higher than those of normal gingival tissues. Furthermore, in SCCs of two patients who had very poor prognosis, the EGFR numbers were extremely high (over 1×10^6 per cell) compared to the other SCCs (Fig. 3a). As to the EGFR numbers assayed in SCCs of the oral region, no significant variation has so far been noted depending on either their histological differentiation type or the potential of lymphatic metastasis (category N of the TNM system) (Figs. 3b and 3c). The elevation of the EGFR number, on the other hand, showed significant correlations with increasing size and/or local extent of the primary tumor (category T of the TNM system) and the grade of stage (Figs. 3d and 3e).

DISCUSSION

Quantitative assay methods for the detection of EGFRs in tumor tissues have been proposed by several groups. Gullick *et al.*[24] have reported an RIA method for measuring ^{125}I-labeled EGF binding to EGFR trapped on affinity beads coated with anti-EGFR MoAbs. Libermann *et al.*[23] have evaluated the EGFR content in tumor tissues by measuring the level of ^{32}P incorporated into EGFRs. Sunada *et al.*[25] have used the ^{32}P-autophosphorylated EGFRs prepared from A431 cells as a standard to evaluate the receptor number in cells or tissues in a rather indirect fashion. Nicholson *et al.*[26] have reported the Scatchard analysis of multipoint assay performed by using a displacement method with ^{125}I-labeled EGF. However, these methods do not seem suitable for estimation of EGFR numbers, because of the probable difference in EGF-binding affinities among EGFRs of tested tissues or possible changes of affinity even during the membrane preparation.

Recently, Doekeritz *et al.*[27] have proposed a simplified solid-phase assay method for EGFR in cultured cell lines using ^{125}I-labeled MoAbs. Without the need for radioisotope-labeling, our LAB method developed on the basis of the solid-phase assay method is satisfactorily applicable to the quantitation of EGFR in normal and tumor tissues. The validity of the estimation of receptor numbers by our method is secured by the sufficient binding capacity of MoAbs added in excess, thus assuring reproducibility by overcoming the problem caused by the variable affinities of EGFRs. In this LAB method, we took a tissue sample from each of three tumor-inoculated nude mice, and estimated the CV value to be below 35% (Table II). Thus, the present assay conditions are considered to be suitable for quantitation of EGFR number in tumor tissues.

Though the roles of EGF and EGFR still remain to be elucidated, particularly in relation to the carcinogenic status of human tissues, several studies have been attempted. Filmus *et al.*[36] reported that tumor growth generated by MDA-468 human breast cancer cell line

(EGFR number: 1.5×10^6 per cell) in nude mice was significantly faster than that generated by MDA-468 variants with decreased EGFR numbers ($1.6–3.6\times10^4$ per cell). Similar results were also obtained with A431 and its variant cells[37] and other SCCs.[38] Correlations between depth of tumor invasion and elevated EGFR expression were observed in gastric[21,22] and bladder[39] carcinomas. Further, the presence of abundant EGFR seems to correspond to a group of more aggressive tumors endowed with higher metastatic potential.[15,16,19,20]

The present study has indicated that increased EGFR expression is associated with the growth enhancement of SCCs of the oral region. We found apparent correlations between elevated EGFR numbers in tumor cells and increasing size and/or local extent of primary tumors (category T of the TNM system and the clinical stage: Figs. 3d and 3e). At present, it is considered to be premature to propose a simple correlation of EGFR numbers in cells with malignancy of SCCs of the oral region. Analyses with more carcinoma samples are needed for a final clarification of the correlation between elevated EGFR numbers and malignancy of SCCs.

The most important application of the present assay is the prediction of tumors with a poor prognosis. Sainsbury *et al.*[16] have suggested EGFR expression as a predictor of relapse and death in patients with primary operable breast cancers. Nicholson *et al.*[17,18] have reported that EGFR status was significantly associated with a lack of any response to primary endocrine therapy, and further with recurrence and death after salvage surgery for progressive endocrine disease patients. As summarized in Table III and Fig. 3a, the EGFR numbers of two patients with poor prognosis, when compared to other patients, exhibited aberrant EGFR expression of over 1×10^6 per cell. From the data presented here, the sensitivity (11/14) and the specificity (7/8) are, with the assumed cut-off level of EGFR number of 1.5×10^5 per cell, estimated tentatively to be 79 and 88%, respectively. The present SCCs of the oral region were mostly in advanced stages of the disease, and most patients providing tumor samples had received chemotherapy or radiation prior to tumor excision. It is thus necessary to consider the effects of these pretherapies in the prognostic evaluation of tumors based on EGFR measurements.

Though the correlation presented here between the high expression of EGFR in cells and tumor aggressiveness is restricted to SCCs, the quantitative analysis of EGFRs of cancer cells by our LAB method may provide a clue to elucidate the control mechanism of tumor growth.

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