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Epidermal Growth Factor Receptors in Cancer Tissues of Esophagus, Lung, Pancreas, Colorectum, Breast and Stomach

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The levels of epidermal growth factor (EGF) receptors were investigated in surgically resected tumors of various origins including esophagus (n=33), lung (n=14), pancreas (n=9), colorectum (n=10), breast (n=23) and stomach (n=8). The ^{125}I-EGF binding capacities of squamous cell carcinomas of esophagus and lung were exceptionally higher than those of the other cancer tissues. Immunohistochemical staining with an anti-EGF receptor monoclonal antibody detected EGF receptors in the basal cells and parabasal cells of normal esophageal epithelium and in all the cancer cells of squamous cell carcinoma tissues of esophagus and lung. DNA replicating cells were examined by the bromodeoxyuridine staining method and it was found that the basal cells and parabasal cells of normal epithelium and peripheral cells of cancer pearls are proliferating. Contrary to this, a tumor antigen TA-4, known as a specific marker for squamous carcinoma, was detected in the differentiated cancer cells and in middle-layer squamous cells. These results strongly suggest that the increase in EGF receptor levels may be associated with the development of human squamous cell cancers of esophagus and lung. Thus, measurement of EGF receptor expression in tumor tissues has diagnostic value and should prove useful for the development of new therapies.

Key words: Epidermal growth factor receptor — Squamous cell carcinoma — Bromodeoxyuridine — TA-4

Epidermal growth factor (EGF)[*4] stimulates the growth and proliferation of a variety of cell types *in vitro* and *in vivo* through interaction with a specific cell surface receptor.[1,2] It has been found that EGF receptor is homologous to the product of the avian erythroblastosis virus oncogene v-*erb*,[3] and it has been suggested that the v-*erb* B oncogene product is a truncated form of EGF receptor which is produced from the host cell proto-oncogene c-*erb* B.[4,5] The relationship between the EGF/EGF receptor system and cancer has thus been a focus of attention.

EGF receptor levels of various cultured cells and tumor tissues have been measured by radioreceptor assay or immunohistochemical staining. Many squamous cell carcinoma cell lines and some cell lines derived from other tumors express a large number of EGF receptors.[6–9] Elevated levels of EGF receptors have been demonstrated in some gliomas,[10] breast tumors,[11,12] bladder tumors,[13] thyroid tumors,[14] sarcomas,[15] gynecological tumors,[16] lung tumors,[17] esophageal tumors[18] and gastric, colonic carcinomas.[19] EGF receptor levels of established cell lines may not reflect those of the original individual cells in tumors because cell lines may suffer certain selection during the establishment of culture or because the characteristics of cell lines may gradually change with repeated transfer. The measurement of EGF receptor in tumors is thus significant. However, EGF receptor assay techniques vary, and the EGF receptor levels of various tumors have not been measured in the same manner. Moreover, the analysis of normal tissues remains incomplete. It is therefore important to measure the EGF receptor levels of many tumors and normal

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[*4] Abbreviations: EGF, epidermal growth factor; EBSS, Earle's balanced salt solution; ABC, avidin-biotin-peroxidase complex; TBS, Tris-buffered saline; NHS, normal horse serum; NGS, normal goat serum.

tissues using ^{125}I-EGF binding assay and immunohistochemical staining.

In the surgical field, esophageal cancer is a representative squamous cell tumor. Although various treatments including surgical removal, radiotherapy and chemotherapy have been developed, esophageal cancer still has a poor prognosis. To develop an improved treatment for this disease, it is necessary to clarify the distinctive characteristics of various squamous cell carcinomas in terms of the EGF receptors. Though the EGF receptor is involved in cell growth, the relationship between EGF receptors and cancerous growth in tissue is not clear. Recently, new cell kinetic studies have been done using the anti-bromodeoxyuridine (BrdU) monoclonal antibody method.[20-22] This has made it easier to detect S-phase cells in tissues which incorporate BrdU into DNA and react to anti-BrdU monoclonal antibody. A new tumor antigen (TA-4) first prepared from human uterine cervical squamous cell carcinoma was found to be a glycoprotein with a molecular weight of 48,000 daltons.[23] Serum TA-4 concentration is useful for predicting the extent[24] and prognosis[25] of uterine cervical cancer, detecting recurrence,[26] and monitoring the effects of therapy.[27] Its utility has also become clear in lung and esophageal cancer as well as in uterine cervical cancer.

In this study, the EGF receptor levels of several different tumor types were measured for comparison, and the cellular localizations of EGF receptors and TA-4 antigens were examined in detail.

Materials and Methods

Materials Mouse EGF (ultra pure grade) was obtained from Toyobo (Osaka). ^{125}I-EGF was prepared with Iodobeads (Pierce, Rockford, IL) as described previously.[28] The B4G7 mouse monoclonal antibody specific to human EGF receptor has been described.[29] BrdU, anti-BrdU mouse monoclonal antibody, and anti TA-4 rabbit polyclonal antibody were purchased from Takeda Chemical Ind. (Osaka), Becton Dickinson (Mountain View, CA), and Dainabot Ltd. (Tokyo), respectively. Biotinylated horse anti-mouse IgG, biotinylated goat anti-rabbit IgG, normal horse serum, normal goat serum, and Vectastain ABC kit were obtained from Vector Labo. Inc. (Burlingame, CA). Other agents used were of analytical grade. Tissues from 33 cases of esophageal cancers, 14 lung cancers (squamous cell carcinoma), 23 breast cancers, 10 colorectal cancers, 9 pancreatic cancers, 8 gastric cancers, 33 normal esophageal mucosas, and 10 normal breast glands were obtained from patients who had undergone surgery at Keio University Hospital. These patients had never received radiotherapy or anti-cancer chemotherapy. A431 tumors were removed from nude mice that had received a subcutaneous injection of these cells.

EGF Binding Assay This assay was performed as described previously.[18] Tissues were frozen on dry ice shortly after surgical removal and stored at -80° until use. Approximately 100 mg of tissues was minced and suspended in 1 ml of ice-cold Earle's balanced salt solution (EBSS) containing 5mM Hepes buffer (pH 7.4) and 1mM phenylmethylsulfonyl fluoride. The suspended tissue fragments were then homogenized using a Polytron with 4 intermittent 15-sec bursts at medium speed. The homogenate was filtered through nylon mesh and then #300 metal mesh. The protein concentration was adjusted to 1 mg/ml with the above buffer. About 400 μl of tissue homogenate was incubated at 4° for 2 hr with $4\times10^{-10}M$ ^{125}I-EGF in the presence or absence of $4\times10^{-7}M$ unlabeled EGF. The reaction mixtures were then filtered through 0.2-μm EG-type filters (Millipore, Bedford, MA). The filters were washed twice with ice-cold EBSS buffer and the remaining radioactivity was counted in a Beckman gamma counter. Specific bindings were calculated by subtracting nonspecific binding from total binding, and results were expressed as a percent of input, that is, pmoles/mg protein. A431 tumor was adopted as a control.

Immunohistochemical Staining EGF receptors, TA-4 and BrdU were detected by the avidin-biotin-peroxidase complex (ABC) method. EGF receptor staining was performed as described previously.[18] Tissues were embedded in O.C.T. Compound (Miles, Naperville, IL) and snap-frozen in acetone/dry ice. They were then cut into 6-μm cryostat sections and air-dried. The sections were then fixed in acetone for 20 min at 4° and washed 3 times with 50mM Tris-buffered saline (TBS), pH 7.6. The sections were covered with normal horse serum (NHS, diluted 10 times with TBS) as a blocking agent and kept at room temperature for 30 min. The sections were then incubated successively with the B4G7 antibody (1 μg/ml in 10% NHS), biotinylated horse anti-mouse IgG (diluted 200 times with 10% NHS), and avidin-biotin-peroxidase complex for 30 min each at room temperature. After each step, sections were washed 3 times with TBS. After the final wash, the sections were stained by incubation with diaminobenzidine (0.5 mg/ml) in 0.01% H_2O_2 for 5–10 min. The sections were then washed with water, counter-

stained with methylgreen, dehydrated and mounted. Normal mouse IgG (Sigma, St. Louis, MO) was used as a negative control.

For TA-4 staining, normal goat serum (NGS, diluted 10 times with TBS) as a blocking agent, anti-TA-4 rabbit polyclonal antibody (diluted 50 times with 10% NGS) as a first antibody, biotinylated goat anti-rabbit IgG (diluted 200 times with 10% NGS) as a second antibody, and normal rabbit IgG (Sigma, St. Louis, MO) as a negative control were used. Except for the above reagents, TA-4 was stained in the same way as EGF receptor.

BrdU Incorporation and Staining A dose of 20 mg/kg of BrdU dissolved in physiological saline was administered intravenously to patients operated on for esophageal cancer according to the previous report.[22] This study was carried out with the informed consent of the patients. After a 1–2 hr pretreatment, resected specimens were fixed in 70% ethanol, embedded in paraffin, and cut into 3 μm sections. Then the sections were deparaffinized, treated in 2*N* HCl for 30 min to denature DNA, and neutralized for 10 min in 0.1*M* $Na_2B_4O_7$. Intrinsic peroxidase was blocked by incubation for 30 min in methanol containing 0.3% H_2O_2. The sections were covered with 10% NHS as a blocking agent for 30 min and incubated with anti-BrdU monoclonal antibody (diluted 100 times with 10% NHS) at 4° overnight. The subsequent steps were the same as in EGF receptor staining. The labeling indices were obtained in the following manner: 2,000 cells (for esophageal cancer tissue, 2,000 cancer cells; for normal esophageal mucosa, 2,000 squamous epithelial cells) were counted and the labeling indices were calculated as the percentages of the labeled cells among 2,000 labeled and unlabeled cells.

Results

EGF Binding Capacities EGF binding capacities were measured using homogenates of small tissue specimens under our established conditions.[18] Figure 1 shows the EGF binding capacities of various cancer tissues. The EGF binding capacities vary in broad ranges but it is clear that esophageal and lung squamous cell carcinomas possess substantially higher binding capacities. One of the esophageal cancer tissues exhibited a binding value as high as that of A431 tumors. The EGF binding capacities of esophageal cancer tissues were far higher than those of the normal tissues adjacent to the tumors. The EGF binding capacities of pancreatic, colorectal, breast and gastric cancers are relatively lower and the degree of binding capacity decreases in this order. It is important to note that the breast cancers have low EGF binding capacities relative to the other cancers but significantly higher capacities than the normal adjacent tissues.

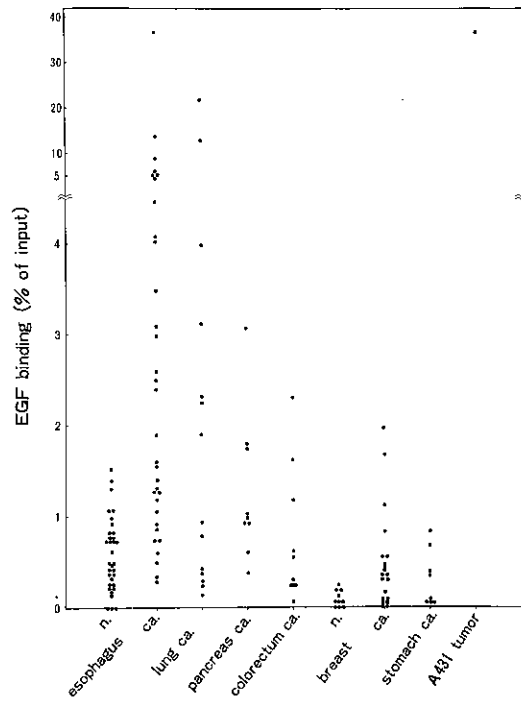

Fig. 1. EGF binding capacities in various cancer tissues. n: Normal tissues adjacent to the cancer tissues.

Immunohistochemical Staining of EGF Receptors The EGF receptor distribution in tumor tissues was examined using monoclonal antibody to human EGF receptors. This analysis revealed that cell membranes of all the esophageal squamous carcinoma cells were uniformly stained, but the stromas were not stained (Fig. 2A). The intensity of EGF receptor staining gradually diminished towards the center of cancer pearls. EGF receptor distribution in the lung squamous carcinomas was similar to that in the esophageal squamous carcinomas (data not shown). Interestingly, basal cells and parabasal cells in normal esophageal epithelium were stained, and the intensity became weaker towards the superficial squamous layer (Fig. 2B). In pancreatic cancer tissues, cancer cells were

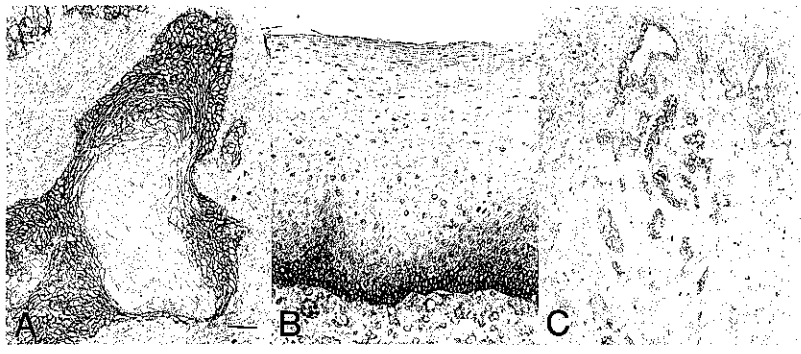

Fig. 2. Immunohistochemical staining of EGF receptors. A, Esophageal squamous cell carcinoma; B, esophageal mucosa; C, pancreatic carcinoma. The bar (Fig. A) indicates 50 μm.

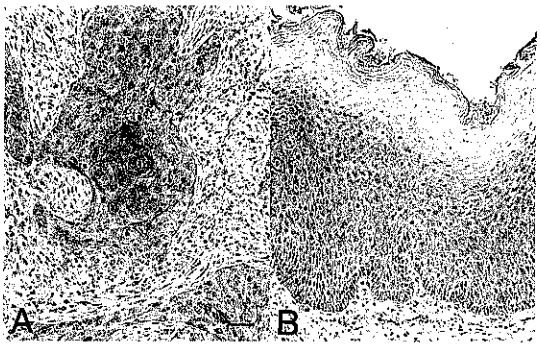

Fig. 3. Immunohistochemical staining of TA-4. A, Esophageal squamous cell carcinoma; B, esophageal mucosa. The bar (Fig. A) indicates 50 μm.

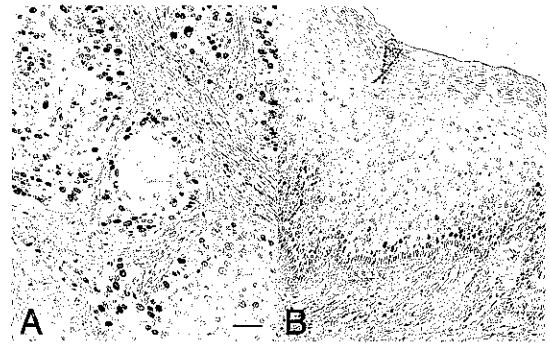

Fig. 4. Immunohistochemical staining of BrdU incorporated into DNA. A, Esophageal squamous cell carcinoma; B, esophageal mucosa. The bar (Fig. A) indicates 50 μm.

weakly but definitively stained with anti-EGF receptor antibody (Fig. 2C). EGF receptors in other cancer tissues were not detected by this immunohistochemical staining method.

Immunohistochemical Staining of a Tumor Antigen TA-4 The distribution of a differentiation marker was examined using antibody to a tumor antigen, TA-4. In this case, cytoplasms of all the esophageal squamous carcinoma cells were stained but cell membranes were not stained (Fig. 3A). The intensity of TA-4 staining gradually increased towards the center of cancer pearls in contrast to EGF receptor staining. The squamous cells in the middle layer of normal esophageal epithelium were positive for TA-4, but neither basal cells nor parabasal cells were stained (Fig. 3B).

Immunohistochemical Detection of DNA Replicating Cells Finally, the distribution of proliferating cells was examined by the BrdU staining method. This analysis revealed that peripheral cells of cancer pearls are active for incorporating BrdU into DNA (Fig. 4A). In normal esophageal epithelium, basal cells and parabasal cells were positive for BrdU staining (Fig. 4B). The labeling index of esophageal cancer was $16.1+6.6\%$ ($n=12$), which is much higher than that of normal esophageal epithelium, $3.2+0.9\%$ ($n=12$), indicating more active proliferation of tumor cells.

Discussion

In this study, we examined the EGF binding capacities of various tumors and found that EGF receptors are expressed in higher numbers in squamous cell carcinomas of lung and esophagus than in other adenocarcinomas of pancreas, colorectum, breast and stomach. Lung adenocarcinomas were studied, but their EGF binding capacities were not high (data not shown). Elevated levels of EGF receptors have been reported in a number of squamous carcinoma cell lines[6-8] and in biopsy specimens of squamous cell carcinomas from lung, head, neck, skin and cervix.[30] This study provides additional evidence to support these previous findings.

In the present study, the distribution of EGF receptor expressing cells was examined in normal esophageal epithelium and squamous cell carcinoma, and compared with the distribution of DNA replicating cells and TA-4 antigen expressing cells. In normal esophageal epithelium, a significant number of EGF receptors was expressed in basal cells and parabasal cells and the number decreased towards the superficial squamous layer. These basal cells and parabasal cells were often found to be proliferating and not to express the TA-4 antigen. The TA-4 antigen was expressed in the squamous cells in the middle layer of normal esophageal epithelium. Thus, there is an inverse correlation between EGF receptor expression and cell differentiation in normal esophageal epithelium.

In esophageal and lung squamous carcinomas, EGF receptors were localized on the membranes of all cancer cells. The number of EGF receptors gradually decreased towards the center of cancer pearls. In contrast to the EGF receptor, the tumor antigen TA-4 was found in the cytoplasms of all cancer cells and the degree of TA-4 expression increased towards the center of cancer pearls. Furthermore, peripheral cells of cancer pearls were active for proliferation. Thus, tumor antigen TA-4 was expressed in more differentiated esophageal cancer cells and the EGF receptor-expressing cells were more proliferative.

In some pancreatic cancer tissues, cancer cells were stained weakly but definitively with anti-EGF receptor antibody. An elevated level of EGF receptors has been observed in an established cell line (UCVA-1) from a pancreatic tumor.[31] Thus, an elevated level of EGF receptors appears to be involved in the development of adenocarcinomas as well as squamous carcinomas.

A correlation between EGF receptor level and biological behavior has been reported for some types of cancer. In breast cancer, there is a significant inverse relationship between EGF receptor and estrogen receptor status, and a significant association between metastatic potential and poor prognosis.[12, 32] We have also observed that EGF receptor expression in breast cancer is relatively low as compared to other cancers, but is significantly higher than in normal adjacent tissue. In bladder cancer, the presence of EGF receptors is associated with poor differentiation and active invasion.[13] In the case of esophageal cancer, we found no definite correlation between elevated EGF receptor levels and pathological findings.[18] Other types of cancer showed no pathological relations, either (data not shown). However, elevated level of EGF receptors was definitely associated with poor prognosis (unpublished data). Thus, in investigating the malignant potential of squamous cell carcinoma, the EGF receptor appears to be a more useful marker than TA-4, although serum TA-4 levels are more easily monitored. Furthermore, the detection of EGF receptor expression in biopsy sections by immunohistochemical staining offers precise diagnostic information of the involvement of squamous cells in the mucoepidermoid cell carcinoma.

The EGF receptor has been successfully used as the target of radioimmunoscintigraphy using ^{111}In-labeled anti-receptor monoclonal antibody.[33] Anti-receptor monoclonal antibodies have also been proven useful for suppressing the growth of A431 tumors in nude mice.[34] In addition, patients with gliomas have been treated with administration of ^{131}I-labeled anti-receptor monoclonal antibody.[35] We have recently succeeded in selectively killing squamous carcinoma cells by using an immunotoxin that specifically recognizes the EGF receptor.[36] The above-mentioned approaches and the determination of prognosis via EGF receptor level measurement may allow a significant improvement in

the treatment of patients with squamous cell carcinomas in the near future.

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