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# Immunoreactive Transforming Growth Factor Alpha Is Commonly Present in Colorectal Neoplasia

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***Surgical specimens from 19 patients with invasive colorectal cancers and 12 specimens of normal mucosa from the same patients were examined immunohistochemically for the production of the immunoreactive (IR-) transforming growth factor (TGF)-α and IR-epidermal growth factor (EGF) with an anti-TGF-α monoclonal antibody (MAb) OAL-MTG01 and anti-EGF MAb KEM-10. Immunoreactive TGF-α was detected in 16 (84.2%) of 19 colorectal cancers. In contrast, there was no IR-TGF-α in the gland cells of normal mucosa. Immunoreactive EGF was detected in 7 (36.8%) of 19 colorectal cancers and 1 (8.3%) of 12 cases of normal mucosa. The production of both IR-TGF-α and IR-EGF in colorectal cancer did not differ by histologic type and Dukes' stage. Immunoreactive TGF-α was detected at significantly higher incidence than IR-EGF in colorectal cancer. These results indicate that IR-TGF-α should prove valuable as a possible tumor marker in colorectal cancers, and it may be very useful in understanding the biology of colorectal cancer. (Am J Pathol 1991, 139:123–129)***

Recently the incidence of colorectal cancers has increased in Japan. It is thus important to understand potency of malignancy and prognosis. One significantly malignant and prognostic factor for primary colorectal cancers is the cellular growth. The transforming growth factor (TGF)-α, produced by cancer cells, may stimulate their own growth.[1,2] We previously demonstrated that colon cancer cell lines produce TGF-α, which functions as a possible autocrine growth factor.[3–5] The production of TGF-α may correlate with malignant potential and thus serve as a reliable tumor marker in colorectal cancer.

In several colorectal cancers, both TGF-α mRNA and IR-TGF-α have been detected, as well as other cancers.[4,6–11] A few immunohistochemical studies for IR-TGF-α production in colorectal cancer have been reported. In the present study, immunohistochemical analysis was performed for examining the production of TGF-α with surgical specimens of invasive colorectal cancers and normal mucosa from the same patients. Recently epidermal growth factor (EGF), which is structurally and functionally related to TGF-α and binds to a common receptor,[12] has been detected in some cancer cells.[13,14] Thus an immunohistochemical study for EGF was also performed using the same materials.

## Materials and Methods

### Cell Line and Tissues

A375 (a human melanoma cell line producing TGF-α[5]) was obtained from the American Type Culture Collection and used as the control for TGF-α immunostaining. It was fixed in 10% neutral formalin at 4°C overnight before immunostaining.

Specimens of 19 invasive colorectal carcinomas and 12 corresponding normal colorectal mucosa of the resection margins from the same patients were examined. The invasive colorectal carcinomas included 7 well-differentiated adenocarcinomas, 11 moderately differentiated adenocarcinomas, and 1 poorly differentiated adenocarcinoma. All were surgical specimens resected at Hiroshima University Hospital from 1987 to 1989. They were fixed immediately in 10% neutral formalin and embedded in paraffin. Sections of normal human submandibular gland, which produces EGF, were used as controls for EGF immunostaining. These were also fixed in formalin and embedded in paraffin.[15]

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### *Antibodies*

The anti–TGF-α monoclonal antibody (MAb) OAL-MTG01 was provided by the Otsuka Assay Laboratory (Tokushima, Japan). The development of this MAb will be described elsewhere (Akamatsu et al, manuscript in preparation). Briefly, recombinant human (h-) TGF-α (1–50) (Earth Chemical Inc., Akoh, Japan) conjugated with Ascaris extracts was used as immunogens. BALB/C mice were immunized three times with the immunogen containing 33 μg of recombinant h-TGF-α (1–50), and the MAb was developed by the method described previously.[16] We characterized OAL-MTG01 by radioimmunoassay (RIA) with recombinant h-EGF and h-TGF-α (Earth Chemical, Akoh, Japan) as described previously[17] and by immunocytochemical examination of the A375 cell line. This antibody was used at a concentration of 32 μg/ml (about 1:100 dilution) in 0.01 mol/l (molar) phosphate-buffered saline (PBS) for immunohistochemistry.

The anti-EGF MAb KEM-10 was provided by Wakunaga Pharm. (Kohda, Japan). It showed no cross-reactivity with h-TGF-α, as described previously,[5] and was used at a concentration of 50 μg/ml (about 1:20 dilution) in 0.01 mol/l PBS for immunohistochemistry.

### *Immunohistochemistry*

Immunohistochemical analysis for TGF-α was performed by the avidin-biotin-peroxidase complex (ABC) method using an ABC *Elite* kit (Vector Laboratories, Burlingame, CA) with some modification.[18] The assay buffer consisted of 0.01 mol/l PBS with 1 mol/l NaCl. The sections were incubated with buffer containing 2% Tween 20 for 20 minutes to block nonspecific binding. Biotinylated anti-mouse gamma G immunoglobulin (IgG) diluted 1:400 was used as the second antibody. Epidermal growth factor staining was performed by the same method for TGF-α, differences being that the assay buffer was 0.01 mol/l PBS and biotinylated anti-mouse IgG diluted 1:100 was used as the second antibody.

The specificity of immunostaining with OAL-MTG01 was verified by its absorption test and by replacing this antibody with the same concentration of recombinant mouse IgG1. Absorption test was performed by preincubation with 200 μl (32 μg/ml) of OAL-MTG01 and 5 μg of the recombinant h-TGF-α at 4°C overnight. The specificity of immunostaining with KEM-10 was confirmed in the same way.

Immunoreactivity of tissue specimens was classified by the percentage of positive staining tumor cells in the section divided into four grades as follows: + + +; 100% to 70% positive, + +; 70% to 30% positive, +; 30% to 0% positive; and −, negative.

The data obtained were evaluated by the chi-square test.

## *Results*

### *Cross-reactivity of OAL-MTG01*

The standard curve for TGF-α using OAL-MTG01 is shown in Figure 1. This RIA system was specific for TGF-α. At the h-EGF concentration of less than 12 μg/ml, the antibody showed no cross-reactivity.

### *Specificity of Antibodies in Immunocytochemical and Immunohistochemical Reactions*

The cell line A375, which produces TGF-α, showed positive control staining with OAL-MTG01 (Figure 2a). Immunoreactive TGF-α was detected diffusely in cell cytoplasma. Recombinant mouse IgG1 did not react with A375 (Figure 2b). The absorption test for this antibody in A375 is shown in Figure 2c, where IR–TGF-α disappeared. The surgical specimen of IR-TGF-α positive staining is shown in Figure 3a. Immunoreactive TGF-α was present diffusely in the cytoplasma of cancer cells. Recombinant mouse IgG1 did not react with this section (Figure 3b). In the absorption test, IR–TGF-α also disappeared (Figure 3c). Immunoreactive TGF-α–positive specimens in each histologic grade are shown in Figure 4. The same results of absorption test as for KEM-10 were obtained, using a tissue specimen of normal human submandibular gland as an EGF-positive control and a colorectal cancer specimen. Immunoreactive EGF was present diffusely in the cytoplasm as well as IR-TGF-α (Figure 5).

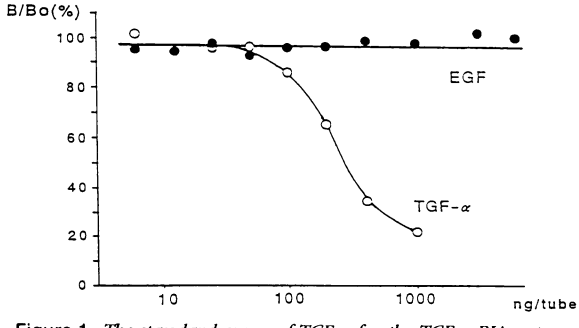


**Figure 1.** *The standard curve of TGF-α for the TGF-α RIA system. Recombinant h-TGF-α was used as the assay standard and labeled antigen. It was radioiodinated by the lactoperoxidase method. EGF showed no cross-reactivity with OAL-MTG01. Binding is expressed as percent of maximal binding after background subtraction as described in Materials and Methods. Data represent means of duplicate determinations.*

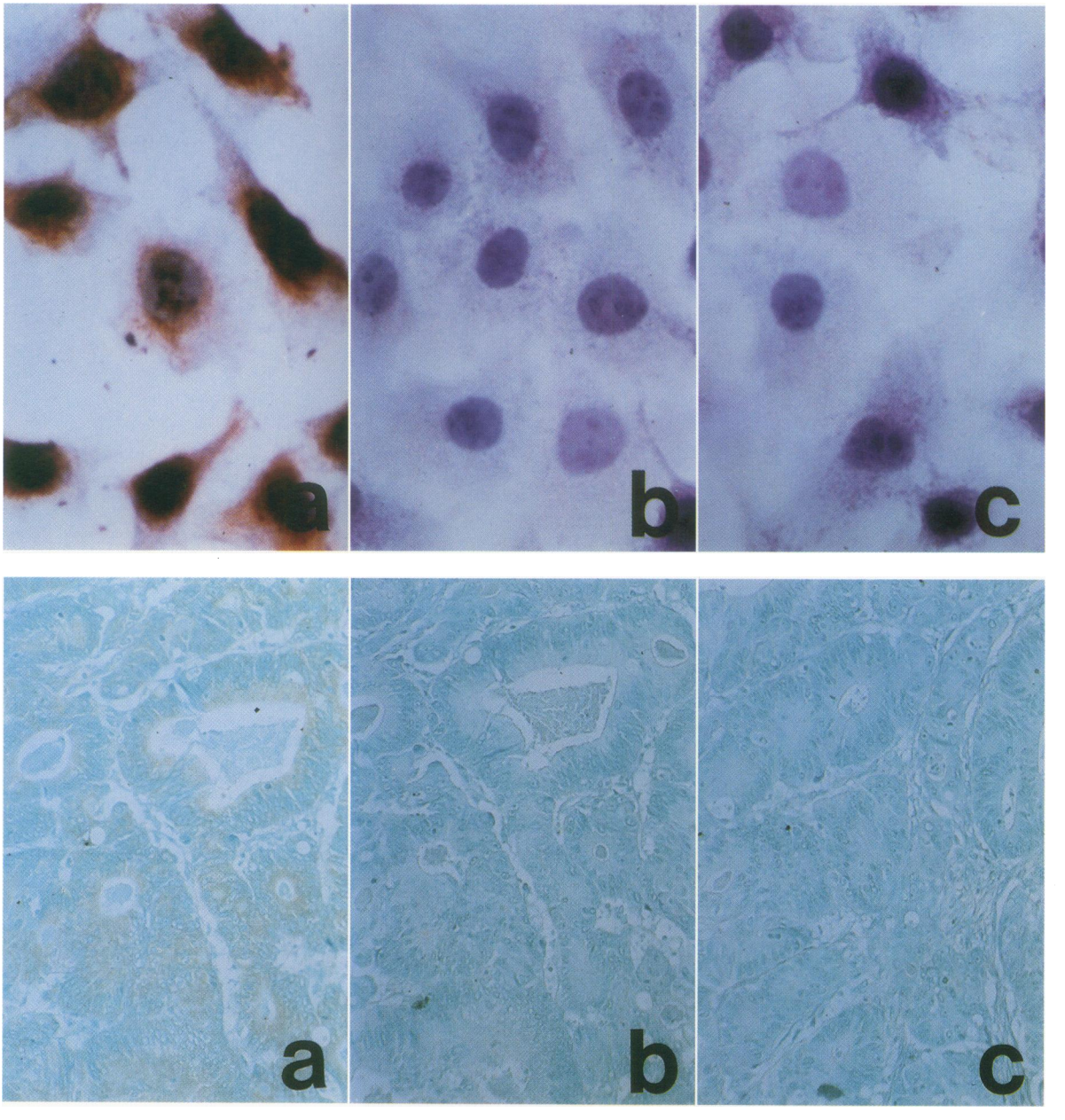


**Figure 2.** *Immunocytochemical staining of the A375 for TGF-α (high-power view).* **a**: *OAL-MTG01 reacted with the cytoplasma of A375 cells.* **b**: *Recombinant mouse IgG1 did not detect IR–TGF-α.* **c**: *Absorption of OAL-MTG01 with an excess amount of recombinant TGF-α did not detect IR–TGF-α.*

**Figure 3.** *Immunohistochemical staining of the colorectal cancer specimen for TGF-α (middle-power view). This specimen showed a moderately differentiated adenocarcinoma and its staining intensity grade was + + +.* **a**: *OAL-MTG01 reacted with the cytoplasma of cancer cells. IR-TGF-α was not detected in connective tissue of the specimen.* **b**: *Recombinant mouse IgG1 did not detect IR–TGF-α as well as A375.* **c**: *Absorption of OAL-MTG01 with an excess amount of recombinant TGF-α did not detect IR–TGF-α as well as A375.*

## *Immunohistochemical Analysis of Colorectal Cancers and Normal Colorectal Mucosa*

The incidence of IR–TGF-α and IR-EGF positivity is shown in Table 1. Immunoreactive TGF-α was detected in 16 of 19 invasive carcinomas. Although IR–TGF-α could not be detected in normal mucosa. Immunoreactive EGF was found in 7 of 19 invasive adenocarcinomas and in only 1 of 12 normal mucosa. Relationships of IR–TGF-α and IR-EGF production in the same specimens are shown in Table 2. All of IR-EGF–positive colorectal cancers showed production of IR–TGF-α. The specimens of the stronger IR-EGF staining intensity grades had a tendency for stronger IR–TGF-α staining intensity grades. Neither the incidence nor grade of IR–TGF-α var-

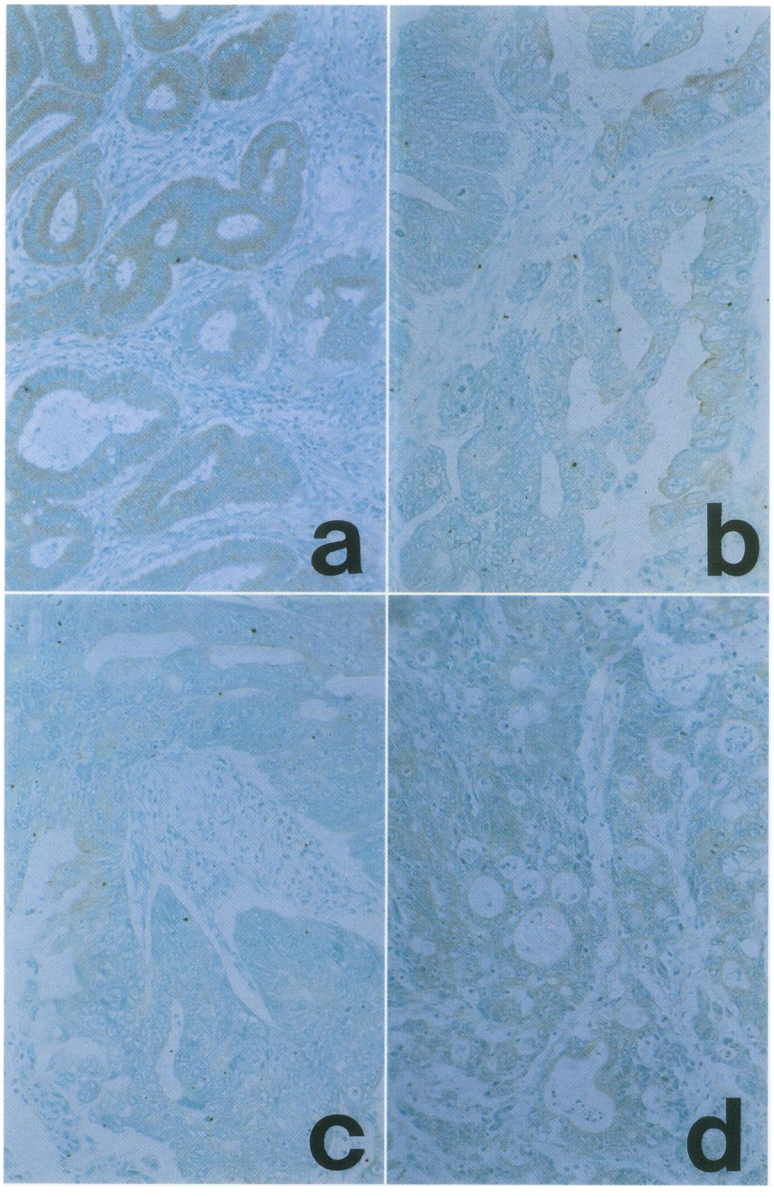


**Figure 4.** *IR-TGF–α positive staining of colorectal cancer specimens with each histologic grade (middle-power view). In all histologic grades IR–TGF-α was detected in the cytoplasma of cancer cells and was not detected in connective tissue of the specimen.* **a**: *Well-differentiated adenocarcinoma. Staining intensity grade of this specimen was + + +.* **b**: *Moderately differentiated adenocarcinoma. Staining intensity grade of this specimen was + +.* **c**: *Moderately differentiated adenocarcinoma. Staining intensity grade of this specimen was +.* **d**: *Poorly differentiated adenocarcinoma. Staining intensity grade of this specimen was + + +.*

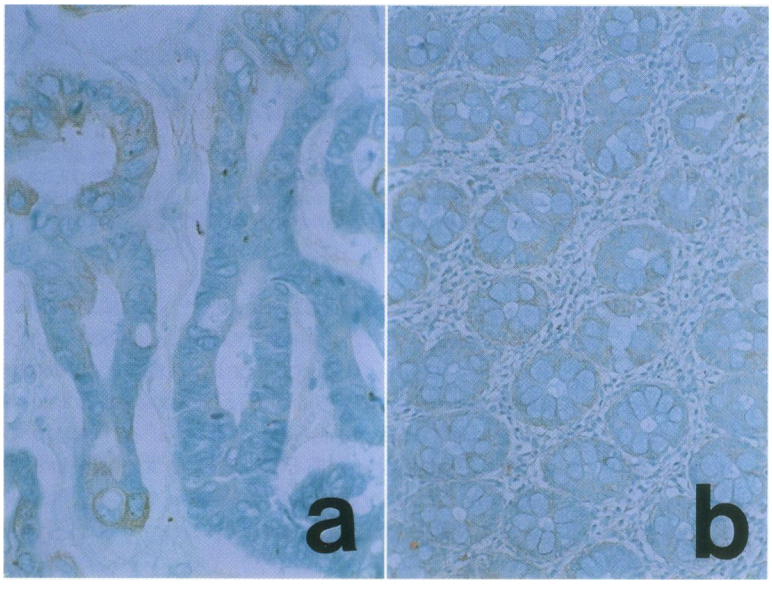


**Figure 5.** *Immunohistochemical staining of IR-EGF–positive specimens (middle-power view).* **a**: *This showed a well-differentiated adenocarcinoma, and its staining intensity grade was + +. IR-EGF was detected in the cytoplasma of cancer cells as well as IR–TGF-α.* **b**: *IR-EGF in normal mucosa. IR-EGF was detected in the cytoplasma of gland cells as well as cancer cells and was not detected in connective tissue.*

**Table 1.** *Incidence of Cases with Positive Staining for IR-TGF-α and IR-EGF*

| Histology | Number of cases | Immunoreactivity in cancer cells | |
|---|---|---|---|
| | | IR–TGF-α (%) | IR–EGF (%) |
| Invasive cancers | 19 | 16 (84.2)* | 8 (42.1)† |
| Well | 7 | 6 (85.7)‡ | 3 (42.9)§ |
| Moderately | 11 | 9 (81.3)‖ | 5 (45.5)# |
| Poorly | 1 | 1 (100) | 0 (0.00) |
| Normal mucosa | 12 | 0 (0.00)¶ | 1 (8.3)** |

* and †, ‡ and §, ‖ and #: $P < 0.01$.
* and ¶, † and **: $P < 0.01$.

ied with histologic type, as was also the case for the incidence and grade of IR-EGF. There was no correlation between IR–TGF-α or IR-EGF production and the Dukes' stage.

## Discussion

In the present study, we demonstrated that OAL-MTG01, which recognized recombinant h–TGF-α, did not cross-react with recombinant h-EGF in an RIA system. This antibody detected IR-TGF-α immunohistochemically in the A375 cell line that produces TGF-α.[4] In contrast, recombinant mouse IgG1 did not react with A375. An excess amount of h–TGF-α inhibited, by competition, binding of this antibody to the IR–TGF-α produced by A375. The characteristics of KEM-10 were described previously.[5] Using human submandibular gland tissue, known to produce EGF, we also confirmed immunohistochemically that KEM-10 has specificity for EGF. Given their specificity profiles, these antibodies were useful for determining the productions of IR–TGF-α and IR-EGF.

Immunohistochemical analysis was performed using these antibodies. Immunoreactive TGF-α was detected in 16 (84.2%) of 19 invasive colorectal cancers. The gland cells of normal mucosa, however, had no IR–TGF-α. Thus the high incidence of production of IR–TGF-α in colorectal cancers is one characteristic. Recently TGF-α mRNA has been found to be expressed in normal tissues of the human gastrointestinal tract as well as in colorectal cancers.[9,10] A small amount of IR–TGF-α detected by RIA was also reported.[19] The reasons for the discrepancy between those and our findings may be explained as follows: 1) Transforming growth factor α mRNA in normal mucosa might not be translated into mature protein. 2) Transforming growth factor α in normal mucosa might be expressed in forms not recognizable by this antibody. 3) Transforming growth factor α in normal mucosa might be present in extremely small amounts, thereby preventing detection by this technique.

As for the expression of EGF receptor, a common receptor both for TGF-α and EGF, a recent study indicated the detection of IR-EGF receptor expression in almost all human colon cancers.[20] Increased expression both of TGF-α and EGF receptor mRNA in several cancer cells has also been shown.[21] These findings along with ours support the possibility that interactions between TGF-α and EGF receptor contribute to colorectal cancer cell growth.

Regarding EGF, immunostaining demonstrated IR-EGF to be present in 7 (36.8%) of 19 invasive colorectal

**Table 2. Production of IR-TGF-α and IR-EGF in Colorectal Invasive Cancers**

| No. | IR–TGF-α | IR-EGF | Histology | Dukes' stage |
|---|---|---|---|---|
| 1 | + + + | + + + | Well | A |
| 2 | + + + | + + | Moderately | B |
| 3 | + + + | + + | Moderately | C |
| 4 | + + + | − | Moderately | C |
| 5 | + + + | − | Poorly | A |
| 6 | + + + | − | Well | A |
| 7 | + + | + | Moderately | C |
| 8 | + + | + | Moderately | C |
| 9 | + + | + | Moderately | C |
| 10 | + + | − | Well | C |
| 11 | + + | − | Moderately | A |
| 12 | + | + | Well | A |
| 13 | + | + | Well | A |
| 14 | + | − | Moderately | C |
| 15 | + | − | Moderately | C |
| 16 | + | − | Well | A |
| 17 | − | − | Moderately | C |
| 18 | − | − | Well | C |
| 19 | − | − | Moderately | A |

Classification of staining intensity grade: + + +: 100%–70%, + +: 70%–30%, +:30–0% positive. −: negative.

cancers and 1 (8.3%) of 12 normal colorectal mucosa. The incidence of the production of IR-EGF in invasive colorectal cancer was higher than that in normal colorectal mucosa. Epidermal growth factor may take part in the regeneration and repair of non-neoplastic tissue as well as the cancer cell growth, and thus IR-EGF in normal colorectal mucosa may function similarly.[22] Comparing IR–TGF-α with IR-EGF, the incidence of IR–TGF-α production was significantly higher than that of IR-EGF production in colorectal cancers. In the synchronous productions of IR–TGF-α and IR-EGF in the same cancer specimen, all the IR-EGF–detectable specimens demonstrated the production of IR–TGF-α. Thus IR–TGF-α may be more important than IR-EGF in the growth of colorectal cancers.

In histologic analysis of colorectal cancers as to IR–TGF-α and IR-EGF production, differences could not be found in incidence and grade. One reason for this may be that almost all colorectal cancers are differentiated adenocarcinomas.

In summary, IR–TGF-α was detected frequently in colorectal cancers and was present in a greater percentage of colorectal cancer specimens than was IR-EGF. Thus IR–TGF-α may be valuable as a possible tumor marker of colorectal cancer and may play an important role in their growth. Furthermore our findings may be very useful in understanding the biology of colorectal cancer. Finally the expression of these growth factors has been reported to occur in several human cancer cells. Recent progress, however, has determined several homologous substances to EGF family protein (Amphiregulin, etc.).[23] It will be important to examine the production of these homologous substances in colorectal cancer.

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