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Expression of *cripto*, a Novel Gene of the Epidermal Growth Factor Family, in Human Gastrointestinal Carcinomas

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The expression of mRNA for *cripto* gene, a novel transforming gene of the epidermal growth factor family, was examined in 20 alimentary tract carcinoma cell lines, 60 surgically resected tumor tissues and their adjacent normal mucosas. Although the *cripto* mRNA was not detected in esophageal carcinomas or in normal mucosas, it was detected in gastric and colorectal carcinomas. In gastric carcinomas, 2.2 kb *cripto* mRNA was detected in one cell line, all the gastric carcinoma tissues and their adjacent normal mucosas. Of 23 gastric tumor tissues 8 (34.8%) exhibited a higher mRNA level than normal gastric mucosas. *cripto* mRNA was detected in 2 out of 6 colorectal carcinoma cell lines. Interestingly, 18 (81.8%) out of 22 colorectal carcinoma specimens expressed a higher level of *cripto* mRNA than that in normal mucosas. The level of the expression was higher than that in gastric carcinoma tissues. The expression was also correlated to tumor stage of colorectal carcinomas.

Key words: *cripto* — Gastrointestinal carcinoma — Growth factor

Human gastrointestinal tumors express a variety of growth factors that evidently regulate the growth of cancer cells.[1-3] We have previously reported that EGF[6] and TGF-α produced by tumor cells have a crucial role in the growth of gastrointestinal carcinomas in an autocrine fashion.[4-6] Recently a novel human growth factor-related gene, *cripto*, was cloned from an undifferentiated human teratocarcinoma cell line NTERA2 clone D1 (NT2D1). The *cripto* cDNA is 2020 bp in length with a 564 bp open reading frame, encoding a protein of 188 amino acids, of which the central portion shares structural homology with human EGF, human TGF-α and human amphiregulin in that it contains a cysteine-rich motif.[7] Moreover, *cripto* exhibits transforming activity in transfected mouse NIH3T3 fibroblasts[7] and in mouse NOG-8 mammary epithelial cells,[8] like EGF or TGF-α. We report here the expression of mRNA for the *cripto* gene in gastrointestinal carcinomas.

Six esophageal, 8 gastric, and 6 colorectal carcinoma cell lines were routinely grown in RPMI-1640 (Nissui Co., Tokyo) supplemented with fetal bovine serum (Whittaker M. A. Bioproducts Inc., Maryland). The cell lines we used were as follows; 6 human esophageal carcinoma cell lines (TE-1, well differentiated squamous carcinoma; TE-2 and TE-5, poorly differentiated squamous carcinoma; TE-7, adenocarcinoma; TE-8 and TE-12, moderately differentiated squamous carcinoma) which were kindly provided by Dr. T. Nishihara (Tohoku University, Sendai). TMK-1 cell line (poorly differentiated gastric adenocarcinoma) was established in our laboratory. KATO-III and HSC-39 cell lines established from signet ring cell carcinoma were kindly provided by Dr. Sekiguchi (University of Tokyo, Tokyo) and Dr. K. Yanagihara (Hiroshima University, Hiroshima),[9] respectively. The other five human gastric carcinoma cell lines (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28 and MKN-74, well differentiated adenocarcinoma; MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima). Five human colorectal carcinoma cell lines (DLD-1, LoVo, CoLo201, CoLo320DM, SW837) were provided by the Japanese Cancer Research Resources Bank (JCRB). TCO colon carcinoma cell line was established in our laboratory from a colon carcinoma.

Fifteen primary esophageal, 23 primary gastric, and 22 primary colorectal carcinomas were used. Tumor specimens were frozen in liquid nitrogen immediately after

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[6] The abbreviations used are: EGF, epidermal growth factor; TGF-α, transforming growth factor-α; cDNA, complementary DNA; poly(A)$^+$, polyadenylated; kb, kilobase; T/N ratio, tumor/normal ratio.

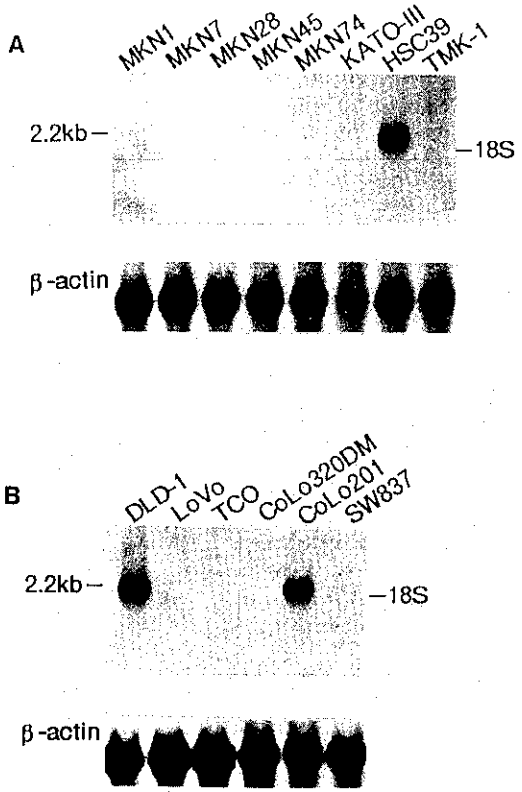

Fig. 1. *cripto* mRNA levels determined by Northern hybridization in gastric (A) and colorectal (B) carcinoma cell lines. The 2.2 kb mRNA was determined in HSC-39 gastric carcinoma cell line and DLD-1 and CoLo201 colorectal carcinoma cell lines. β-Actin probe was applied as an internal control.

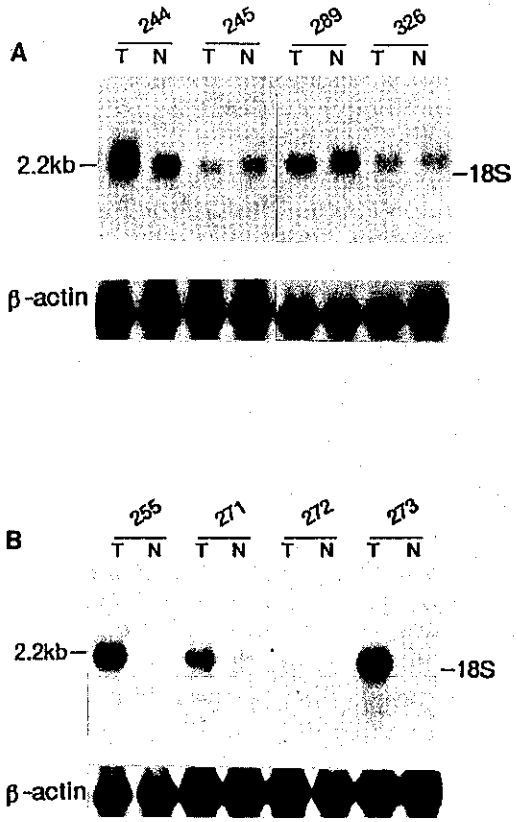

Fig. 2. *cripto* mRNA levels determined by Northern hybridization in gastric (A) and colorectal (B) carcinomas. Numbers above the lanes are sample numbers (T: tumor specimen, N: normal mucosa). Histological types of the cases were as follows. In gastric carcinomas: 244 and 245, well differentiated adenocarcinoma; 289, poorly differentiated adenocarcinoma; 326, scirrhous carcinoma. In colorectal carcinomas: 271 and 273, well differentiated adenocarcinoma; 255 and 273, moderately differentiated adenocarcinoma. β-Actin probe was applied as an internal control.

surgical removal and stored at −80°C until use. Adjacent normal mucosa excluding muscle layer was taken from the same patient and used as a normal control. It was confirmed microscopically that normal mucosa did not contain tumor cells by cryostat sectioning.

RNAs were extracted by the guanidine isothiocyanate/cesium chloride method.[10] Five to 10 μg of poly(A)$^+$-selected RNA was electrophoresed on 1.0% agarose/formaldehyde gels and blotted onto nylon membrane. Filters were baked for 2 h at 80°C under vacuum. Hybridization and washing procedures were performed as described previously.[11] Filters were autoradiographed overnight at −80°C with Kodak XAR-5 filters with an intensifying screen.

A 0.9 kb human *cripto* cDNA was used for Northern blot analysis.[7] β-Actin probe was purchased from Oncor, Gaithersburg, MD.

We initially examined esophageal, gastric and colorectal carcinoma cell lines for the expression of the *cripto* gene as shown in Fig. 1. Although *cripto* expression was not detected in esophageal carcinoma cells, one gastric cell line, HSC-39, out of the 8 gastric cell lines showed a 2.2 kb *cripto* transcript. Of 6 colorectal carcinoma cell lines, 2 cell lines, DLD-1 and CoLo201, expressed *cripto* mRNA.

As shown in Table I, we examined a small panel of esophageal, gastric and colorectal primary carcinomas and their corresponding normal mucosas by Northern blotting for *cripto* mRNA expression. Although no expression of *cripto* mRNA was observed in any of the esophageal carcinoma specimens (data not shown), gastric carcinoma tissues showed various levels of expression of the 2.2 kb *cripto* transcript, as did the correspond-

Table I. Expression of *cripto* Gene and Clinical Features of 23 Gastric and 22 Colorectal Carcinomas

Case No.	Age	Sex	Histology[a]	Stage[b]	Relative expression[c] (T/N) of *cripto* gene	Case No.	Age	Sex	Histology[a]	Stage[b]	Relative expression[c] (T/N) of *cripto* gene
Gastric carcinomas						Colorectal carcinomas					
216	71	F	Sci	III	0.3	255	74	M	Mod	B	4.1
218	63	M	Sci	III	0.3	271	73	M	Well	C	2.8
219	27	F	Sci	IV	1.5	272	63	M	Mod	B	1.0
220	63	F	Sci	III	0.5	273	79	F	Well	B	3.5
226	59	M	Well	I	0.9	279	70	M	Mod	A	1.0
243	79	M	Poor	I	0.7	281	80	F	Well	B	2.5
244	72	M	Well	II	1.4	282	57	M	Poor	B	1.3
245	51	M	Well	II	1.3	288	50	F	Mod	B	1.1
246	44	M	Well	II	2.1	401	56	F	Mod	C	1.2
247	48	F	Sci	III	0.3	402	56	F	Well	C	2.4
248	73	M	Well	II	0.2	403	73	F	Well	B	1.3
249	45	F	Poor	IV	1.0	404	56	F	Well	C	1.5
289	79	F	Poor	IV	0.8	405	72	M	Well	A	1.8
291	62	M	Poor	III	0.1	406	72	F	Well	C	2.9
292	72	F	Poor	II	0.8	407	63	M	Mod	C	4.6
294	37	F	Sci	IV	0.8	408	62	M	Muc	C	1.4
326	74	M	Sci	IV	1.0	409	69	F	Poor	B	1.3
327	50	M	Well	III	1.9	410	69	M	Mod	B	1.0
332	75	M	Poor	II	1.5	411	64	F	Mod	C	1.5
333	63	F	Poor	II	1.2	412	70	F	Well	C	1.6
334	71	F	Sci	III	0.2	413	63	F	Mod	B	1.0
335	58	M	Poor	II	0.2	414	56	M	Mod	C	1.2
339	62	M	Well	III	2.9						

a) In gastric carcinomas, according to the criteria of the Japanese Research Society for Gastric Cancer. Well, well differentiated adenocarcinoma including papillary and tubular adenocarcinoma; poor, poorly differentiated adenocarcinoma including signet ring cell carcinoma and mucinous adenocarcinoma; sci, scirrhous gastric carcinoma. In colorectal carcinomas, according to the criteria of the Japanese Research Society for Cancer of Colon, Rectum and Anus. Well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma; muc, mucinous carcinoma.
b) In gastric carcinomas, according to the criteria of the Japanese Research Society for Gastric Cancer. In colorectal carcinomas, according to Dukes' classification.
c) The ratio (tumor/normal) was calculated from autoradiographic signal intensities of the bands on the Northern blots using densitometric scanning, normalized by internal control.

ing normal gastric mucosas (Fig. 2A). Interestingly, most specimens of colorectal carcinomas exhibited higher levels of *cripto* mRNA than those of the corresponding normal mucosas, which expressed very low levels of this gene (Fig. 2B).

The relationship between *cripto* gene expression and histology of gastric and colorectal carcinomas is summarized in Table II. Expression of the *cripto* gene was found in all cases of primary gastric carcinomas and 8 of them (34.8%) showed a higher level of *cripto* mRNA expression than the corresponding normal gastric mucosas. Histologically, 5 (71.4%) of 7 well differentiated gastric cancers expressed a higher level of *cripto* mRNA than the corresponding normal mucosa. More importantly, 18 (81.8%) out of 22 colorectal carcinomas showed a higher level of *cripto* mRNA than the corresponding normal mucosas. The level of *cripto* mRNA expression in colorectal carcinomas was found to be 2.7 times higher than that in gastric carcinomas as determined from the signal intensity of the bands on the Northern blots using densitometric scanning.

The high incidence of *cripto* mRNA expression in colorectal carcinoma tissues led us to examine the relationship between expression and clinical staging. Ten (100%) out of 10 cases in Dukes' stage C revealed a higher expression of *cripto* than corresponding normal

Table II. Expression of *cripto* Gene in Primary Human Gastric and Colorectal Carcinomas

Histological type[a]	Number of cases	Level of *cripto* mRNA[b] T>N	T=N	T<N
Gastric carcinoma				
well	7	5 (71.4%)	0	2 (28.6%)
poor	8	2 (25.0%)	1 (12.5%)	5 (62.5%)
sci	8	1 (12.5%)	1 (12.5%)	6 (75.0%)
total	23	8 (34.8%)	2 (8.7%)	13 (56.5%)
Colorectal carcinoma				
well	9	9 (100%)	0	0
mod	10	6 (60.0%)	4 (40.0%)	0
others[c]	3	3	0	0
total	22	18 (81.8%)	4 (18.2%)	0

a) See footnote to Table I.
b) According to relative expression of *cripto* gene (see Table I). T>N, more than 1.0; T=N, equal to 1.0; T<N, less than 1.0.
c) Two poorly differentiated carcinoma cases and one mucinous carcinoma case.

Table III. Relationship between Expression of *cripto* and Tumor Progression in Colorectal Carcinomas

Stage[a]	Number of cases	Level of *cripto* mRNA[b] T>N	T=N	T<N
A	2	1 (50%)	1 (50%)	0
B	10	7 (70%)	3 (30%)	0
C	10	10 (100%)	0	0

a) Dukes' classification.
b) See footnote to Table II.

colorectal mucosa (Table III). The expression level of *cripto* was correlated with the degree of tumor invasion in colorectal carcinomas.

Several additional normal human tissues were also examined to determine the level of expression of *cripto* mRNA in these nonpathologic tissues. In ileum mucosa and kidney, expression of *cripto* mRNA was detected, as well as in gastric mucosas (data not shown).

In the present study, most of the colorectal carcinomas expressed a high level of *cripto* mRNA, whereas no esophageal carcinomas showed *cripto* expression. In fact, the expression was correlated with tumor staging in colorectal cancer. Since *cripto* is overexpressed in a majority of colorectal carcinomas, it may play an important role in the growth and progression of colorectal cancer. Conversely, expression of *cripto* mRNA was higher in well differentiated gastric cancers and their mean T/N ratio of *cripto* mRNA levels was similar to that of colorectal carcinomas. Therefore, it is likely that well differentiated gastric cancers might develop by a genetic pathway similar to that of colorectal carcinoma. This possibility is supported by aspects of genetic alterations in gastric carcinomas, in that well differentiated gastric cancers as well as colorectal cancers are more frequently associated with allelic losses on chromosome 5q and 17p (p53 locus).[12]

Various levels of *cripto* mRNA were detected not only in carcinoma tissue but also in normal tissues such as gastric mucosa. This is quite different from the level and distribution of EGF expression examined previously in gastric tissues. Approximately one-third of gastric carcinomas express EGF mRNA, whereas it is not detected in normal gastric mucosas.[5] TGF-α mRNA is also detected in normal mucosas of the esophagus, small intestine and colon.[13] In contrast, *cripto* mRNA was not expressed in esophageal mucosa. It was found in ileal mucosa. Interestingly, 10 (83.3%) out of the 12 gastric noncancerous mucosas which contained a high degree of intestinal metaplasia expressed high levels of *cripto* mRNA. Thus, *cripto* mRNA expression in the gastric noncancerous mucosa might account for the metaplastic change of the gastric mucosa.

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