Elongation factor 1 γ mRNA expression in oesophageal carcinoma

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Abstract

Elongation factor 1 γ (EF1 γ) is known to be a subunit of EF1, one of the G proteins that mediate the transport of aminoacyl tRNA to 80S ribosomes during translation. As little is known regarding the expression of EF1 γ in human oesophageal carcinoma, this study looked at its expression using a northern blot analysis. Thirty six cases of oesophageal carcinoma and 15 oesophageal carcinoma cell lines were studied. The EF1 γ mRNA overexpression at a level of twofold or more was seen in five (14%) of 36 carcinomatous tissues compared with the normal counterparts. All five overexpressed cases showed severe lymph node metastases compared with the non-overexpressed cases, and the difference was significant ($p=0.028$). The stage of the disease of these five cases was far advanced compared with the non-overexpressed cases ($p=0.012$). All 15 oesophageal carcinoma cells expressed EF1 γ mRNA relatively lower than the gastric or pancreatic carcinoma cell lines, in which EF1 γ was originally isolated. As the expression of EF1 γ mRNA could be detected even in the biopsy specimens, its overexpression in tumour tissue may provide preoperative useful information for predicting the aggressiveness of tumours.

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In patients with carcinoma of the oesophagus, more than 90% of patients are detected at an advanced stage of the disease.¹⁻³ This carcinoma carries a poor prognosis, and the five year survival rates are only 15 to 20%.⁴ It is therefore important to identify a good biological marker that could be useful in predicting the aggressiveness of the tumour.

cDNA for elongation factor 1 γ (EF1 γ) was isolated from a cDNA library of the human pancreatic adenocarcinoma cell line, CAPAN2.⁵ EF1 γ mRNA was overexpressed in the malignant tissue of the pancreas (78%) in comparison with corresponding normal pancreatic tissue,⁵ and similar findings were seen in adenocarcinomas⁶ and adenomas⁷ of the colon. We recently reported that EF1 γ mRNA was also overexpressed in gastric carcinomatous tissues compared with normal counterparts in 73% of the cases.⁸

To our knowledge, there have been no previous reports studying the EF1 γ gene expression in human oesophageal carcinomas.

The purpose of this study was thus to discover if EF1 γ mRNA was or was not expressed in oesophageal carcinoma tissues. We also studied the EF1 γ mRNA overexpression as a marker of biological behaviour for patients with oesophageal carcinoma. In addition we examined the relation between the expression of EF1 γ and the growth rate in the oesophageal carcinoma cell lines to determine the significance of the EF1 γ in growth of oesophageal carcinoma cells.

Methods

PREPARATION OF EF1 γ CDNA PROBE

The probe for the EF1 γ sequence was constructed by a polymerase chain reaction (PCR) on human cDNA libraries. Briefly, after PCR amplification using specific primers (5'-GCTGATTCCGATATATAGTGCC-3', 5'-AAAGGATGACACTGGCGAAG-3'), the amplified 784 bp fragment was cloned into a pUC118 vector and thereafter certified by nucleotide sequencing as described previously.⁸ The nucleotide sequences were identical with that of human EF1 γ cDNA registered at EMBL (accession no X63527). This clone was labelled with [α -³²P] deoxycytidine 5'-triphosphate (20 μ Ci, 3000 Ci/m mol) using a random primed DNA labelling kit.

SURGICAL SPECIMENS

Thirty six cases of oesophageal squamous cell carcinoma were analysed. All the tissue samples were obtained during the period from September 1991 to July 1994 at Saitama Cancer Centre and Oita Prefectural Hospital. The patients included 34 men and two women with a mean age of 63 years (range, 48-81). The tumour was located in the upper oesophagus (n=2), the middle oesophagus (n=24), and the lower oesophagus (n=10). Seven tumours were well differentiated, 14 were moderately differentiated, and 11 were poorly differentiated squamous cell carcinomas. The depth of invasion of the tumour was as follows, eight within the proper muscular layer, 24 invading the adventitia, and four invading the adjacent organs. The cases with lymph node metastases were classified into three groups: the non-metastatic group (n0), the mild metastatic group (n1 or n2), and the severe metastatic group (n3 or n4), according to the guidelines for the clinical and pathological studies on carcinoma of the oesophagus.⁹ Of these three groups, the severe lymph node metastasis group was defined as follows¹⁰: (1) lymph node metastasis exists below the lower thoracic paraoesophagus,

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Figure 1: Representative cases; a northern blot analysis of EF1 γ mRNA expression in oesophageal carcinoma tissue (T), and normal corresponding tissue (N). The bands of 1.7 kb in length are seen in all lanes. As an internal control, a cDNA clone coding a partial sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used. The ratio of the EF1 γ expression per GAPDH expression was shown in the lower row.

while the primary tumour is located on the upper oesophagus. (2) It also exists below the lesser curvature or left gastric artery, while the primary tumour exists in the middle oesophagus. (3) It exists above the common hepatic artery and below the supra pylorus, when the primary tumour is in the lower oesophagus. The cases were classified into four stages as follows, three were stage I, one was stage II, 16 were stage III, and 16 were stage IV.

ENDOSCOPIC BIOPSY SPECIMENS

To determine the usefulness of biopsy specimens for evaluating the mRNA expression by northern blot, three and six endoscopic biopsy specimens were obtained using biopsy forceps from oesophageal carcinoma tissue and the adjacent normal tissue, respectively. This examined case with oesophageal carcinoma was independent of the 36 analysed cases.

CARCINOMA CELL LINES

Fifteen oesophageal cancer cell lines were studied. Of these 15, 13 were the TE series obtained from Tohoku University¹¹; and two were the KSE series produced in our university. Pancreatic (RF1, CRL1739, CAPAN2) and gastric (CRL1420, CRL1687) carcinoma cell lines were used as a control. These cell lines were maintained in D-MEM (TE series) and RPMI1640 (KSE series) medium containing 10% fetal calf serum and antibiotics.

Twelve oesophageal carcinoma cell lines (1, 2, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14) from the TE

series were incubated at 37°C for 72 hours to calculate the doubling time of cell population number from the growth curve in the logarithmic phase.^{12 13}

TOTAL CELLULAR RNA PREPARATION

About 500 mg of tumour tissue and normal mucosal tissue were obtained in each surgical specimen and were frozen at -80°C. The total RNA was extracted from the specimens according to the method of Chirgwin *et al.*¹⁴ Each tissue in guanidinium isothiocyanate solution was homogenised with homogeniser (HG30 Homogenizer, HITACHI) so that it could be dissolved in a liquid. The total RNA was extracted with ultracentrifugation through a cesium chloride solution at 32 000 rpm for more than 20 hours, and then the pellet was purified.

On the other hand, the extraction of total RNA from the biopsy specimen was according to the previous method of acid guanidinium thiocyanate/phenol/chloroform extraction.¹⁵ In this process, we treated all the samples in Eppendorf tubes (Eppendorf, Germany). We then measured the concentration at a wavelength of 260 nm using spectrophotometer. The total cellular RNA was extracted from 15 carcinoma cell lines using the same procedures as the frozen tissue samples.

NORTHERN BLOT HYBRIDISATION

The methods used have all been described elsewhere.¹⁶ Briefly, equal amounts of 20 μ g of

Clinicopathological factors and the expression of EF1 γ in 36 cases of oesophageal carcinoma

	Expression of EF1 γ		
	T/N>2	T/N $\leq$ 2	
Age	63.2	63.1	NS
Sex			NS
Male	5	29	
Female	0	2	
Location			NS
Upper	0	2	
Middle	3	21	
Lower	2	8	
Histology*			NS
Well	2	5	
Moderately	1	13	
Poorly	2	9	
Depth of tumour invasion			NS
Within the proper muscle layer	0	8	
Invading the adventitia	4	20	
Invading the adjacent organs	1	3	
Lymphatic vessel invasion			NS
Absent	0	2	
Present	5	29	
Vascular vessel invasion			NS
Absent	1	2	
Present	4	29	
Lymph node metastasis			<0.05†
n0	0	5	
n1	0	3	
n2	0	12	
n3	3	5	
n4	2	6	
Stage†			<0.05‡
I	0	4	
II	0	1	
III	0	16	
IV	5	10	

T/N=tumour/normal ratio. *Four undifferentiated tumours are excluded. †According to the guidelines for clinical and pathological studies on carcinoma of the oesophagus (see ref 9). ‡This difference is seen between the mild lymph node metastasis group n1/n2 (n=15) and the severe lymph node metastasis group n3/n4 (n=16). §This difference is seen between stage I/II/III (n=21) and stage IV (n=15).

Figure 2: A northern blot analysis of EF1 γ in cell lines of two gastric carcinomas (RF1, CRL1739), three pancreatic carcinomas (CAPAN2, CRL1420, CRL1687), and 15 oesophageal carcinoma (13 were TE series, two were KSE series). The expression ratio of EF1 γ in oesophageal or gastric carcinoma cell lines relative to that of CAPAN2 was shown.

total RNA from 36 pairs of surgical specimens and 15 carcinoma cell lines, and equal amounts of 15 μ g of total RNA from three biopsy samples were loaded onto each lane of 1.0% agarose-formaldehyde gels and electrophoresed for eight hours. Those RNA were then transferred onto Hybond-N nylon membranes. Using prepared cDNA probes, overnight hybridisation at 42°C was done. After hybridisation, followed by washing, the membranes were exposed to imaging plates. The hybridisation signals were quantified using a Fuji Image Analyzer (BAS1000, Fuji Photo Film Ltd). Then the tumour-normal ratio of mRNA expression for each pair was calculated. Next, the membranes were dehybridised to be used for another hybridisation with a GAPDH probe as an internal control.¹⁷ After the equal amount of mRNA extracted from carcinoma cell lines was confirmed with the ethidium bromide solution, it was then hybridised with the EF1 γ probe.

STATISTICAL ANALYSIS

The data were analysed by the Student's *t* test. The correlation coefficient between doubling time of cell number and the expression of EF1 γ was analysed by a linear regression analysis.

Results

NORTHERN BLOT ANALYSIS

Surgical specimens

A northern blot analysis showed that the expression of EF1 γ mRNA signal was recognised in both the tumour and the normal corresponding tissue of every specimen, and some representative cases and the ratio of EF1 γ expression are shown (Fig 1). In this

study, more than a twofold of tumour-normal (T/N) signal ratio was implicated as a case of overexpression. In five (13.9%) of 36 cases, an overexpression of EF1 γ mRNA was seen in oesophageal carcinoma, in comparison with the corresponding normal mucosa. In the other 31 (86.1%) cases, the expression of EF1 γ mRNA was less than twofold. The Table summarises the clinicopathological characteristics of five overexpressed (T/N>2) cases and 31 non-overexpressed (T/N<2) cases. All five overexpressed cases exhibited severe (n3 or n4) lymph node metastases while 11 (35%) of 31 non-overexpressed cases showed the same grade metastasis, and the difference was significant ($p=0.028$). Concerning the stage of the disease, stage IV was seen in all five overexpressed cases while it was only seen in 10 of 31 non-overexpressed cases ($p=0.012$). There was no difference between the overexpressed and non-overexpressed cases among the other clinicopathological factors such as, age, sex, tumour location, depth of invasion, histological type, or vascular invasion.

Carcinoma cell lines

Figure 2 shows the expressions of EF1 γ mRNA in all 15 oesophageal carcinoma cell lines, two gastric carcinoma cell lines, and three pancreatic carcinoma cell lines. The expression of EF1 γ mRNA was seen in all oesophageal carcinoma cell lines. The expression of EF1 γ in oesophageal carcinoma cell lines was relatively lower than that in the gastric or pancreatic carcinoma cell lines (Fig 2).

In addition, the relation between cell growth rate, evaluated by the doubling time of cell population number, and the expression of EF1 γ in 12 of 15 oesophageal carcinoma cell lines, were both analysed to show that no correlation existed between them.

Biopsy specimens

The amount of total cellular RNA extracted from biopsy specimens of both the tumour tissue and the normal mucosa was ample, and good quality results were obtained by means of a northern blot analysis. The EF1 γ expression per GAPDH expression was 1.33 (Fig 3).

Discussion

Elongation factor 1 (EF1) consists of four subunits (α , β , γ , and δ), and plays an important part in protein synthesis with an elongation of the polypeptide chains. In the control of cell cycle, EF1 γ is important as one of the protein synthetic apparatus. EF1 γ is responsible for the binding of aminoacyl tRNA to the ribosome with hydrolysis of guanosine triphosphate (GTP). EF1 $\beta\gamma$ complex catalyses the guanosine diphosphate (GDP) to GTP exchange on EF1 α . In the regulation of translation, EF1 γ is phosphorylated as a major substrate for P34, one of the maturation promoting factors, which is related to the product of the gene *cdc2* controlling cell division.¹⁸⁻²⁰ Recent reports also expressed EF1 γ mRNA in

Figure 3: A northern blot analysis of the EF1 γ mRNA extracted from endoscopic biopsy specimens of a case with carcinoma of the oesophagus. We used equal amount of 15 μ g of total RNA from one tumour and two normal samples for northern blotting. In tumour tissue, 77 μ g of total cellular RNA was extracted from three biopsy specimens. While, in the normal mucosa, 34 μ g and 69 μ g of total cellular RNA was extracted from three and six biopsy specimens, respectively. The ratio of the EF1 γ expression per GAPDH expression is 1.33.

various human carcinomas as follows: seven (78%) of nine pancreatic carcinoma specimens expressed higher values of EF1 γ mRNA than the corresponding normal appearing adjacent pancreatic tissue.⁵ An overexpression was seen in the colorectal carcinoma tissue in comparison with the normal adjacent colonic tissue,⁶ and was also seen in 14 (56%) of 25 adenomas.⁷ We examined the expression of EF1 γ mRNA in gastric carcinoma, and an overexpression was seen in 22 (73%) of 30 gastric carcinoma in comparison with the normal corresponding tissue. Interestingly, its overexpression in gastric carcinomas correlated well with the vascular permeation of carcinoma cells. Fifteen (68%) of 22 overexpressed cases were positive for vascular permeation.⁸

In this study, the incidence of overexpression of EF1 γ mRNA in oesophageal carcinoma was five (13.9%) of 36. There were two important features recognised in the clinicopathological factors of overexpressed cases. One feature is that all overexpressed cases disclosed severe lymph node metastases, and the other was that these cases were distributed into the most advanced stages (stage IV). Similar findings have also been reported in colorectal carcinomas; in Dukes's B2 and C cases, an overexpression was seen in 73% and 94%, respectively.⁶

There was a remarkable difference in the incidence of overexpression of EF1 γ between oesophageal carcinoma and colorectal or gastric carcinoma. The expressions of EF1 γ in oesophageal carcinoma cell lines were also relatively lower than other carcinoma cell lines as shown in Fig 2. We cannot explain this discrepancy at the present time. All the examined oesophageal carcinomas were histologically confirmed squamous cell carcinomas, while all the examined colon or gastric carcinomas were adenocarcinomas. This histological discrepancy may thus be one of the factors causing a discrepancy among the oesophageal, gastric or colonic carcinomas.

The significance of overexpression of EF1 γ gene in carcinoma tissues has been unclarified. Jung *et al* reported that in squamous cell carcinoma, the enhance expression of EF1 δ , which binds to EF1 β complex loosely, precedes G₂ arrest in the exposure of radiation. EF1 β γ δ complex is responsible for G₂-M cell cycle check point. Therefore, Jung *et al* considered that the activation of cell cycle, caused by overexpression of EF1 γ , may thus induce the overgrowth of neoplastic tissue.²¹ Lew *et al*, however, suggested that the overexpression of EF1 γ was not simply because of an increase in growth rate.⁵ Our data showed that there was no relation between the expression of EF1 γ and the growth rates of the oesophageal carcinoma cells. These controversial findings imply that further studies should be performed to clarify these findings at the genetic level.

There are several clinicopathological factors that are effective in evaluating the malignant potential of oesophageal carcinomas. These factors, however, are usually obtained post-operatively by a pathological examination of the resected specimen. Our study showed that EF1 γ mRNA expression may also be effectively used for biopsy specimens. Information on the EF1 γ status can be obtained preoperatively. A careful examination of the EF1 γ mRNA expression may therefore be another useful factor for providing information on the malignant potentiality of oesophageal carcinomas.

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- 1 Sugimachi K, Watanabe M, Sadanaga N, Ikebe M, Kitamura K, Mori M, *et al*. Recent advances in the diagnosis and surgical treatment of patients with carcinoma of the esophagus. *J Am Coll Surg* 1994; 178: 363-8.
- 2 Mori M, Ohno S, Tsutsui S, Matsuura H, Kuwano H, Sugimachi K. Esophageal carcinoma in young patients. *Ann Thorac Surg* 1990; 49: 284-6.
- 3 Mori M, Adachi Y, Matsushima T, Matsuda H, Kuwano H, Sugimachi K. Lugol staining pattern and histology of esophageal lesions. *Am J Gastroenterol* 1993; 49: 284-6.
- 4 Mannel A, Becker P. Evaluation of the results of oesophagectomy for oesophageal cancer. *Br J Surg* 1991; 78: 36-40.
- 5 Lew Y, Jones D, Frazier M. Expression of elongation factor-1 gamma-related sequence in human pancreatic cancer. *Pancreas* 1992; 7: 144-52.
- 6 Chi K, Jones D, Frazier M. Expression of an elongation factor 1 γ -related sequence in adenocarcinomas of the colon. *Gastroenterology* 1992; 103: 198-202.
- 7 Ender B, Lynch P, Kim Y, Inamdar N, Cleary K, Frazier M. Overexpression of an elongation factor-1-hybridizing RNA in colorectal adenomas. *Mol Carcinog* 1993; 7: 18-20.
- 8 Mimori K, Mori M, Tanaka S, Sugimachi K. The overexpression of elongation factor 1 gamma mRNA in gastric carcinoma. *Cancer* 1995; 75: 1446-9.
- 9 Japanese Society for Esophageal Diseases. Guidelines for the clinical and pathologic studies on carcinoma of the esophagus. *Jpn J Surg* 1976; 6: 69-78.
- 10 Akiyama H, Tsurumaru H, Udagawa H, Kajiya Y. Radical lymph node dissection for cancer of the thoracic esophagus. *Ann Surg* 1994; 220: 364-73.
- 11 Nishihira T, Hashimoto Y, Katayama M, Mori S, Kuroki T. Molecular and cellular features of the esophageal cancer cells. *J Cancer Res Clin Oncol* 1993; 119: 441-9.
- 12 Fonagy A, Swiderski C, Ostrovsky AM, Bolton WE, Freeman JW. Effect of nuclear P120 expression on the proliferation capacity of breast cancer cells. *Cancer Res* 1994; 54: 1859-64.
- 13 Terano A, Nakada R, Mutoh H, Hiraishi H, Ota S, Shiina S, *et al*. Characterization of newly established cell line (JR-St) derived from human gastric signet ring cell cancer, producing tumor markers. *Gastroenterol Jpn* 1991; 26: 7-13.
- 14 Chirgwin J, Przybyla A, MacDonald R. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979; 18: 5294-9.
- 15 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. *Ann Biochem* 1987; 162: 156-9.

- 16 Mori M, Bernard G, Staniunas R, Jessup J, Steel G, Chen L. Prothymosin- α mRNA expression correlates with that of c-myc in human colon cancer. *Oncogene* 1993; **8**: 2821–6.
- 17 Saiki R, Scharf S, Faloona F, Mullis K, Horn G, Erlich H, *et al.* Enzymatic amplification of β -globin genomic sequences and restriction site analysis for the diagnosis of sickle cell anemia. *Science* 1985; **230**: 1350–4.
- 18 Janssen G, Morales J, Schipper A, Labbe J, Mulner-Lorillon O, Belle R. A major substrate of maturation promoting factor identified as elongation factor 1 $\beta\delta\gamma$ in *Xenopus* leaves. *J Biol Chem* 1991; **266**: 14885–8.
- 19 Moreno S, Nurse P. Substrates for P34 cdc2: in vitro veritas? *Cell* 1990; **61**: 549–51.
- 20 Mulner-Lorillon O, Pohlhe R, Cormier P, Labbe J, Doree M, Belle R. Purification of a p47 phosphoprotein from *Xenopus laevis* oocytes and identification as an in vivo and in vitro p34cdc2 substrate. *FEBS Lett* 1989; **251**: 219–24.
- 21 Jung M, Kondratyev A, Dritschilo A. Elongation factor 1 δ is enhanced following exposure to ionizing radiation. *Cancer Res* 1994; **54**: 2541–3.