Elongation factor 1γ mRNA expression in oesophageal carcinoma

K Mimori, M Mori, H Inoue, H Ueo, K Mafune, T Akiyoshi, K Sugimachi

Abstract
Elongation factor 1γ (EF1γ) 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 EF1γ 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 EF1γ 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 EF1γ mRNA relatively lower than the gastric or pancreatic carcinoma cell lines, in which EF1γ was originally isolated. As the expression of EF1γ 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.

Keywords: elongation factor 1γ, oesophageal carcinoma, northern blot analysis, biopsy specimens.

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 1γ (EF1γ) was isolated from a cDNA library of the human pancreatic adenocarcinoma cell line, CAPAN2.5 EF1γ mRNA was overexpressed in the malignant tissue of the pancreas (78%) in comparison with corresponding normal pancreatic tissue,6 and similar findings were seen in adenocarcinomas7 and adenomas8 of the colon. We recently reported that EF1γ mRNA was also overexpressed in gastric carcinomatous tissues compared with normal counterparts in 73% of the cases.9

To our knowledge, there have been no previous reports studying the EF1γ gene expression in human oesophageal carcinomas.

The purpose of this study was thus to discover if EF1γ mRNA was or was not expressed in oesophageal carcinoma tissues. We also studied the EF1γ mRNA overexpression as a marker of biological behaviour for patients with oesophageal carcinoma. In addition we examined the relation between the expression of EF1γ and the growth rate in the oesophageal carcinoma cell lines to determine the significance of the EF1γ in growth of oesophageal carcinoma cells.

Methods
PREPARATION OF EF1γ cDNA PROBE
The probe for the EF1γ sequence was constructed by a polymerase chain reaction (PCR) on human cDNA libraries. Briefly, after PCR amplification using specific primers (5'-GCTGATTTCCGATATAGTGCC-3'), the amplified 784 bp fragment was cloned into a pUC118 vector and thereafter confirmed by nucleotide sequencing as described previously.8 The nucleotide sequences were identical with that of human EF1γ cDNA registered at EMBL (accession no X63527). This clone was labelled with [α-32P] deoxyctydine 5'-triphosphate (20 μCi, 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 follows:10 (1) lymph node metastasis exists below the lower thoracic paraoesophagus,
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 μg of

Clinicopathological factors and the expression of EF1γ in 36 cases of oesophageal carcinoma

<table>
<thead>
<tr>
<th>Expression of EF1γ</th>
<th>T/N&gt;2</th>
<th>T/N&lt;2</th>
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<tbody>
<tr>
<td>Age</td>
<td>63.2</td>
<td>63.1</td>
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<tr>
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<tr>
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<td>29</td>
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<tr>
<td>Female</td>
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<tr>
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</tr>
<tr>
<td>Middle</td>
<td>3</td>
<td>21</td>
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<tr>
<td>Lower</td>
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<td>8</td>
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<tr>
<td>Histology*</td>
<td>2</td>
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<tr>
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<td>Poorly</td>
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<td>9</td>
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<td>Depth of tumour invasion</td>
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<tr>
<td>Within the proper muscle layer</td>
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<td>Invading the adventitia</td>
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<td>Invading the adjacent organs</td>
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<td>Lymphatic vessel invasion</td>
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<td>Vascular vessel invasion</td>
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<td>IV</td>
<td>5</td>
<td>10</td>
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</table>

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 III/IV (n=21) and stage IV (n=15).
Figure 2: A northern blot analysis of EF1γ in cell lines of two gastric carcinomas (RF1, CRL1739), three pancreatic carcinomas (CAPAN2, CRL1420, CRL1667), and 15 oesophageal carcinoma (13 were TE series, two were KSE series). The expression ratio of EF1γ in oesophageal or gastric carcinoma cell lines relative to that of CAPAN2 was shown.

Discussion

Elongation factor 1 (EF1) consists of four sub-units (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, EF1γ is important as one of the protein synthetic apparatuses. EF1γ is responsible for the binding of aminoacyl tRNA to the ribosome with hydrolysis of guanosine triphosphate (GTP). EF1βγ complex catalyses the guanosine diphosphate (GDP) to GTP exchange on EF1α. In the regulation of translation, EF1γ 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 EF1γ mRNA in total RNA from 36 pairs of surgical specimens and 15 carcinoma cell lines, and equal amounts of 15 µg 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 EF1γ 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 EF1γ was analysed by a linear regression analysis.

Results

NORTHERN BLOT ANALYSIS

Surgical specimens
A northern blot analysis showed that the expression of EF1γ mRNA signal was recognised in both the tumour and the normal corresponding tissue of every specimen, and some representative cases and the ratio of EF1γ 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 EF1γ mRNA was seen in oesophageal carcinoma, in comparison with the corresponding normal mucosa. In the other 31 (86-1%) cases, the expression of EF1γ 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 (n5 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 EF1γ mRNA in all 15 oesophageal carcinoma cell lines, two gastric carcinoma cell lines, and three pancreatic carcinoma cell lines. The expression of EF1γ mRNA was seen in all oesophageal carcinoma cell lines. The expression of EF1γ 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 EF1γ 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 EF1γ expression per GAPDH expression was 1:33 (Fig 3).
The significance of overexpression of EF1\(\gamma\) gene in carcinoma tissues has been unclarified. Jung et al reported that in squamous cell carcinoma, the enhance expression of EF1\(\beta\), which binds to EF1\(\beta\) complex loosely, precedes G\(_2\) arrest in the exposure of radiation. EF1\(\beta\) complex is responsible for G\(_2\)-M cell cycle check point. Therefore, Jung et al considered that the activation of cell cycle, caused by overexpression of EF1\(\gamma\), may thus induce the overgrowth of neoplastic tissue.\(^{21}\) Lew et al, however, suggested that the overexpression of EF1\(\gamma\) was not simply because of an increase in growth rate.\(^{5}\) Our data showed that there was no relation between the expression of EF1\(\gamma\) 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 postoperatively by a pathological examination of the resected specimen. Our study showed that EF1\(\gamma\) mRNA expression may also be effectively used for biopsy specimens. Information on the EF1\(\gamma\) status can be obtained preoperatively. A careful examination of the EF1\(\gamma\) mRNA expression may therefore be another useful factor for providing information on the malignant potentiality of oesophageal carcinomas.

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