ORIGINAL ARTICLE

Correlation between cathepsin D expression and p53 protein nuclear accumulation in oesophageal squamous cell carcinoma

M Ikeguchi, T Sakatani, T Ueta, K Fukuda, S Oka, K Hisamitsu, K Yamaguchi, S Tsujitani, N Kaibara

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Aim: The lysosomal protease cathepsin D has been reported to be associated with tumour progression in malignant tumours. Expression of the gene encoding cathepsin D is known to be stimulated by oestrogen in mammary cancer cells. Recent experiments revealed that a p53 DNA binding site is located in the promoter region of the cathepsin D gene. This fact indicates that cathepsin D expression may correlate with p53 protein expression. The purpose of this study is to evaluate the expression patterns of the cathepsin D and p53 proteins in oesophageal squamous cell carcinoma (SCC).

Methods: In 154 patients with oesophageal SCC, expression of the cathepsin D and p53 proteins was measured in tumours by means of immunohistochemistry using monoclonal antibodies against cathepsin D (clone, 1C11) and p53 (clone, BP53–12).

Results: Cathepsin D was detected in tumour cells, although it was not found in normal oesophageal epithelium adjacent to carcinoma. High cathepsin D expression (positive tumour cells > 10%) was detected in 76 of 154 cases (49%) and high p53 nuclear expression (positive tumour cells > 50%) was detected in 70 cases (46%). High cathepsin D expression was significantly associated with invasive tumour growth ($p = 0.002$), poor prognosis ($p = 0.049$), and nuclear accumulation of p53 protein ($p = 0.001$). Overexpression of both p53 and cathepsin D was seen in 45 of the 154 cases (29.2%). In addition, there was a positive correlation between the cathepsin D index (percentage of cathepsin D positive tumour cells) and Ki-67 labelling index (percentage of Ki-67 positive tumour cells) in 154 oesophageal SCCs ($p = 0.257$; $p = 0.009$). However, in multivariate survival analysis, cathepsin D expression by the tumours was not an independent prognostic factor in patients with oesophageal SCC ($p = 0.236$).

Conclusions: The expression of cathepsin D by cancer cells may play an important role in the invasive growth of oesophageal SCC. Overexpression of both p53 and cathepsin D was seen frequently in tumours; p53 gene abnormalities may correlate with cathepsin D overexpression in oesophageal SCC.

See end of article for authors' affiliations

Correspondence to:
Dr M Ikeguchi, Department
of Surgery I, Faculty of
Medicine, Tottori
University, 36–1 Nishi-cho,
Yonago 683–8504,
Japan;
surgery1@
grape.med.tottori-u.ac.jp

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Malignant tumour cells invade the surrounding matrix and penetrate the basement membrane. This process is a fundamental characteristic of cancer cells. Proteases are thought to play an important role in tumorous invasion and metastasis because they degrade extracellular matrices and basement membranes.^{1,2} An extracellular matrix consists of multiple components, which include collagens, glycoproteins, and proteoglycans. Thus, extracellular matrix degradation must involve the action of a complex family of proteases.

Cathepsin D is a lysosomal acidic protease that was first identified as a 52 kDa oestrogen dependent glycoprotein in MCF-7 cells.³ Overexpression of human cathepsin D in rat embryo cells correlates with increased metastatic ability of these cells in athymic nude mice.⁴ Three forms of the enzyme are known. The 52 kDa procathepsin D is enzymatically inactive and can be secreted by cells. This molecule is converted to an active intermediate form, with a molecular mass of 48 kDa, which is then cleaved into active, mature 34 kDa and 14 kDa dimer forms.⁵ These mature forms of cathepsin D proteolytically degrade extracellular matrices and proteoglycans. Moreover, cathepsin D can activate cathepsin B and urokinase-type plasminogen activator, and these enzymes initiate the proteolytic cascade that may be responsible for the breakdown of basement membrane proteins.⁶ Thus, cathepsin D is thought to be the key enzyme in this process, and it has attracted con-

siderable attention because of its potential role in tumour invasion and metastasis.

"High expression of cathepsin D has been reported to correlate closely with poor prognosis and tumour progression in breast cancer, colorectal cancer, and thyroid cancer"

The clinical importance of cathepsin D expression has been discussed in various tumours. High expression of cathepsin D has been reported to correlate closely with poor prognosis and tumour progression in breast cancer,^{7–10} colorectal cancer,¹¹ and thyroid cancer.¹² However, the biological abnormality that regulates cathepsin D expression remains unclear. Recently, Wu *et al* reported the presence of two p53 DNA binding sites in the promoter sequence of the gene encoding cathepsin D and they revealed that either site could be bound specifically by p53 protein.¹³ These results provide evidence for a direct relation between the p53 protein and cathepsin D expression.

Abbreviations: CD, cathepsin D; CI, confidence interval; HR, hazards ratio; LI, labelling index; PBS, phosphate buffered saline; SCC, squamous cell carcinoma

Oesophageal cancer is now thought to arise through the accumulation of inactivating mutations in tumour suppressor genes, such as the p53 gene. The p53 gene product is important in the control of the cell cycle and apoptosis. Frequent mutation of the p53 gene and overexpression of the p53 protein have been found in oesophageal squamous cell carcinoma (SCC) and a significant correlation between p53 overexpression and tumour progression or poor survival has been reported in oesophageal SCC.^{14 15}

Thus, to understand the mechanism of tumour progression in oesophageal SCC, we investigated the correlation between the expression of cathepsin D and p53 in oesophageal SCC.

METHODS

Tissues

Formalin fixed and paraffin wax embedded tissues were obtained from 154 patients with oesophageal SCC who had undergone oesophagectomy between 1981 and 1997 at Tottori University Hospital. The patients comprised 138 (90%) men and 16 (10%) women, and their mean age at surgery was 64.3 years (SD, 8.8; median, 66; range, 45–84). All of the 154 tumours were diagnosed as SCC. The grades of tumour differentiation were as follows: nine tumours were identified as well differentiated SCC (G1), 66 as moderately differentiated SCC (G2), and 79 as poorly differentiated SCC (G3). The depth of tumour invasion of 46 tumours was diagnosed as pTis and pT1, that of 24 tumours as pT2, that of 49 tumours as pT3, and that of 35 tumours as pT4. Lymph node metastasis was detected in 82 cases. Liver metastasis was detected in one case at the time of surgery. The histopathological stage of the tumours in these 154 patients was diagnosed by UICC TNM classification.¹⁶ The stages of the tumours were as follows: stage 0, four; stage I, 33; stage IIA and IIB, 48; stage III, 58; and stage IV, 11. The pattern of tumour infiltration into the surrounding tissue was classified into two subgroups (invasive growth and expanding growth). Tumours with invasive growth show an indistinct border with the surrounding tissue and those with expanding growth show a distinct border with the surrounding tissue.¹⁷

Patients

None of the 154 patients had received preoperative radiotherapy or chemotherapy. Transthoracic oesophagectomy was performed on 108 patients by right sided anterolateral thoracotomy and laparotomy. Intrathoracic and perigastric lymph nodes were dissected during this procedure. Transhiatal oesophagectomy without thoracotomy was performed on 36 patients. Lower oesophagectomy through the transabdominal approach was performed on 10 patients. Curative oesophagectomy was performed on 121 patients and non-curative oesophagectomy was performed on 33 patients (liver metastasis, one; extended lymph node metastasis, seven; local invasion, 22; and cancer cells positive at oral cut margin, three). All patients were followed either at our hospital or our affiliated hospitals until December of 2000. The median follow up period of the 154 patients was 19 months (range, 2–237). Causes of death were determined from the clinical findings. The types of cancer recurrence were established by diagnostic imaging, cytology, and biopsy. Thirteen patients died from operative complications after surgery, and the in hospital mortality rate was 8.4%. Fifty patients are alive at the end of 2000, and a total of 104 patients had died by the end of 2000. Twenty eight patients died from diseases other than oesophageal SCC, and 76 patients died from a recurrence and relapse of oesophageal SCC (lymph node metastasis, 31; liver, lung, bone, and brain metastasis, 27; local recurrence, 17; and pleural dissemination, one).

Immunohistochemical staining of tumour specimens

Specimens were routinely fixed in 10% buffered formalin and embedded in paraffin wax. Four serial paraffin wax embedded

Figure 1 Cathepsin D cytoplasmic staining was detected in tumour cells only and not in normal oesophageal epithelium adjacent to carcinoma (cathepsin D immunostaining; original magnification, $\times 100$).

sections (4 μ m thick), containing tumour and non-cancerous epithelium, were prepared for immunostaining and for a negative control in each case. The sections were dewaxed using xylene and then transferred to alcohol. The slides were then placed in a citric acid buffer (10mM) and heated in a microwave oven (700 W) for 12 minutes to expose the antigens. Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide in methanol for 30 minutes. Slides were then washed three times in phosphate buffered saline (PBS) and incubated in 10% normal goat serum for 20 minutes to reduce non-specific antibody binding. After washing with PBS, four slides from each case were incubated overnight at 4°C with the monoclonal antibody against cathepsin D (clone, 1C11; already diluted; Zymed Laboratories, South San Francisco, California, USA), with the monoclonal antibody against p53 (BP53-12; diluted 1/50; Novocastra Laboratories, Newcastle, UK), with the monoclonal antibody Ki-67 (MIB-1; diluted 1/50; Immunotech International, Cedex, France), and with non-specific mouse IgG1 as a negative control. The monoclonal antibody against cathepsin D identifies both the 34 kDa and the 48 kDa forms of cathepsin D. Breast cancer tissue strongly expressing cathepsin D was used as a positive control. Oesophageal cancer tissues with strong expression of p53 and Ki-67 were used as positive controls for p53 and Ki-67 expression. After the slides were washed with PBS, biotinylated antibodies against mouse immunoglobulin were applied as second layer antibodies (Histofine ABC Kit; Nichirei, Tokyo) for 30 minutes. The reaction products were visualised with diaminobenzidine as the chromogen and the slides were counterstained with methyl green.

Evaluation

Each sample was examined under the same magnification ($\times 200$, Vanox-S; Olympus, Tokyo, Japan) by two independent pathologists (TS and TU). The cathepsin D positive, p53 positive, and Ki-67 positive tumour cells were counted by two pathologists who moved the microscopic field randomly across the section. In each sample, approximately 2000 tumour cells were examined. The results of the cathepsin D and p53 staining were classified into two groups (positive and negative). The cut off points of the two groups were based on the median percentages of positive stained tumour cells in all cases. Tumours with more than 10% of cancer cells showing strong staining for cathepsin D were classified as cathepsin D positive.¹⁸ In the case of p53 immunostaining, we decided that tumours with more than 50% of tumour cells showing strong nuclear reactivity of p53 were p53 positive.¹⁹ This p53 protein overexpression was interpreted as mutant p53 protein.^{20 21} Strong cathepsin D and p53 immunostaining was seen in all cathepsin D positive and p53 positive cases and no significant

Table 1 Correlation between clinicopathological findings and cathepsin D (CD) expression

Variables	CD positive cases (%)	p Value	p53 positive cases (%)	p Value
Tumour size (cm)*				
Small (≤ 5 , n=79)	37 (47)	0.522	38 (48)	0.498
Large (>5 , n=75)	39 (52)		32 (43)	
Histopathological grading				
G1 (n=9)	7 (78)	0.29	5 (56)	0.253
G2 (n=66)	32 (49)		25 (38)	
G3 (n=79)	37 (47)		40 (51)	
Depth of tumour invasion				
pTis, pT1 (n=46)	18 (39)	0.15	17 (37)	0.346
pT2 (n=24)	10 (42)		13 (54)	
pT3 (n=49)	26 (53)		21 (43)	
pT4 (n=35)	22 (63)		19 (54)	
Lymph node metastasis				
Absent (n=72)	33 (46)	0.413	30 (42)	0.376
Present (n=82)	43 (52)		40 (49)	
Distant metastasis				
Absent (n=146)	73 (50)	0.491	67 (46)	0.643
Present (n=8)	3 (38)		3 (38)	

*The 154 patients were divided into two subgroups according to the median tumour size (5 cm).

differences in staining intensity were seen among the cases. Tumour infiltrating macrophages (stromal cells) express cathepsin D. The percentages of cathepsin D positive stromal cells in and around the tumour were compared with those seen in the non-cancerous area in each case.

Statistical analysis

The χ^2 test and Fisher's exact probability test were used to compare frequencies. The differences in the numerical data between the two groups were evaluated by the Mann-Whitney U test. The relation between the percentage of cathepsin D positive tumour cells and that of Ki-67 positive tumour cells was analysed using the Spearman rank correlation test. Survival rates were calculated by the Kaplan-Meier method. Corrected survival rates were used; that is, only deaths caused by oesophageal SCC were taken as outcome events and all other deaths were considered as censored events. The Mantel-Cox log rank test was used to compare the survival curves. A multivariate survival analysis was performed using Cox's proportional hazard model. All statistical analyses were performed using the StatView-5.0 software package for Macintosh (Abacus Concepts, Berkeley, California, USA). Only p values of less than 0.05 were considered to be significant.

RESULTS

Cathepsin D and p53 expression in oesophageal SCC

Cathepsin D staining had a fine granular appearance and was localised to the cytoplasm of tumour cells; cathepsin D expression was not detected in normal oesophageal epithelium adjacent to the carcinoma (fig 1). The mean percentage of cathepsin D positive cancer cells in the 154 tumours was 11.4% (range, 0.3–69.9%; median, 10%) and that of p53 positive cancer cells was 53.7% (range, 0–92%; median, 50%). Table 1 shows the correlation between cathepsin D and p53 expression and clinicopathological findings in the 154 oesophageal SCCs. Even though cathepsin D expression did not correlate with the depth of tumour invasion, only one of four tumours

with carcinoma in situ was cathepsin D positive, whereas cathepsin D positive tumour cells were detected in seven of 11 tumours that had invaded the lamina propria.

It was difficult to determine the growth pattern of the tumours in the 15 cases with epithelial oesophageal SCCs or tumours that had only invaded the lamina propria. Thus, we divided the remaining 139 oesophageal SCCs, which had invaded the submucosal layer (or deeper), into two subgroups (the invasive growth group, 61; and the expanding growth group, 78). Cathepsin D expression was detected more frequently in the invasive growth group than in the expanding growth group (table 2). A considerable number of cathepsin D

Figure 2 (A) Invasive growth oesophageal squamous cell carcinoma (SCC) (haematoxylin and eosin staining; original magnification, $\times 66$). (B) A considerable number of cathepsin D positive cancer cells were seen in invasive growth oesophageal SCC (cathepsin D immunostaining; original magnification, $\times 100$).

Table 2 Cathepsin D (CD) expression and growth patterns of oesophageal squamous cell carcinomas

Growth pattern	N	CD positive cases (%)	p Value
Invasive growth	61	39 (64)	0.002
Expanding growth	78	29 (37)

Figure 3 (A) Expanding growth oesophageal squamous cell carcinoma (SCC) (haematoxylin and eosin staining; original magnification, $\times 66$). (B) Only a few cathepsin D positive cancer cells were detected in the cancer nests of expanding growth oesophageal SCC (cathepsin D immunostaining; original magnification, $\times 100$).

Table 3 Correlation between cathepsin D (CD) expression and p53 overexpression

p53 expression	N	CD positive cases (%)	p Value
Negative	84	31 (37)	0.001
Positive	70	45 (64)	

positive cancer cells were detected in the tumours with invasive growth (fig 2). In contrast, only a few cathepsin D positive cancer cells were detected in the centre of cancer nests in the tumours with expanding growth (fig 3).

Reticulum cells in the lymph follicle or stromal cells expressed cathepsin D. The cathepsin D staining intensity and positivity of stromal cells in and around tumours was stronger than that of stromal cells in non-cancerous areas of the oesophagus. However, the expression of cathepsin D in stromal cells around the tumours did not differ among the cases.

Correlation between cathepsin D, p53 protein nuclear accumulation, and proliferative activity of tumour cells

There was a significant positive correlation between p53 protein nuclear accumulation (abnormal p53 overexpression) and cathepsin D expression (table 3). Expression of both cathepsin D and p53 was detected in 45 tumours (29.2%). Strong p53 protein nuclear accumulation was detected in 37 of 61 tumours with invasive growth, although it was detected in only 28 of 78 tumours with expanding growth ($p = 0.038$). However, in some cases of expanding growth, p53 nuclear accumulation was seen in cancer cells located within the cancer nests, whereas cathepsin D was detected in tumour cells around the cancer nests (fig 4).

Figure 4 (A) Expanding growth oesophageal squamous cell carcinoma (SCC) (haematoxylin and eosin staining; original magnification, $\times 66$). (B) p53 nuclear accumulation was observed in cancer cells located within the cancer nests (p53 immunostaining; original magnification, $\times 100$). (C) Cathepsin D expression was detected in invading cancer cells around the nests (cathepsin D immunostaining; original magnification, $\times 100$).

The proliferative activity of cancer cells was assessed by the Ki-67 labelling index (Ki-67 LI, percentage of Ki-67 positive cancer cells). The mean Ki-67 LI of the 154 oesophageal SCCs was 62% (range, 11–92%). A significant positive correlation between the percentage of cathepsin D positive cancer cells and the Ki-67 LI in the 154 oesophageal SCCs was detected ($p = 0.257$; $p = 0.009$). The mean Ki-67 LI of the 76 cathepsin D positive tumours (66%) was higher than that of 78 cathepsin D negative tumours (58%; $p = 0.024$). In addition, the mean Ki-67 LI of the 70 p53 positive tumours (67%) was significantly higher than that of the 84 p53 negative tumours (56%; $p = 0.012$).

Prognostic significance of cathepsin D expression in oesophageal SCC

The disease specific, five year survival rate of the 154 patients with oesophageal SCC was 41%. The disease specific, five year survival rate of the 76 patients with cathepsin D positive tumours (32%) was significantly poorer than that of the 78

Table 4 Univariate and multivariate analysis of prognostic factors of patients with oesophageal squamous cell carcinoma

Variable	HR	95% CI	p Value
Univariate analysis			
Invasion to adventitia			
Present v absent	4.184	2.451 to 7.143	<0.001
Lymph node metastasis			
Present v absent	4.926	2.841 to 8.547	<0.001
Distant metastasis			
Present v absent	8.403	3.745 to 18.868	<0.001
Cathepsin D expression			
Positive v negative	1.583	1.002 to 2.502	0.049
p53 expression			
Positive v negative	1.502	0.67 to 1.651	0.827
Multivariate analysis			
Invasion to adventitia			
Present v absent	2.762	1.575 to 4.854	<0.001
Lymph node metastasis			
Present v absent	3.546	1.992 to 6.289	<0.001
Distant metastasis			
Present v absent	4.525	2.004 to 10.204	<0.001
Cathepsin D expression			
Positive v negative	1.325	0.832 to 2.111	0.236

HR, hazard ratio; CI, confidence interval.

patients with cathepsin D negative tumours (50%; $p = 0.049$). However, the disease specific, five year survival rate of the 70 patients with p53 positive tumours (39%) was not different from that of the 84 patients with p53 negative tumours (43% $p = 0.827$). According to the multivariate survival analysis, depth of tumour invasion, lymph node metastasis, and distant metastasis were recognised as independent prognostic factors of patients with oesophageal SCC, but cathepsin D expression was not ($p = 0.236$; table 4).

DISCUSSION

In our study, the expression of cathepsin D was detected in tumour cells but was not seen in normal oesophageal epithelium. Moreover, cancer cells that expressed cathepsin D were often detected in tumours showing invasive growth and at the invasive front of the tumour or around the cancer nests in expanding growth oesophageal SCCs. In contrast, tumour cells at the centre of cancer nests of tumours showing expanding growth remained cathepsin D negative. These findings have been reported in other epithelial carcinomas, such as gastric carcinoma,²² oral carcinoma,²³ and colorectal carcinoma.²⁴ In addition, Vigneswaran *et al* reported that in oral carcinoma, high amounts of cathepsin D immunoreactivity were detected in the cytoplasm and at the cell surface at the invasive front of tumours.²³ These results indicate that localisation of cathepsin D in tumour cells may shift from the lysosome to the plasma membrane at the invasive tumour front, and that such tumour cells may be involved in the digestion of extracellular matrix components.²³

"The expression of the proteolytic enzyme cathepsin D, which can directly digest the extracellular matrix, may be essential for the initial progression of epithelial carcinoma"

Moreover, only a few tumours with carcinoma in situ showed strong cathepsin D expression; however, more than 60% of tumours that invaded the lamina propria showed strong cathepsin D expression. These findings indicate that the expression of the proteolytic enzyme cathepsin D, which can directly digest the extracellular matrix, may be essential for the initial progression of epithelial carcinoma, or may play an important role in invasive tumour growth.

Reticulum cells in the lymph follicle or stromal cells showed cathepsin D expression by immunohistochemical staining. The positivity and intensity of cathepsin D expression was significantly higher and stronger in stromal cells around tumours than in stromal cells in non-cancerous areas of the oesophagus. These findings suggest that counteractions between cancer cells and stromal cells that express cathepsin D may be important. Johnson and colleagues²⁵ and Rogers and colleagues²⁶ reported that although inflammatory cells infiltrating the border of the tumour invasive front act as a defensive mechanism against tumour invasion, the increased cathepsin D activity in stromal components, such as infiltrative inflammatory cells, might degrade the extracellular matrix and might provide an effective microenvironment for the invasion of the carcinoma cells.

However, in our study, the degree of cathepsin D expression in stromal cells in early oesophageal SCCs was similar to that seen in advanced tumours. Thus, although cathepsin D expression may be important in stromal cells in the early stages of tumour invasion, it may not be important for tumour growth in oesophageal SCC.

Significant positive correlations between increased tumour cathepsin D expression and lymph node metastasis have been reported in oral cancer²³ and in breast cancer.⁹ However, in colorectal adenocarcinoma^{27,28} and in gastric carcinoma,²² no such positive correlation was detected. In oesophageal SCC, no positive correlations were found between cathepsin D expression in tumours and lymph node metastasis or distant metastasis. Thus, the metastatic potential of cancer cells cannot be explained just by cathepsin D expression.

In vitro, cathepsin D can act as a mitogen and its overexpression has been shown to increase cell proliferation and decrease contact inhibition between cells.²⁹ Interestingly, a recent study revealed that proliferation rates of tumours measured by Ki-67 labelled fractions showed a significant positive correlation with the expression of cathepsin D in oral cancer.²³ A similar finding was seen in our study. Previously, it was reported that cancer cells with overexpression of mutated p53 protein showed high proliferative activity in oesophageal SCC.^{19,30} The wild-type p53 protein blocks the cell cycle at the G1-S border; however, mutated p53 proteins might not have this ability and might not suppress the progression of the tumour. Recently, Wu *et al* found that cathepsin D mRNA values were increased by direct binding of wild-type p53 to

Take home messages

- The expression of cathepsin D by cancer cells may play an important role in the invasive growth of oesophageal squamous cell carcinoma (SCC)
- p53 gene abnormalities may correlate with cathepsin D overexpression in oesophageal SCC
- Although high cathepsin D expression was significantly associated with tumour growth and prognosis, in multivariate analysis it was not an independent prognostic factor in patients with oesophageal SCC

p53 specific DNA binding sites located in the cathepsin D promoter in vitro.¹³ However, recent reports have revealed a significant positive correlation between cathepsin D expression and p53 nuclear accumulation in various tumours.^{31, 32} In addition, in our present study, we found that oesophageal SCCs with overexpression of p53 also overexpressed cathepsin D. From these results, it is hypothesised that not only wild-type p53 protein but also mutated p53 protein can bind to cathepsin D promoter sites and mutated p53 protein may upregulate the expression of cathepsin D and enhance tumour progression.

In expanding growth oesophageal SCCs, p53 nuclear accumulation was mainly detected in tumour cells located in the cancer nests, whereas increased cathepsin D expression was occasionally detected in tumour cells surrounding the cancer nests. Thus, in expanding growth oesophageal SCCs, the location of p53 positive tumour cells and cathepsin D positive tumour cells was different in some cases. This observation indicates that in some cases, tumour cells with p53 nuclear accumulation may release signals that result in the increased expression of cathepsin D in surrounding cells to degrade the extracellular matrix. The biological mechanisms linking p53 gene abnormality and cathepsin D overexpression in cancer cells should be investigated.

CONCLUSIONS

Cathepsin D expression may be an early event in oesophageal SCC. When epithelial oesophageal SCC invades the lamina propria or submucosa, cathepsin D may play an important role and oesophageal SCCs with overexpressed cathepsin D may develop into tumours with invasive growth. Mutation of the p53 gene may correlate with cathepsin D expression in oesophageal SCC.

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Authors' affiliations

M Ikeguchi, K Fukuda, S Oka, K Hisamitsu, K Yamaguchi, S Tsujitani, N Kaibara, Department of Surgery I, Faculty of Medicine, Tottori University, 36-1 Nishi-cho, Yonago 683-8504, Japan
T Sakatani, Department of Pathology I, Faculty of Medicine, Tottori University, Nishi-cho 86-1, Yonago 683-8503, Japan
T Ueta, Department of Pathology II, Faculty of Medicine, Tottori University, Nishi-cho 86-1, Yonago 683-8503, Japan

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