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Prognostic factors of oesophageal squamous cell carcinoma from the perspective of molecular biology

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Summary Recent developments in molecular biology have revealed that several oncogenes, suppressor genes and adhesion molecules are involved in the development of oesophageal cancer; however, the role of these genes is still unknown. To evaluate which molecular biological factors are related to patients' prognosis and recurrence, we checked p53, p16, p21/Waf1, cyclin D1, Ki-67, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), Mdm2, Bcl2, E-cadherin and MRP1/CD9 by means of immunohistochemical analysis in 116 cases of oesophageal cancer (R0). We also checked the regrowth capability of the primary cultures of the resected tumours and the effect of post-operative treatment. Although univariate analysis revealed that pN (pTNM), pT (pTNM), sex, cyclin D1, Ki-67, VEGF, E-cadherin and cell regrowth capability were prognostic factors, multivariate analysis revealed that pN (risk ratio (RR) 3.17), sex (RR 8.13), cell regrowth capability (RR 3.03) and E-cadherin (RR 0.30) were prognostic factors. Interestingly, step-wise analysis revealed that the following five factors were prognostic factors: pN (RR 5.74), sex (RR 3.14), cyclin D1 (RR 2.29), E-cadherin (RR 0.26) and cell regrowth capability (RR 1.94). Logistic regression analysis revealed that the risk factors of haematogenous recurrence were pN (odds ratio (OR) 8.97), cyclin D1 (OR 4.52) and EGFR (OR 0.18). On the other hand, the risk factor of lymph node recurrence was pN (OR 5.16). With regard to the effect of post-operative treatment, post-operative radiotherapy was a favourable risk factor (RR 0.43) and reduced the haematogenous recurrence (OR 0.18). Our data indicate that combination analysis using pN, sex, cyclin D1, E-cadherin, EGFR and cell regrowth capability may be useful for the prediction of patient survival and recurrence.

Keywords: oesophageal cancer; molecular marker; biological marker; cyclin D1; E-cadherin

Although surgical techniques and perioperative management have progressed, the prognosis for oesophageal cancer patients remains poor. The prognostic clinical characterization of oesophageal carcinoma remains inadequate using conventional histological grading and staging systems. In fact, the biological factors that determine a different individual outcome (recurrence, survival) within an analogous stage of disease are obscure. Recently, various molecular biological factors such as epidermal growth factor receptor (EGFR) (Ozawa et al, 1988), vascular endothelial growth factor (VEGF) (Inoue et al, 1997), p53 (Sarbia et al, 1994; Wang et al, 1994), p16 (Takeuchi et al, 1997), Mdm2 (Shibagaki et al, 1995; Shimada et al, 1997), cyclin D1 (Naitoh et al, 1995; Shinozaki et al, 1996), Ki-67 (Youssef et al, 1995), Bcl2 (Sarbia et al, 1996; Ohbu et al, 1997), AMF (Maruyama et al, 1995), DNA ploidy pattern (Ruol et al, 1990) and E-cadherin (Miyata et al, 1994; Tamura et al, 1996) have been proposed as prognostic indicators for oesophageal cancer. Cell regrowth capability (Shimada et al, 1993, 1996), one of the biological markers of cancer cells, also proposes to be a prognostic factor of oesophageal cancer.

Though various oncogenes, oncogene products, suppressor genes and biological factors have been identified in human oesophageal carcinomas, most papers have focused on only a few factors at one time. Therefore, prediction of the true risk of tumour progression and metastatic spread, and the relationship between these factors and disease outcome remains controversial. Thus, the type of treatment that should be selected, such as an extended surgery, an aggressive multi-disciplinary therapy or a more palliative treatment, remains uncertain. We previously analysed loss of heterozygosity (LOH) of chromosomes, LOH of the retinoblastoma (RB) gene, LOH of the APC gene, LOH of the DCC gene, p53 mutation, Mdm2 amplification, human papillomavirus (HPV) infection, int-2 amplification and cyclin D1 amplification in oesophageal carcinomas using Southern blot analysis, polymerase chain reaction-single–strand conformation polymorphism (PCR-SSCP) and Direct Sequencing Analysis (Wagata et al, 1991, 1993; Kanda et al, 1994; Shibagaki et al, 1994). We proposed that the accumulation of genetic abnormalities may result in poor prognosis (Shimada et al, 1997).

To clarify which factors are the true prognostic factors in oesophageal cancer patients among various genetic biomarkers, we examined the expression of the following 11 selected molecular biological factors: EGFR, VEGF, p53, p16, p21/Waf1, Mdm2, cyclin D1, Ki-67, Bcl2, MRP1/CD9 and E-cadherin. This time, for ease in clinical application, we carried out immunohistochemical analysis. The data was then subjected to multivariate analysis using Cox's proportional hazard model and logistic regression analysis.

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MATERIALS AND METHODS

Oesophageal cancer patients

One hundred and sixteen oesophageal carcinoma patients who underwent curative oesophagectomy (R0), performed by the same

Table 1 Background data of the patients

Term	No. of cases
Total	116
Age	
≥60	71
<60	45
Sex	
Male	98
Female	18
Tumour	
pT1	16
pT2	28
pT3	48
pT4	24
Node	
pN0	26
pN1	90
Metastasis	
pM0	91
pM1a	10
pM1b	15
Lymph node metastasis	14
Organ metastasis	1
Stage	
1	9
2a	12
2b	18
3	52
4a	10
4b	15
Haematogenic recurrence (+)	25
Haematogenic recurrence (–)	91
Lymph nodal recurrence (+)	43
Lymph nodal recurrence (–)	73
Post-operative chemotherapy (+)	30
Post-operative chemotherapy (–)	86
Post-operative radiotherapy (+)	54
Post-operative radiotherapy (–)	62

surgeon (MI) in the Department of Surgery and Surgical Basic Science of Kyoto University from 1987 to 1995, were studied. All patients had pathological squamous cell carcinomas (the incidence of adenocarcinoma in Japan is less than 5%), and each patient's clinical status was classified as Stage 1, 2a, 2b, 3, 4a, or 4b according to the pathological tumour-node-metastasis (pTNM) classification system (Sabin and Wittekind, 1997). Seventy-seven cases (66.4%) were Stage 3, 4a, or 4b, and 90 cases (77.6%) had lymph node metastasis. There was only one case of distant organ metastasis and this metastasis was removed at the time of operation. Mean age was 63.9 ± 10.2 years (Table 1).

The standard surgical method which was used has been previously described (Imamura et al, 1992). In brief, oesophagectomy with lymph node dissection was performed by means of a right thoracotomy, and subsequent reconstruction was carried out by means of an oesophagogastrostomy using a gastric tube through the retrosternal route. Fifty-four patients (46.6%) received post-operative irradiation (50.4 Gy) (Nishimura et al, 1989) and 30 patients (25.9%) received post-operative chemotherapy (mainly CDDP 50 mg × 2). Haematogenous recurrence occurred in 25 cases and lymph node recurrence occurred in 43 cases. The recurrence site is the tissue in which metastasis was first recognized.

Immunohistochemical staining

Immunohistochemical analysis was done retrospectively. Resected oesophageal specimens which included both tumour and normal mucosa were fixed in a 10% formaldehyde solution and embedded in paraffin. To examine many factors simultaneously, consecutive 4-μm sections were cut and mounted on APS-coated glass slides. Immunohistochemical staining was performed using the avidin–biotin method as described previously (Uchida et al, 1998). The antibodies used in this study are as follows: p53 (DO-7; DAKO, Tokyo, Japan), p16 (p16INK4; Phamingen, San Diego, CA, USA), p21/Waf1 (Waf1 Ab-1; Oncogene Science, Cambrige, MA, USA), Bcl2 (anti-human Bcl2, DAKO, Tokyo, Japan), E-cadherin (HECD, kindly gifted by M Takeichi, Kyoto University, Kyoto, Japan), VEGF (anti-human VEGF polyclonal antibody, kindly gifted by T Ishiwata, Nippon Medical School, Tokyo, Japan), EGFR (anti-EGFR; Novocastra, Newcastle, UK), cyclin D1 (anti-cyclin D1/Bcl1; MBL, Nagoya, Japan), MRP1 (M31-15, kindly gifted by M Miyake, Kitano Hospital, Osaka, Japan), Ki-67 (MM1; Novocastra, Newcastle, UK), and Mdm2 (anti-Mdm2; Novocastra, Newcastle, UK).

The unmasking of antigens was carried out by incubation in citrate buffer (pH 6.0) at 95°C for 10 min (microwave) or 120°C for 5 min (autoclave). Sections were incubated overnight at 4°C with each antibody in phosphate-buffered saline containing 1% bovine serum albumin and 5% normal serum. These the sections were counterstained with Mayer's haematoxylin. Anti-rabbit or anti-mouse IgG was used for checking non-specific staining.

Positive criterion of immunohistochemical staining

The intensity of immunohistological staining was evaluated in five areas of the slide section for correlation and confirmation of the tissue analysis. The following definition were made:

- p53, cyclin D1: More than 10% positive staining in nuclei was defined as positive staining (Casey et al, 1996; Takeuchi et al, 1997).
- Bcl2, VEGF: More than 10% positive staining in cytoplasma was defined as positive staining (Ohbu et al, 1997; Uchida et al, 1998). With regard to Bcl2, staining of infiltrating lymphocyte was used as a positive control.
- Ki-67, Mdm2: More than 30% positive staining in nuclei was defined as positive staining (Youssef et al, 1995; Higashiyama et al, 1997).
- p21/Waf1, p16: More than 50% positive staining in nuclei was defined as positive staining (Bukholm et al, 1997). Due to the lower percentage of p16 staining compared to previous report (Takeuchi et al, 1997), 50% staining in nuclei was defined as positive staining.
- MRP1/CD9: More than 50% positive staining in membrane was defined as positive staining (Miyake et al, 1996).
- E-cadherin: More than 90% positive staining in cell membrane was defined as positive staining (Kadowaki et al, 1994).

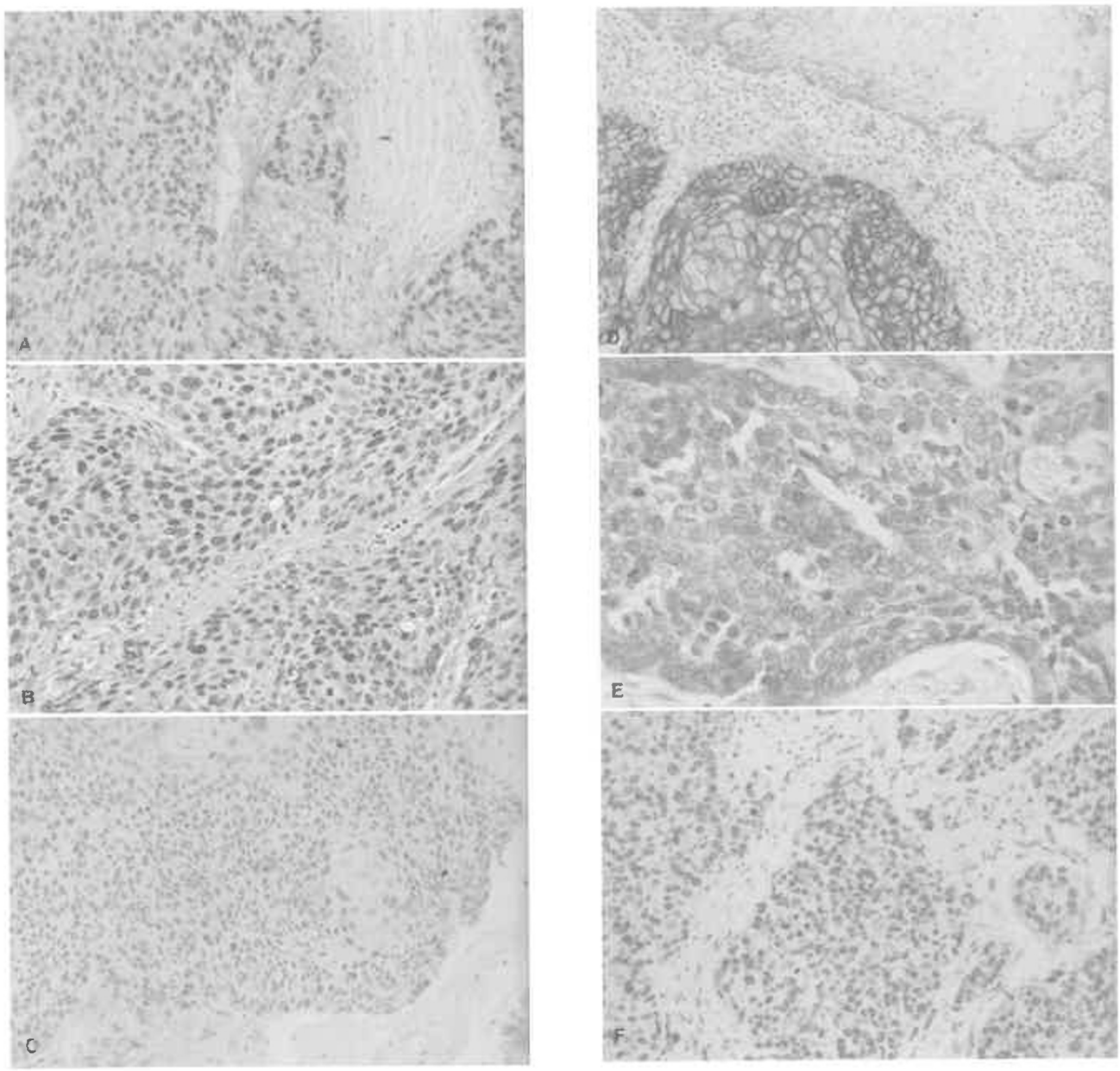


Figure 1 Representative staining of each antibody used in this study. (**A**) p53, (**B**) p21/Waf1, (**C**) cyclin D1, (**D**) EGFR, (**E**) VEGF, (**F**) Mdm2, (**G**) Bcl2, (**H**) E-cadherin, (**I**) MRP1/CD9, (**J**) p16 and (**K**) Ki-67 (original magnification × 200: **A–I**; original magnification × 400: **J** and **K**)

- EGFR: Strong staining in tumour cell membrane or cytoplasma compared to normal mucosa was defined as overexpression (Itakura et al, 1994).

Scoring of the results

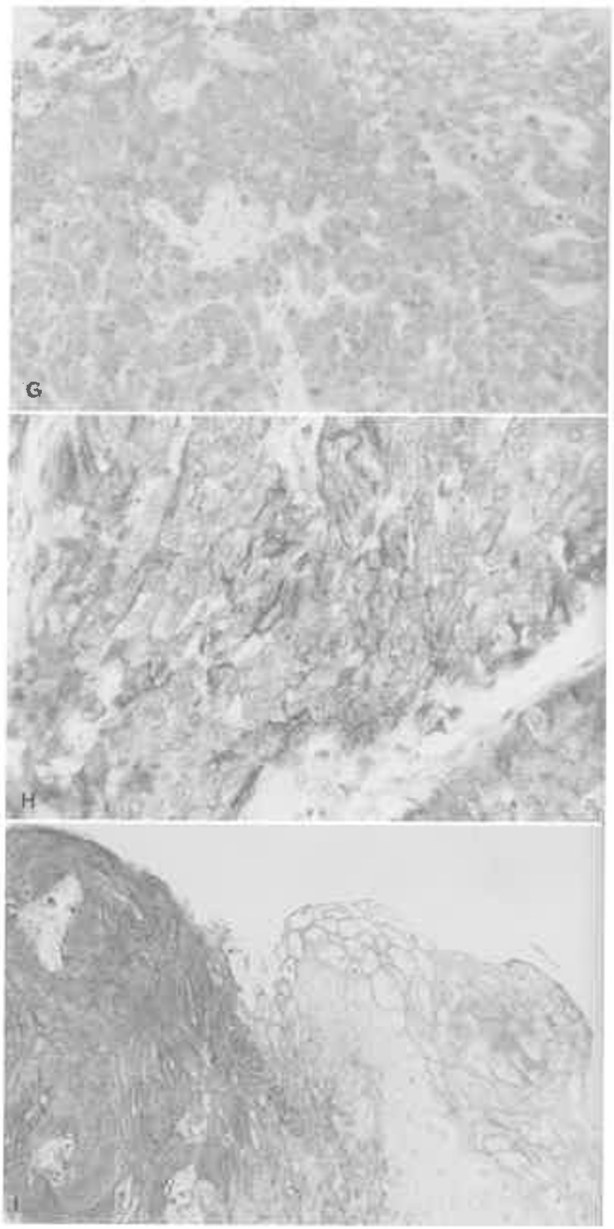
Positive stained or overexpression cases were designated '1' and negative or normal stained cases were designated '0'. pT, age, sex and TNM stage were divided as follows:

- pT1: 0; pT2, pT3, pT4: 1
- Under 59 years old: 0; over 60 years old: 1
- Female: 0; Male: 1
- Stage 1, 2a: 0; Stage 2b, 3, 4: 1.

With regard to post-operative treatment, terms were simply divided into the following two categories: not done: 0; done: 1.

Cell culture

To determine whether any of the cellular biological characteristics were related to prognosis, we checked the monolayer epithelial growth capability of the tumour samples. The resected oesophageal samples were cultured in Petri dishes and culture



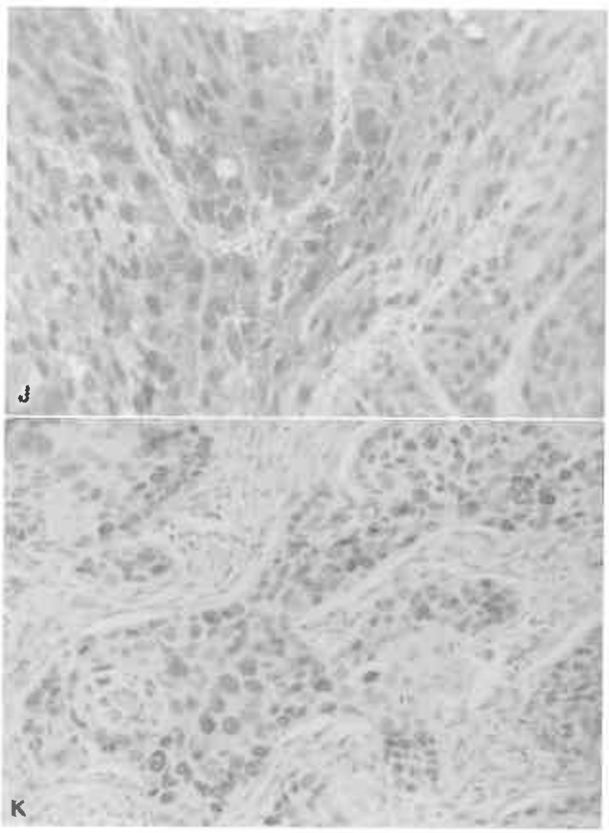


Figure 1 Representative staining of each antibody used in this study (**G**) Bcl2, (**H**) E-cadherin, (**I**) MRP1/CD9, (**J**) p16 and (**K**) Ki-67 (original magnification × 200: **A–I**; original magnification × 400: **J** and **K**)

patterning was checked under phase contrast microscopy as described previously (Shimada et al, 1993, 1996). The growth pattern in which tumour cells migrated out from scattered tumour samples was designated as monolayer epithelial growth.

Statistical analysis

Univariate and multivariate analysis was performed using Cox's proportional hazard model and logistic regression analysis (Cox, 1972). The correlation between clinical and molecular parameters were statistically evaluated using Fisher's exact test. The software used was JMP version 3 for Macintosh (SAS Institute Inc, Cary, NC, USA).

Follow-up period

The follow-up period is one of the most important factors of this type of analysis. The haematogenous and lymph node recurrence of oesophageal cancer appears mainly within 2 years after the operation, therefore the minimal follow-up period of the survival patients in this paper is 2 years and 6 months (mean 5.69, standard deviation (s.d.) 2.23).

RESULTS

Overall, the 5-year survival rate was 25.9% due to the constitutions of advanced cases. The prognostic analyses were carried out using 16 factors: 11 molecular biomarkers, one cell biological marker (cell culture) and four clinicopathological data (sex, age, pT(TNM) and pN(TNM)). Distribution of immmunohistochemical analysis is summarized in Table 2. The representative staining of each antibody was demonstrated in Figure 1.

First, we analysed the data with univariate Cox's proportional hazard model. Univariate analysis revealed that pN, pT, sex, cyclin D1, Ki-67, VEGF, E-cadherin and cell regrowth capability were significant prognostic factors (Table 3). Next, we analysed the data using multivariate Cox's proportional hazard model. Multivariate analysis revealed that pN, sex, cell regrowth capability and E-cadherin expression were prognostic factors (Table 4). Interestingly, step-wise analysis revealed that the following five factors were selected as prognostic factors: pN (risk ratio (RR) 4.5), sex (RR 3.22), cyclin D1 expression (RR 2.42), E-cadherin expression (RR 0.36) and cell regrowth capability (RR 1.97) (Table 5). Finally, we analysed the factors which related to gender, then VEGF expression and p21 reduction significant correlated with male (Table 6).

Table 2 Distribution of molecular and biomarkers

Term	No. of cases	Term	No. of cases
VEGF (–)	36	p53 mutation (–)	66
VEGF (+)	80	p53 mutation (+)	50
E-cadherin (–)*	94	MDM2 (–)	35
E-cadherin (+)	22	MDM2 (+)	81
MRP1/CD9 (–)	101	p16 (–)	97
MRP1/CD9 (+)	15	p16 (+)	19
Culture (–)***	67	Ki-67 (–)	68
Culture (+)	49	Ki-67 (+)	48
Cyclin D1 (–)	70	p21/Waf1 (–)	106
Cyclin D1 (+)	46	p21/Waf1 (+)	10
BCL2 (–)	93	EGFR (–)	63
BCL2 (+)	23	EGFR (+)**	53

(–): negative; (+): positive. *Reduced expression; **overexpression; ***culture: cell regrowth capability.

Table 3 Cox proportional hazard model (univariate) ($n = 116$)

Term	Estimate	RR	95% CI	P
Age	0.226	1.254	0.81–1.98	0.317
Sex	0.932	2.541	1.30–5.73	0.005
pT	0.681	1.976	1.04–4.25	0.037
pN	0.997	2.711	1.52–5.28	0.0004
VEGF	0.594	1.811	1.08–3.18	0.023
E-cadherin	–0.666	0.514	0.24–0.99	0.045
MRP1/CD9	–0.371	0.69	0.30–1.36	0.302
Culture*	0.751	2.141	1.33–3.43	0.002
Cyclin D1	0.546	1.727	1.10–2.68	0.017
Bcl2	–0.41	0.663	0.36–1.14	0.144
p53	0.279	1.322	0.85–2.05	0.217
MDM2	0.312	2.211	0.85–2.26	0.197
p16	–0.133	0.875	0.47–1.51	0.645
Ki-67	0.486	1.626	1.03–2.54	0.036
p21/Waf1	–0.463	0.629	0.24–1.33	0.245
EGFR	0.034	1.035	0.67–1.60	0.877
Radiation	–0.583	0.558	0.36–0.87	0.009
Chemotherapy	0.215	1.239	0.75–1.98	0.391

Culture*: cell regrowth capability. RR, risk ratio; CI, confidence interval.

Table 4 Cox proportional hazard model (multivariate) ($n = 116$)

Term	Estimate	RR	95% CI	P
Age	0.186	1.20	0.61–2.40	0.589
Sex	2.10	8.132	2.01–48.12	0.002
pT	0.843	2.323	0.56–16.21	0.270
pN	1.153	3.167	1.10–10.60	0.031
VEGF	0.462	1.588	0.69–4.04	0.290
E-cadherin	–1.217	0.296	0.09–0.86	0.024
MRP1/CD9	–0.238	0.788	0.22–2.68	0.708
Culture*	1.109	3.032	1.31–7.05	0.010
Cyclin D1	0.608	1.837	0.763–4.47	0.174
Bcl2	0.125	1.133	0.30–3.89	0.846
p53	0.239	1.269	0.53–3.01	0.586
MDM2	0.556	1.744	0.73–4.33	0.214
p16	–0.193	0.825	0.23–2.72	0.756
Ki-67	0.427	1.532	0.66–3.60	0.322
p21/Waf1	1.373	3.946	0.43–30.86	0.214
EGFR	–0.605	0.546	0.23–1.31	0.175
Radiation	–0.767	0.465	0.19–1.03	0.059
Chemotherapy	0.743	2.103	0.88–5.07	0.094

Culture*: cell regrowth capability. RR, risk ratio; CI, confidence interval.

Logistic regression analysis revealed that the risk factors of haematogenous recurrence were pN (odds ratio (OR) 8.97), cyclin D1 expression (OR 4.52) and EGFR expression (OR 0.18). On the other hand, pN (OR 5.16) was the single risk factor of lymph node recurrence; there were no other factors (Tables 7 and 8).

With regard to the effect of post-operative treatment, post-operative radiotherapy was a favourable risk factor (RR 0.43) and reduced the haematogenous recurrence (OR 0.18). However, post-operative chemotherapy could not be used to predict death or recurrence (Tables 5 and 7).

DISCUSSION

Although various factors have already been proposed as prognostic factors of oesophageal squamous cell carcinoma, true predictive factors have not been proposed. In this paper, we selected the following 11 molecular biological markers which were suggested to be related to the prognosis and recurrence of oesophageal cancer:

- Growth factor and receptors – EGFR, VEGF
- Suppressor genes – p53, p21/Waf1, p16
- Oncogenes – Mdm2, cyclin D1
- Proliferation marker – Ki-67
- Apoptosis related protein – Bcl2
- Cell motility marker – MRP1/CD9
- Adhesion molecule – E-cadherin.

We also examined cell regrowth capability using monolayer culture as a cell biological marker.

The patient's outcome and recurrence may be influenced by post-operative treatment. Although there was a selection bias in the treatment choice, we performed the multivariate analysis including post-operative treatment. Post-operative treatment was simply divided into two categories ('done' or 'not done'). The final step-wise regression model revealed that sex, pN, cyclin D1 and cell regrowth capability are significantly worse prognostic factors (RR 1.94–5.74) and E-cadherin expression and post-operative irradiation are favourable prognostic factors (RR 0.26 and 0.43 respectively).

Few analyses have employed combination analysis (cyclin D1 and p16, cyclin D1 and RB etc.) (Takeuchi et al, 1997; Ishikawa et al, 1998). However, to our knowledge, there has been no report regarding multivariate analysis in oesophageal cancer from the perspective of molecular biology. In this study, we used a single antibody for immunohistochemical analysis. However, p53 staining did not always correlate to the results of direct sequencing (Coggi et al, 1997) and many reports already indicated that p53 expression has heterogeneity. Furthermore, cross-reactivity may also exist in certain antibodies. Therefore, the results of immunohistochemical study using a single antibody have limitations on interpretation. Although there are such disadvantages, our results might provide the useful information for the treatment of oesophageal cancer.

Our data indicated that one oncogene (cyclin D1) and one adhesion molecule (E-cadherin) are predictive death factors of oesophageal squamous cell carcinoma. There have already been several reports regarding cyclin D1 in oesophageal carcinoma (Jiang et al, 1992; Naito et al, 1995; Nakagawa et al, 1995; Shinozaki et al, 1996; Shimada et al, 1997; Takeuchi et al, 1997). The cyclin D1 gene encodes a cell-regulatory protein that is expressed at high

Table 5 Step-wise regression analysis

Term	Estimate	RR	95% CI	P
Sex	1.144	3.139	1.33–9.28	0.007
E-cadherin	−1.331	0.264	0.10–0.60	0.0009
pN	1.747	5.736	2.38–16.44	<0.0001
Culture*	0.662	1.938	1.08–3.46	0.026
Cyclin D1	0.826	2.285	1.21–4.31	0.011
Radiation	−0.841	0.431	0.24–0.76	0.004

Culture*: cell regrowth capability. RR, risk ratio; CI, confidence interval.

Table 6 Gender-related factors (female vs male)

Term	χ^2	P*	Odds
VEGF	4.229	0.043	3.16
pN	3.326	0.069	2.646
Ki-67	3.293	0.061	0.392
p21	16.32	0.0009	0.086

*P**: Fisher's exact test.

levels during the G1 phase of the cell cycle (Matsushime et al, 1991). D cyclin binds to the cyclin-dependent kinases (cdk2 and cdk4) and to proliferating cell nuclear antigens, and formation of these complexes has been implicated in the control of cell proliferation (Maex et al, 1994). Antisense mRNA for cyclin D1 inhibits the growth of tumour cells (Zhou et al, 1995) and transfection of the cyclin D1 gene results in the overexpression of other adjacent genes (Zhou et al, 1996). It is therefore likely that an amplification and overexpression of the cyclin D1 gene could lead to uncontrollable cell growth and the proliferation of tumour cells. Our results confirmed and established the predictive value of cyclin D1 in the prognosis of oesophageal squamous cell carcinoma. A relationship between cyclin D1 expression and haematogenous recurrence has already been proposed by Shinozaki et al (1996). In our study logistic regression analysis supports that cyclin D1 is indeed a factor which relates to haematogenous recurrence. The reason why cyclin D1 relates to haematogenous recurrence is unclear. One possible explanation is that VEGFb also located closely to the cyclin D1 locus (Paavonen et al, 1996). However, cyclin D1 amplification and VEGFb amplification do not always co-exist (Paavonen et al, 1996). Our data also indicated that VEGF could not predict haematogenous recurrence, thus the role of cyclin D1 in haematogenous recurrence remains unclear.

E-cadherin has already been proposed as a significant prognostic factor not only in oesophageal cancer (Miyata et al, 1994; Tamura et al, 1996), but also in various carcinomas. Only 19% (22/116) of oesophageal carcinomas preserve E-cadherin expression; this means that most oesophageal cancers have a tendency to release itself from the primary tumour.

Previous papers suggested that EGFR expression revealed poor prognosis (Ozawa et al, 1988; Mukaida et al, 1991; Yano et al, 1991; Itakura et al, 1994); however, some of these results were analysed by univariate analysis. In this study, univariate and multivariate analysis revealed that there was no significant correlation between EGFR expression and the patient's prognosis. However, logistic regression analysis revealed that overexpression of EGFR relates to reduction of haematogenous recurrence. The reason for this remains unclear. EGFR distribution in either the membrane or cytoplasma may also be a different function. However, our evaluation criteria of EGFR was not concerned with heterogeneity (Itakura et al, 1994) and staining type (diffuse or membrane) (Yano et al, 1991). Furthermore, recent reports suggested that EGF reduced E-cadherin expression in experimental model (Shiozaki et al, 1995). Thus, not only EGFR expression, but also EGFR function, is important.

Table 7 Logistic regression analysis (haematologic recurrence)

Term	Estimate	Standard error	χ^2	P	OR	95% CI
pN	1.097	0.6	3.34	0.068	8.97	1.23–198.10
Sex	0.522	0.602	0.75	0.386	2.84	0.35–61.94
E-cadherin	−0.324	0.446	0.53	0.467	0.52	0.08–2.85
Culture*	0.331	0.327	1.02	0.312	1.94	0.54–7.30
Cyclin D1	0.755	0.362	4.35	0.037	4.52	1.16–20.73
EGFR	−0.85	0.362	5.52	0.019	0.18	0.03–0.69
Radiation	−0.864	0.351	6.07	0.014	0.18	0.04–0.65

Culture*: cell regrowth capability. OR, odds ratio; CI, confidence interval.

Table 8 Logistic regression analysis (lymph nodal recurrence)

Term	Estimate	Standard error	χ^2	P	OR	95% CI
pN	0.82	0.405	4.11	0.043	5.16	1.25–35.17
Sex	0.358	0.376	0.91	0.341	2.05	0.50–10.52
E-cadherin	−0.273	0.373	0.54	0.465	0.58	0.11–2.33
Culture*	−0.057	0.261	0.05	0.828	0.89	0.31–2.49
Cyclin D1	−0.272	0.26	1.1	0.294	0.58	0.2–1.59
EGFR	0.149	0.247	0.36	0.546	1.35	0.51–3.61

Culture*: cell regrowth capability. OR, odds ratio; CI, confidence interval.

Monolayer epithelial growth capability in primary cell cultures has also been shown to be related to the malignant potential of oesophageal cancer after curative resection, and this growth capability is strongly related to haematogenous recurrence (Shimad et al, 1994, 1996). This method is useful for detecting dynamic biological tumour characteristics and we consider this factor as a composite assessment of malignant potential.

With regard to conventional factors, sex and pN strongly correlated with the patient's prognosis. Oesophageal carcinoma occur more frequently in men and the prognosis is more favourable in women (VanAndel et al, 1979). Sugimachi et al (1987) suggested that oesophageal carcinoma in women shows a more favourable prognostic factor of DNA aneuploidy patterns reflecting the malignant potential of the tumour. Furthermore, sex hormones such as oestrogen were suggested to suppress the proliferation of oesophageal cancer cells (Utsuki et al, 1989; Ueo et al, 1990). Fisher's exact test revealed that p21/Waf1 protein expressed more frequently ($P = 0.0009$) and VEGF protein expressed less frequently ($P = 0.043$) in women. Furthermore, VEGF protein expression significantly correlates with pN factor ($P = 0.0005$, data not shown). p21/Waf1 expression has recently reported to be a favourable prognostic factor in squamous cell carcinoma of the lung (Komiya et al, 1997) and a certain report revealed that p21 expression resulted in tumour growth arrest of the epidermal cell carcinoma (Ohtsubo et al, 1998). Although p21/Waf1 and VEGF revealed no significant correlation with patients prognosis using Cox's multivariate analysis, these data suggested that p21/Waf1 and VEGF may also be key genes of oesophageal cancer. On the other hand, the p53 gene, which is the most common molecular biological factor in many malignancies, showed no correlation with sex, pN, recurrence and prognosis.

The number of lymph node metastases was probably the strongest predictive death factor (Akiyama et al, 1994; Matsubara et al, 1994). However, cut-off criteria remains controversial, thus, in this study, we selected pN as a representative factor of lymph node metastasis and pN is still the strongest predictive death factor.

With regard to post-operative treatment, multivariate analysis revealed that post-operative irradiation was a significant favourable prognostic factor. Unfortunately, post-operative chemotherapy was not a significant favourable prognostic factor. However, the range of dosage and period of chemotherapy were varied. Therefore, post-operative chemotherapy should be performed using a strict protocol, and be re-analysed.

Finally, with regard to the value of molecular analysis, molecular analysis may not always surpass that of conventional variables (Akselen et al, 1995). However, multivariate analysis using TNM stage (instead of pT and pN) revealed that E-cadherin (RR 0.36), cyclin D1 (RR 2.27) and cell regrowth capability (RR 2.23) were still independent prognostic factors. Therefore, not only conventional staging analysis, but also molecular biological analysis, might predict patient's death and recurrence type.

In conclusion, expression of cyclin D1, expression of E-cadherin, expression of EGFR and cell regrowth capability play an important role in the progression of oesophageal cancer. Although our study gives important information to aid in the selection of treatment choice, this study is a retrospective study and the question remains whether the high-risk cases can obtain long-term survival by aggressive treatment. Therefore, we are now doing a prospective study as to whether these factors are true prognostic factors, and plan to study the treatment of high-risk patients with intensive chemotherapy or chemo-radiotherapy. We also have to determine whether surgical treatment, such as three-field dissection, leads to a positive outcome.

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