Expression of *ECRG4* is an independent prognostic factor for poor survival in patients with esophageal squamous cell carcinoma

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Abstract. In this study, we examined the expression of esophageal cancer-related gene 4 *(ECRG4)* mRNA and evaluated its clinical significance in esophageal squamous cell carcinoma (ESCC). *ECRG4* mRNA expression was quantified by real-time RT-PCR in 63 ESCC and corresponding normal esophageal mucosal samples. *ECRG4* mRNA expression levels were significantly lower in ESCC tissues compared with corresponding normal esophageal mucosa (P<0.0001), in patients with locally invasive T2-4 tumors compared with less invasive T1 tumors (P=0.0229) and in stage 4 tumors compared with stage 0-3 tumors (P=0.0120). Furthermore, low *ECRG4* mRNA expression levels were associated with significantly shorter survival after surgery compared with high *ECRG4* mRNA expression levels (P=0.0150) in ESCC patients. On the basis of multivariate analysis, we conclude that *ECRG4* mRNA expression level could be a candidate for an independent prognostic factor for ESCC patients.

Introduction

In Japan, esophageal squamous cell carcinoma (ESCC) is the ninth most frequent cancer. However, it is the sixth most frequent cause of death from malignant tumors, and the number of deaths due to this cancer has been steadily increasing. ESCC is often diagnosed at an advanced stage and the prognosis remains poor, prompting the search for new treatment strategies. Although pre-operative chemotherapy and chemoradiation therapy are currently used for patients with advanced-stage ESCC, their results are unsatisfactory. Even among patients with early-stage disease, we have observed many who develop locally recurrent tumors or distant metastases within a short period after curative surgery. Molecular biological studies have revealed that ESCC is caused by the accumulation of multiple genetic changes in oncogenes and tumor suppressor genes.

The esophageal cancer-related gene 4 *(ECRG4)*, a novel tumor suppressor gene candidate, was cloned and identified by Su *et al* from normal human esophageal epithelium in 1998 (1). Its function is unknown and its role in ESCC has not been studied. In this study, we investigated the *ECRG4* mRNA expression in ESCC and in its paired normal esophageal mucosa by real-time RT-PCR using a LightCycler system. We analysed the results with reference to the clinicopathological characteristics and the prognosis of the ESCC patients.

Materials and methods

Tissue samples. Tissue samples were obtained from 63 patients with primary ESCC who had undergone radical esophagectomy at the Department of Surgery II, Nagoya City University Medical School, between 1996 and 2001. The study design was approved by the Institutional Review Board of our university hospital, and a written consent was obtained from each patient. The tumors were classified according to the Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus. The patients comprised of 50 males and 13 females, and their mean age was 63.0±8.2 years (range, 46-79 years). All the samples were frozen immediately in liquid nitrogen and stored at -80˚C until use. The clinicopathological factors and the tumor and patient characteristics of the 63 subjects are shown in Table I.

RNA extraction and RT-PCR analysis. Total RNA was extracted from ESCC tissue and from corresponding normal esophageal mucosa taken from apparently non-cancerous mucosa as far away from the tumor as possible, using the Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The concentration of total RNA was adjusted to 200 ng/ml with a spectrophotometer. The reverse transcription reaction was carried out at 42˚C for

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Table I. Correlation of *ECRG4* mRNA expression in esophageal cancer with clinicopathological factors, including patient and tumor characteristics.

Characteristics	No. of patients (n=63)	*ECRG4* expression relative to GAPDH[a]	P-value
Normal	63	176.5±271.4	
Tumor	63	85.4±167.1	<0.0001
Age at surgery			
<65 years	36	95.2±194.6	
≥65 years	27	72.3±123.9	0.8350
Gender			
Male	50	88.6±177.1	
Female	13	73.1±126.8	0.7341
Tumor status			
T1	10	151.1±281.1	
T2	6	72.4±107.4	
T3	32	95.5±163.1	
T4	15	25.3±51.5	0.0651
Lymph node status			
N0	12	70.0±122.6	
N1	11	64.9±94.2	
N2	22	93.1±213.0	
N3	9	166.9±221.0	
N4	7	39.1±78.5	0.1728
Unknown	2		
Pathological stage			
0	3	41.7±6.7	
I	3	53.3±47.3	
II	11	138.2±275.2	
III	23	126.9±183.5	
IV	23	28.6±58.7	0.1432
Histological differentiation			
Well	21	113.9±217.8	
Moderate	31	77.0±151.7	
Poor	7	40.9±32.0	0.3639
Unknown	4		
Lymphatic invasion			
Negative	12	133.7±262.5	
Positive	39	76.2±147.2	0.3070
Unknown	12		
Blood vessel invasion			
Negative	21	109.1±214.0	
Positive	30	76.1±153.4	0.2065
Unknown	12		

[a]Mean ± standard deviation (SD). ECRG4, esophageal cancer-related gene 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

90 min and 95˚C for 5 min, followed by incubation at 72˚C for 15 min, using 1 μg of total RNA and 0.5 μg each of oligo(dT) primer and Superscript II enzyme (Gibco BRL, Gaithersburg, MD). All the samples were quantified after

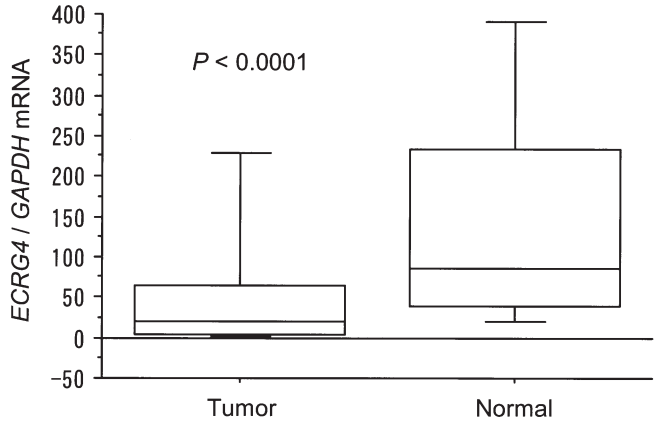

Figure 1. *ECRG4/GAPDH* mRNA expression levels in ESCC tissue were significantly lower than those in the corresponding normal esophageal mucosal tissue (85.4±167.1 vs. 176.5±271.4, P<0.0001; Wilcoxon signed-ranks test).

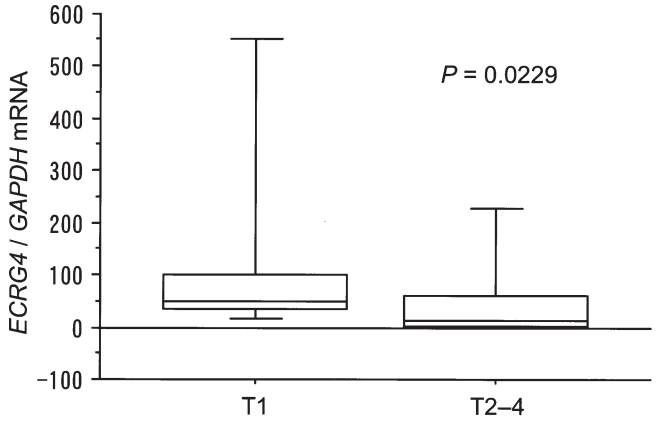

Figure 2. *ECRG4/GAPDH* mRNA expression levels in patients with locally invasive T2-4 tumors were significantly lower than those in less invasive T1 tumors (P=0.0229, Mann-Whitney U test).

PCR amplification using a Lightcycler-Faststart DNA Master SYBR-Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). We used the following set of primers: forward primer ECRG4-F, 5'-AAGTGGCCGTTGATGAG AAT-3'; and reverse primer ECRG4-R, 5'-GGGACCAA TTGCAGAGTCTT-3'. The size of the product was 245 bp. The PCR protocol was: initial denaturation at 95˚C for 10 min, followed by 45 cycles at 95˚C for 10 sec, annealing at 60˚C for 5 sec and extension at 72˚C for 10 sec. The PCR product was quantified on the basis of the intensity of SYBR-Green I at 72˚C.

Statistical analysis. The relative *ECRG4* mRNA expression levels were calculated from quantified data in reference to the expression level of the glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* gene. The data are expressed as the means ± standard deviation (SD). Statistical analysis was performed using the software package StatView (Abacus Concepts, Berkeley, CA). The Wilcoxon signed-ranks test, Mann-Whitney U test and Kruskal-Wallis test were used to evaluate the significance of the differences in the expression

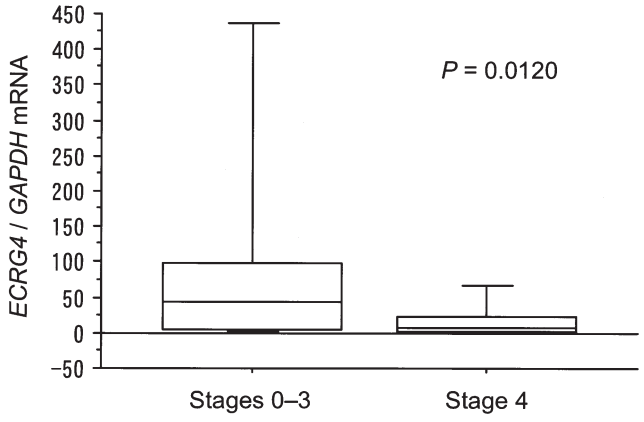

Figure 3. *ECRG4/GAPDH* mRNA expression levels in patients with histological stage 4 tumors were significantly lower than those in histological stages 0-3 tumors (P=0.0120, Mann-Whitney U test).

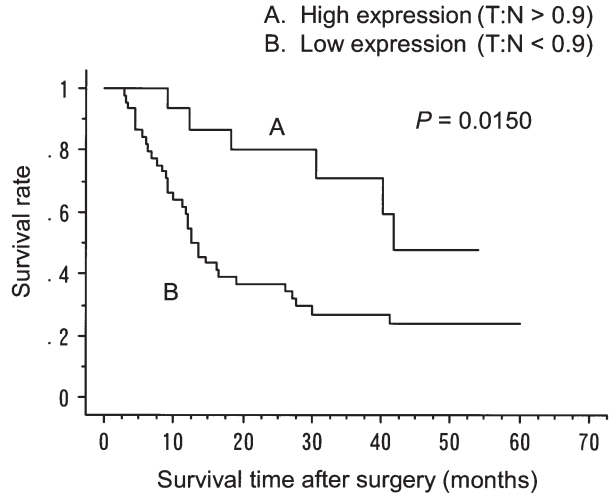

Figure 4. Patients who had low *ECRG4/GAPDH* mRNA expression levels [indicated as the ratio of *ECRG4/GAPDH* mRNA expression in the tumor to that in normal esophageal mucosa (T:N ratio) <0.9, n=47] had a significantly shorter survival (13.0±2.1 months) after surgery compared with patients who had high *ECRG4* mRNA expression levels (T:N ratio >0.9, n=16; 42.0±2.7 months) (P=0.0150, log-rank test).

levels of *ECRG4/GAPDH* mRNA. The survival of the ESCC patients after surgery was examined using the Kaplan-Meier method, and the survival times were compared using the log-rank test. Multivariate analysis was performed using the Cox regression model and a logistic multivariate regression model. In all analyses, P<0.05 was considered statistically significant.

Results

ECRG4/GAPDH mRNA expression was detectable in all the samples of ESCC tissue and non-cancerous esophageal mucosal tissue. The respective levels of expression in ESCC tissue were significantly lower than those in the corresponding normal esophageal mucosa (85.4±167.1 vs. 176.5±271.4, P<0.0001; Wilcoxon signed-ranks test) (Fig. 1 and Table I). We examined the relationship between *ECRG4/GAPDH* mRNA expression in 63 ESCC samples and the patients' clinicopathological factors (Table I). There were no significant differences in *ECRG4/GAPDH* mRNA with respect to age, gender, lymph node status, histological differentiation, lymphatic invasion and blood vessel invasion. The *ECRG4/GAPDH* mRNA expression levels in patients with locally invasive T2-4 tumors were significantly lower than those in the less invasive T1 tumors (P=0.0229, Mann-Whitney U test) (Fig. 2). The *ECRG4/GAPDH* mRNA expression levels in patients with stage 4 tumors were significantly lower than those in the tumors in stages 0-3 (P=0.0120, Mann-Whitney U test) (Fig. 3).

We investigated the correlation between the *ECRG4/GAPDH* mRNA expression levels and the survival of ESCC patients after surgery (median follow-up, 22.8 months). Patients who had low *ECRG4/GAPDH* mRNA expression levels [indicated as the ratio of *ECRG4/GAPDH* mRNA expression in the tumor to that in normal esophageal mucosa (T:N ratio) <0.9, n=47] had a significantly shorter survival (13.0±2.1 months) after surgery compared with patients who had high *ECRG4/GAPDH* mRNA expression levels (T:N ratio >0.9, n=16; 42.0±2.7 months) (P=0.0150, log-rank test) (Fig. 4). Univariate analysis revealed that among the clinicopathological factors, the local invasiveness (Tumor status) (risk ratio, 4.566; P<0.0001), lymph node metastasis (Node status) (risk ratio, 3.690; P=0.0022), lymphatic invasion (risk

Table II. Univariate analysis of the factors that influence the prognosis of ESCC patients.

Parameter	Risk ratio	95% CI	P-value
Age at surgery			
<65 years	1		
≥65 years	1.669	0.871-3.194	0.1129
Gender			
Male	1		
Female	1.080	0.509-2.290	0.8381
Tumor status			
T1-3	1		
T4	4.566	2.179-9.523	<0.0001
Lymph node status			
N0-3	1		
N4	3.690	1.497-9.090	0.0022
Lymphatic invasion			
Negative	1		
Positive	6.711	1.575-28.57	0.0027
Blood vessel invasion			
Negative	1		
Positive	2.793	1.199-6.536	0.0123
ECRG4 expression (T:N)			
High	1		
Low	2.801	1.163-6.757	0.0150

CI, confidence interval.

Table III. Multivariate analysis of the factors that influence the prognosis of ESCC patients.

Parameter	Risk ratio	95% CI	P-value
Tumor status			
T1-3	1		
T4	2.985	0.810-10.989	0.1002
Lymph node status			
N0-3	1		
N4	5.208	1.013-27.027	0.0482
Lymphatic invasion			
Negative	1		
Positive	6.849	1.256-37.037	0.0262
Blood vessel invasion			
Negative	1		
Positive	0.971	0.344-2.740	0.9549
ECRG4 expression (T:N)			
High	1		
Low	2.740	0.990-7.576	0.0490

CI, confidence interval.

ratio, 6.711; P=0.0027), blood vessel invasion (risk ratio, 2.793; P=0.0123) and *ECRG4* expression (T:N ratio) (risk ratio, 2.801; P=0.0150) were statistically significant prognostic factors (Table II). Multivariate analysis revealed that the extent of lymph node metastasis (P=0.0482), lymphatic invasion (P=0.0262) and *ECRG4* expression (T:N ratio) (P=0.0490) were independent prognostic factors (Table III).

Discussion

We examined *ECRG4* mRNA expression in ESCC tissue and the corresponding normal esophageal mucosa. The *ECRG4* gene was expressed abundantly in adult esophageal epithelium but was down-regulated in ESCC. This suggests that the *ECRG4* gene might be involved in the development of ESCC. This gene is located on chromosome 2q 12.2 and has four exons. The mechanisms that down-regulate *ECRG4*, including DNA methylation, mutation and loss of heterozygosity (LOH), were not examined in this study. However, LOH in 2q has been detected in esophageal cancer in Chinese patients, and this region was suggested to harbour the putative tumor suppressor gene within this region (2).

Many tumor suppressor genes are down-regulated by promoter methylation during the development and progression of cancer, and hypermethylation of gene-promoter regions is being revealed as one of the most frequent mechanisms responsible for the loss of gene function; therefore, the detection of CpG methylation is an important step in understanding the gene regulation of cancer. It has been reported that the expression of certain tumor suppressor genes, such as *p15INK4b, p16INK4a, fragile histidine triad (FHIT)* and *E-cadherin*, are commonly down-regulated by CpG island hypermethylation in ESCC (3-6). Yue *et al* reported that aberrant methylation of CpG islands in the core promoter of the *ECRG4* gene was a frequent molecular event in ESCC, and proved for the first time that the loss or lower expression of *ECRG4* was associated with ECRG4-CpG island methylation. Further, they found that there were CpG islands in the promoter region of exon 1 and part of intron 1 of the gene. These results indicate that the inactivation of the *ECRG4* gene by hypermethylation may be involved in the carcinogenesis of ESCC (7).

In our study, the *ECRG4* mRNA expression levels correlated significantly with the local invasiveness (tumor status), pathological stages and the prognosis of ESCC patients. The patients whose tumors expressed higher levels of *ECRG4* mRNA survived longer than those with lower levels of expression. Thus, the down-regulation of *ECRG4* may contribute to tumor growth in ESCC.

Moreover, the expression of this gene in the T1 and T2-4 tumors was significantly different. It has been reported that the prognosis of ESCC in patients with T1 tumors who underwent chemoradiation therapy or surgery was similar (8). Therefore, it is increasingly more important to select for surgery only those patients who will benefit from the same. *ECRG4* mRNA expression may identify tumors of a more malignant nature despite demonstrating similar clinical stages.

In ESCC patients, many prognostic markers such as *cyclin D1, E-cadherin* and *murine double-minute type 2 (MDM2)* have been proposed (9,10). It has also been reported that the expression of *survivin* (11), *DNA fragmentation factor 45 (DFF45)* (12), *pituitary tumor transforming gene 1 (PTTG1)* (13), *checkpoint with FHA and ring finger (chfr)* (14), *peroxisome proliferator activated receptor γ (PPARγ)* (15), *excision repair cross complementing 3 (ERCC3)* (16), *acid phosphatase 6, lysophosphatidic (ACP6)* (17), *poly(A) binding protein, cytoplasmic 1 (PABPC1)* (18) and *N-myc downstream regulated gene 1 (NDRG1)* (19) may be prognostic markers of ESCC. *ECRG4* has now been added as a possible prognostic indicator in ESCC patients.

Although the precise molecular mechanism of the down-regulation of *ECRG4* expression needs to be clarified, our data indicate that *ECRG4* may be a good molecular prognostic marker for patients with ESCC. Elucidating the function of *ECRG4* may lead to a better understanding of the carcinogenic mechanism of tumor progression in patients with ESCC.

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