The loss of *CASP4* expression is associated with poor prognosis in esophageal squamous cell carcinoma

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Abstract. Esophageal squamous cell carcinoma (ESCC) has high biological malignant potential among the various digestive tract cancers and is associated with a poor prognosis. To identify novel genes involved in tumor progression, the present study analyzed the genetic and transcriptional alterations in two clinical cohorts, totaling 157 cases of ESCC (78 cases from the discovery set and 79 cases from the validation set). From the discovery set, gene expression and copy number profiles were analyzed using expression arrays and array-comparative genomic hybridization, respectively. Notably, a copy number loss of caspase-4 (*CASP4*) was observed in 82% of ESCC cases and *CASP4* expression levels were significantly associated with copy number levels. Gene set enrichment analysis demonstrated that the upregulation of *CASP4* expression levels was associated with the signaling pathways involved in apoptosis, inflammatory responses and immune responses. The present study demonstrated that *CASP4* expression levels were significantly associated with the expression levels of the endoplasmic reticulum (ER) stress marker glucose-regulated protein 78, indicating that *CASP4* has a role in cell death induced by ER stress in ESCC. In the survival analysis the *CASP4* low expression group exhibited a poor prognosis, compared with the *CASP4* high expression group in the discovery set (P=0.003); this observation was reproduced in the validation set (P=0.037). Therefore, the results of the current study suggest that *CASP4* may function as a tumor-suppressor gene and may have applications as a biomarker for the prediction of the prognosis in ESCC.

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Introduction

The incidence of esophageal squamous cell carcinoma (ESCC) was ~24.4 males and ~4 females per 100,000 individuals in Japan in 2004; 3.4% of mortalities from all malignant neoplasms are due to ESCC (1). ESCC has high biological malignant potential amongst digestive tract cancers due to the frequent involvement of lymph node metastasis and tumor invasion of adjacent organs at the early stages (2). Therefore, it is crucial to identify biomarkers for predicting the malignant potential of ESCC.

DNA copy number alterations, which are associated with the dysregulation of gene expression, are considered to have a critical role in the pathogenesis of human cancers (3). The amplification and overexpression of human epidermal growth factor receptor 2 *(HER2)* are associated with poor prognosis in patients with breast cancer, and *HER2* is an effective therapeutic target for trastuzumab (4). In ESCC, integrative analysis of copy number and gene expression profiles may facilitate the identification of genes associated with tumor progression (5-7); the current study aimed to investigate the genetic and transcriptional alterations in ESCC.

The caspase-4 *(CASP4)* gene encodes a protein involved in immunity and inflammation (8). Previous studies into cell death have demonstrated that endoplasmic reticulum (ER) stress induces *CASP4*-mediated cell apoptosis (9-11). Although pro-apoptotic caspases are downregulated in certain cancers (12), few previous clinical studies have focused on *CASP4*. Notably, *CASP4* expression is suppressed, and is associated with poor prognosis, in head and neck squamous cell carcinoma (13,14). Loss-of-function *CASP4* mutations are rarely observed in colorectal cancers (15); however, the clinical significance and role of *CASP4* have yet to be evaluated in ESCC. The aim of the present study was to elucidate the clinical role of *CASP4* expression in ESCC. The association between the expression levels and copy number profiles of *CASP4* in tumor samples was investigated. Furthermore, gene set enrichment analysis (GSEA) (16) was performed to identify the signaling pathways involved in *CASP4* expression in ESCC; the association between the expression levels of ER stress markers and *CASP4* was also analyzed. Finally, the present study assessed the significance of *CASP4* expression levels in the prognosis of ESCC.

Materials and methods

Sample collection, DNA extraction and RNA extraction. ESCC tumor and adjacent tissue samples were collected from a total of 157 patients diagnosed with ESCC who underwent surgical resection at the following five institutions: Juntendo University Hospital (Tokyo, Japan), National Cancer Center (Tokyo, Japan), Kurume University Hospital (Kurume, Japan), Saitama Cancer Center (Saitama, Japan), and Kagoshima University Medical and Dental Hospital (Kagoshima, Japan). The mean age of the patients was 65.5 years (age range, 40-83 years), with a 137:17 male:female ratio (clinical information was available for 154/157 patients). ESCC tissues samples with tumor stages T1–T4 were included in the study. The tissue samples were randomly divided into two sets, with 78 samples included in the discovery set (clinical information including survival profile was available for 75/78 cases) and 79 samples in the validation set (clinical information was available for 79 cases); each dataset included the above-mentioned institutions. In the discovery set, gene expression profiles were analyzed using human gene expression microarrays (Whole Human Genome Microarray kit; 4x44; cat. no. G4112F; Agilent Technologies, Inc., Santa Clara, CA, USA). Array-comparative genomic hybridization (CGH) was also performed in 57/78 cases in the discovery set, as genomic DNA was available in these cases. In the validation set, *CASP4* expression levels were analyzed using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Cells were isolated using a laser microdissection system (LMD; Leica Microsystems GmbH, Wetzlar, Germany) and DNA and RNA samples were purified and extracted using a QIAamp DNA Micro kit (Qiagen GmbH, Hilden, Germany) and an RNeasy Micro kit (Qiagen GmbH). Written informed consent was obtained from all patients and the study protocol was reviewed and approved by the internal review board of Kyushu University (Fukuoka, Japan).

Array-CGH and copy number analysis. Genomic DNA samples from 57 tumor specimens were analyzed using array-CGH. The genomic DNA of 3 samples from the normal esophageal mucosa was also analyzed and used as a reference for the array-CGH. DNA labeling and hybridization were performed using a Genome Microarray kit 244K (Agilent Technologies, Inc.). The data were evaluated using a microarray scanner (Agilent Technologies, Inc.) and analyzed using the Feature Extraction software version 9.1 (Agilent Technologies, Inc.).

Expression array. An expression array was performed using the 78 tumor samples in the discovery set. The total RNA extracted using the LMD technique was reverse transcribed to generate double-stranded cDNA. Amplification was then performed using a T7 RNA polymerase (Agilent Technologies, Inc.), and the product was converted to cyanine-labeled cRNA. The labeled cRNA was fragmented and hybridized to an oligonucleotide microarray (Whole Human Genome Microarray kit, 4x44; G4112F; Agilent Technologies, Inc.). Fluorescence intensities were obtained using an Agilent DNA microarray scanner and subjected to quantile normalization.

GSEA. GSEA was performed using the gene expression data from the discovery set to investigate how signaling pathways were differentially regulated depending on the expression levels of *CASP4* in ESCC. The present study used a continuous-type class file with the *CASP4* profile to phenotype labels in GSEA; the expression level of *CASP4* in each sample was used as an input data in GSEA. The gene sets extracted from the Broad Institute (Cambridge, MA, USA) database (http://software.broadinstitute.org/gsea/msigdb/collections.jsp) included gene sets based on the gene ontology pathway (apoptosis_go, inflammatory_response, and immune_response), gene sets annotated by the reactome pathway (reactome_apoptosis and reactome_innate_immune_system) and a gene set based on the biocarta pathway, biocarta_inflam_pathway.

RT-qPCR. RT-qPCR was performed using a LightCycler system (Roche Diagnostics, Indianapolis, IN, USA) and a LightCycler 480 Probes Master kit (Roche Diagnostics) according to the manufacturer's protocol. The following thermal cycling conditions were used: Initial denaturation at 95˚C for 10 min, followed by 45 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 30 sec, and extension at 40˚C for 30 sec. The *CASP4* primers were as follows: Forward, 5'-TTCCTGGCAATTGAAAATGG-3', and reverse, 5'-TGCAAGCTGTACTAATGAAGGTG-3'. The concentrations of *CASP4* in each sample were calculated by plotting their crossing points against the standard curve from a single experiment. The *CASP4* mRNA level was normalized to the internal standard, glyceraldehyde 3-phosphate dehydrogenase assessed using the following primers: *GAPDH* forward, 5'-AGCCACATCGCTCAGACAC-3', and reverse, 5'-GCCCAATACGACCAAATCC-3'.

Statistical analysis. Student's t-tests and Fisher's exact tests were used to determine significant differences between the groups. The Kaplan-Meier method was used to evaluate the survival rates and the survival curves were compared using log-rank tests. Overall survival was calculated from the date of surgical resection to date of death/final follow-up. Statistical analysis was performed using JMP version 5 software (SAS Institute, Buckinghamshire, UK). $P<0.05$ was considered to indicate a statistically significant result.

Results

Copy number loss inhibits CASP4 expression in ESCC. The copy number alterations accompanying changes in *CASP4* gene expression were analyzed using the array-CGH and the expression array data from the ESCC discovery set. The present study demonstrated that *CASP4* copy number loss occurred in 47/57 ESCC samples and that there was a significant association between the copy number and expression levels (Spearman's correlation, rho=0.37; P=0.005; Fig. 1). In ESCC, copy number loss appeared to suppress *CASP4* gene expression, suggesting that *CASP4* may act as a tumor-suppressor gene.

Higher CASP4 expression levels were significantly associated with the signaling pathways involved in apoptosis, inflammatory responses, and immune responses in ESCC. GSEA of the 78 ESCC samples from the discovery set revealed that certain gene sets involved in the apoptotic signaling pathway were significantly upregulated in ESCCs expressing high

Table I. *CASP4* mRNA expression and clinicopathological factors in the validation set.

Factor	High *CASP4* expression n=39	Low *CASP4* expression n=40	P-value
Age (mean ± SD)	65.92±7.87	63.38±8.43	0.169[a]
Gender			
Male	35	36	0.973[b]
Female	4	4	
Tumor differentiation			
Well	15	14	0.818[b]
Mod or poor	24	26	
Depth			
T1	4	5	1.000[b]
T2-4	35	35	
Lymph node metastasis			
Negative	12	10	0.622[b]
Positive	27	30	
Lymphatic invasion			
Negative	12	2	0.003[b,c]
Positive	27	38	
Venous invasion			
Negative	7	4	0.348[b]
Positive	32	36	

CASP4, caspase-4; SD, standard deviation; Well, well differentiated squamous cell carcinoma; Mod, moderately differentiated squamous cell carcinoma; Poor, poorly differentiated squamous cell carcinoma. [a]Student's t-tests and Fisher's exact tests. [b]Fisher's exact tests. [c]P<0.05, indicates a statistically significant result.
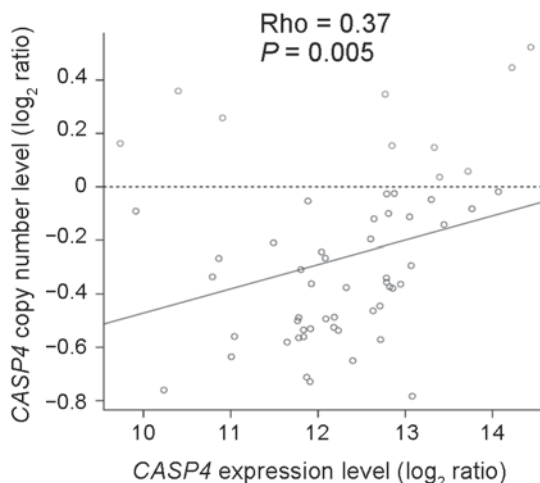

Figure 1. Dot plots of *CASP4* copy numbers and expression levels in ESCC. The horizontal dotted line represents the threshold of copy number loss/amplification. Copy number loss of *CASP4* was observed in 47/57 ESCC cases in the discovery set. The *CASP4* expression levels were significantly positively associated with copy number levels (P=0.005). ESCC, esophageal squamous cell carcinoma; *CASP4*, caspase-4.

levels of *CASP4* as follows: Apoptosis_go, P<0.001, false discovery rate (FDR)=0.025; reactome_apoptosis P=0.031, FDR=0.103 (Fig. 2A). The present study also demonstrated that higher *CASP4* expression was significantly associated

with the enrichment of gene sets involved in the inflammatory response, including inflammatory_response (P<0.001; FDR=0.012) and biocarta_inflam_pathway (P=0.004; FDR=0.024; Fig. 2B), and of gene sets involved in the immune response, including immune_response (P=0.004; FDR=0.017) and reactome_innate_immune_system (P<0.001; FDR=0.031; Fig. 2C). These data were consistent with *CASP4* being an inflammatory caspase and a critical mediator of the innate immune response (8). The current study subsequently evaluated the association between the expression levels of *CASP4* and the ER chaperone glucose-regulated protein (*GRP*) 78, which is used as an ER stress marker (9-11,17). *CASP4* expression levels were positively associated with *GRP78* expression levels (Spearman's correlation, rho=0.29; P=0.009; Fig. 3), demonstrating that *CASP4* has a role in regulating ER stress-induced cell death in ESCC.

CASP4 expression levels predict the prognosis of patients with ESCC. To examine the clinical significance of *CASP4* expression in ESCC, a survival analysis was performed. The present study identified that low *CASP4* expression levels were significantly associated with a poor overall survival rate in the discovery set (P=0.003; Fig. 4A). To investigate the clinical significance of *CASP4* expression levels in ESCC, the validation set was also analyzed. Low *CASP4* expression levels were observed to be significantly associated with lymphatic invasion (P=0.003; Table I). The overall survival rate for the validation set was also analyzed, and revealed no significant differences
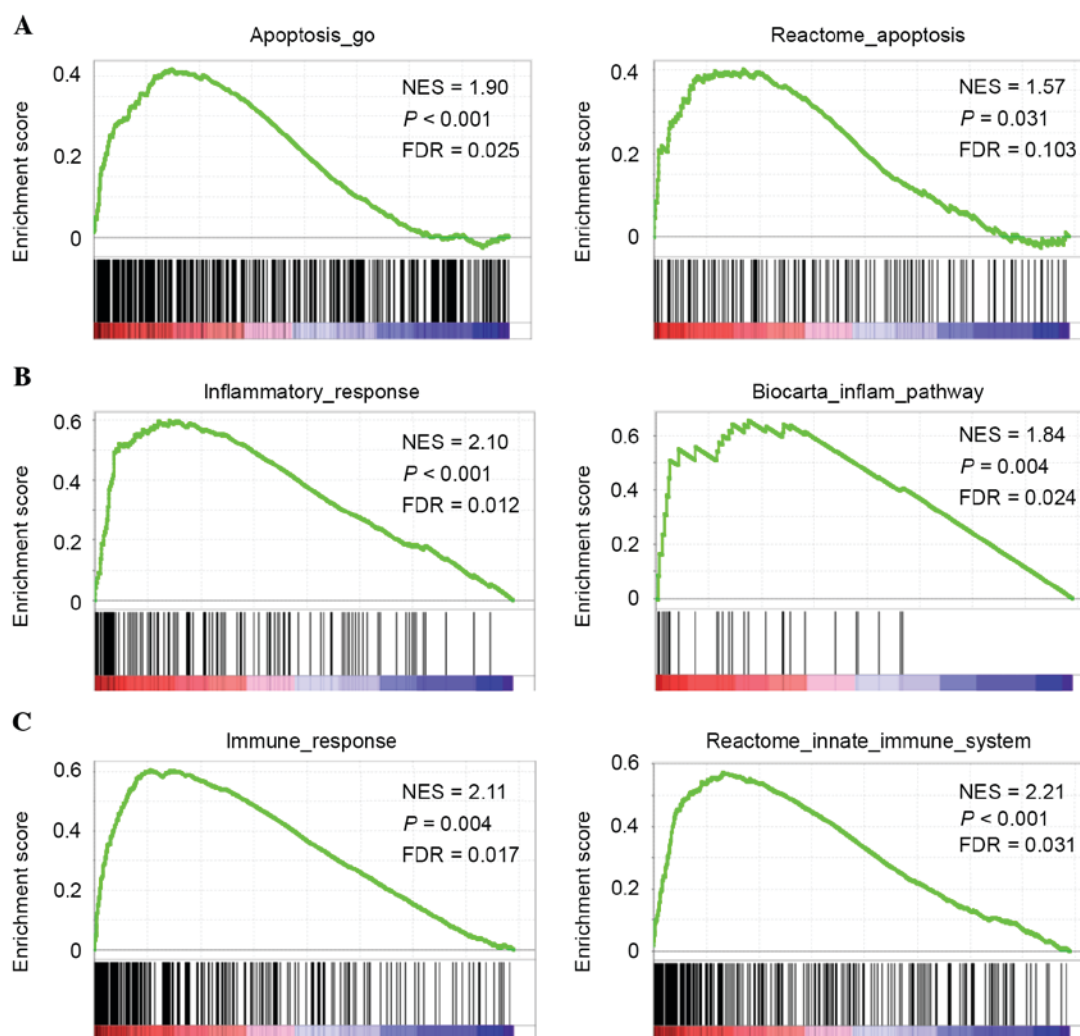
Figure 2. Gene set enrichment analysis in the discovery set demonstrated that higher *CASP4* expression levels were significantly associated with the signaling pathways involved in (A) apoptosis, (B) inflammatory responses and (C) immune responses. Each gene set was based on the Gene Ontology pathway (left) or the Reactome and BioCarta pathways (right). The barcode plot indicates the relative positions of genes in each gene set; red and blue colors indicate positive and negative Pearson correlations with *CASP4* expression, respectively. *CASP4*, caspase-4; NES, normalized enrichment score; FDR, false discovery rate.
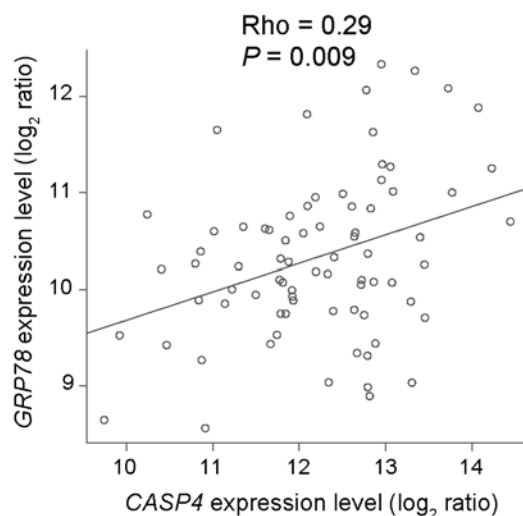

Figure 3. *CASP4* expression levels were positively associated with the expression levels of the endoplasmic reticulum stress marker *GRP78* in esophageal squamous cell carcinoma. The analysis was performed using expression array data from the discovery set. Results were assessed by Spearman's correlation. *CASP4*, caspase-4; GRP78, glucose-regulated protein 78.

in the overall survival rate between the discovery and validation sets (P=0.805). Concordant with the discovery set data, the *CASP4* low expression group had a significantly poorer overall survival rate, compared with the *CASP4* high expression group, in the validation set (P=0.037; Fig. 4B). These results suggest that *CASP4* may have a tumor suppressor role in ESCC.

Discussion

The current study demonstrated that the downregulation of *CASP4* expression levels is associated with ESCC progression. The gene expression and copy number profiles of clinical tissue samples were analyzed using a bioinformatics approach and demonstrated that the copy number loss of *CASP4* was associated with the decreased expression levels of *CASP4* observed in ESCC. The *CASP4* low expression group had a poor prognosis, compared with the *CASP4* high expression group; these results were reproducible in each clinical ESCC cohort. These results suggest that *CASP4* may function as a tumor-suppressor gene and may be a useful biomarker for predicting the prognosis in ESCC.
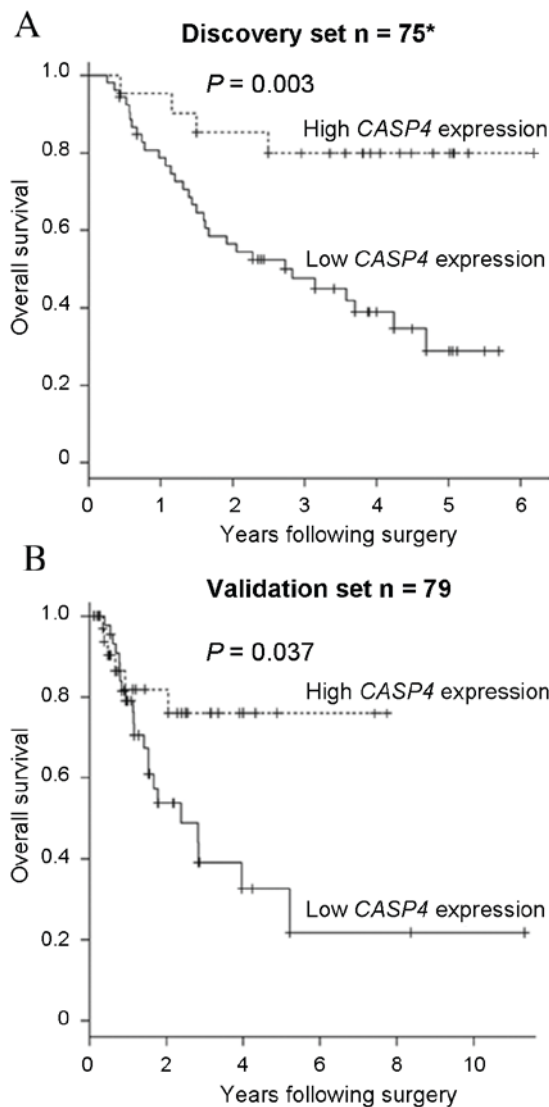
Figure 4. *CASP4* mRNA expression levels predicted the prognosis of patients with esophageal squamous cell carcinoma treated with surgical resection. (A) Kaplan-Meier overall survival curves based on *CASP4* expression levels in the discovery set. *The survival profiles were available for 75/78 patients. (B) Overall survival curves based on *CASP4* expression levels in the validation set. The survival profiles were available for all 79 patients. Overall survival was calculated from the date of surgical resection to date of death/final follow-up. Survival curves were analyzed using the log-rank test. *CASP4*, caspase-4.

CASP4 has been previously reported to induce cell death (9-11). A few studies have identified that *CASP4* is involved in ER stress-induced apoptosis in neurodegenerative disorders (9), muscular dystrophy (10) and retinal pigment epithelial cells (11). Concordantly, the present study demonstrated that *CASP4* expression levels were significantly associated with the apoptotic signaling pathway and the expression levels of *GRP78* in ESCC. Furthermore, *CASP4* encodes a protein involved in inflammation and immune responses (8). Kobayashi *et al* (18) reported that *CASP4* is involved in the innate immune response and inflammatory cell death in bacterial infection. Similarly, the current study indicated that *CASP4* was significantly associated with the inflammatory and immune responses that may contribute to the inhibition of ESCC progression.

The data demonstrates that low *CASP4* expression is significantly associated with lymphatic invasion. We hypothesized that *CASP4* expression may contribute to the early phase of ESCC progression, as cell death, including apoptosis, is more frequent during the early phase of tumor progression, and lymphatic invasion occurs earlier than lymph node metastasis. Therefore, it is possible that *CASP4* expression levels may predict not only the prognosis, but also early phase tumor progression in ESCC.

In conclusion, the current study indicated that *CASP4* may be a tumor-suppressor gene associated with the signaling pathways underlying apoptosis, inflammatory responses and immune responses in ESCC. The results also suggested that ER stress induces *CASP4*-mediated apoptosis. *CASP4* expression may therefore be a useful clinical biomarker for predicting the prognosis of patients with ESCC.

Acknowledgements

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