


Caspase recruitment domain family member 9 expression is a promising biomarker in esophageal squamous cell carcinoma

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Abstract

Aim: Esophageal squamous cell carcinoma (ESCC) is a refractory digestive organ cancer that requires better treatment strategies. We have recently reported that the antidiabetic drug metformin exerts antitumor effects on ESCC by inhibition of nuclear factor kappa B (NF- κ B) nuclear translocation. In the present study, we focused on caspase recruitment domain family member 9 (CARD9), an essential signal adapter in NF- κ B activation to examine whether it can be used as a prognostic factor in ESCC.

Methods: We investigated CARD9 expression immunohistochemically in clinical samples obtained from 93 patients with ESCC who underwent curative esophagectomy. CARD9 expression was analyzed for correlation with clinicopathological characteristics and ESCC prognosis. The molecular effects were investigated by knocking down ESCC cells. Comprehensive RNA expression changes in these ESCC cells were detected by next-generation sequencing (NGS).

Results: High CARD9 expression is significantly correlated with advanced tumor depth ($P < .001$), positive lymph node metastasis ($P = .005$) and advanced stage ($P = .001$). Kaplan-Meier method and the log-rank test showed that overall survival (OS) and disease-free survival (DFS) were significantly poor in the high CARD9 expression group (OS: $P = .027$, DFS: $P = .005$). Univariate and multivariate analysis showed that high CARD9 expression is a significant poor prognostic factor for DFS. Cell proliferation and migration were suppressed by CARD9 knockdown. NGS detected altered the expression of some RNAs including maternally expressed 3 (MEG3).

Conclusion: High CARD9 expression is significantly associated with poor prognosis. Therefore, CARD9 expression may be a prospective prognostic biomarker in ESCC.

KEYWORDS

caspase recruitment domain protein 9, esophageal squamous cell carcinoma, high-throughput nucleotide sequencing

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1 | INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is a refractory digestive organ cancer that shows high morbidity in East Asia. It remains difficult to treat despite multidisciplinary therapy that includes tumor excision, chemotherapy and radiotherapy.¹ Hence, a new treatment strategy for ESCC is required. We recently reported that metformin, which is an oral biguanide for treating type 2 diabetes mellitus, exerts antitumor effects in ESCC by inhibiting nuclear translocation of nuclear factor kappa B (NF- κ B).²

In the present study, we focused on caspase recruitment domain family member 9 (CARD9), which functions as an essential signal adapter in the activation of NF- κ B.^{3,4} CARD9 is known to work mainly in the innate immune system.⁵ CARD9 seems to be strongly associated with inflammation, as it reportedly has strong correlation with inflammatory bowel disease.⁶ Moreover, CARD9 has been reported to promote colon tumors in the adenomatous polyposis coli (APC^{min}) mouse model; surprisingly, this activity was specifically shown only in male mice.⁷ And, in experimental colitis, CARD9 signaling promotes gut fungi-mediated inflammasome activation, which restricts colitis and colon cancer.⁸ Furthermore, correlations between CARD9 and other types of cancer have been reported. For example, CARD9 overexpression is associated with the development or progression of gastric B-cell lymphoma.⁹ In hepatocellular carcinoma, mRNA level of CARD9 is significantly higher and, therefore, CARD9 expression could be used as a diagnostic marker.¹⁰ Moreover, in renal cell carcinoma, high CARD9 expression is associated with an increased risk of recurrence and shorter recurrence-free survival.¹¹

From these reports, it can be concluded that CARD9 plays an important role in NF- κ B-mediated inflammatory response in cancers and exacerbates cancer by inducing inflammatory response. It has already been reported that ESCC with activated NF- κ B has poor sensitivity to chemotherapy and that increased expression of NF- κ B is associated with poor prognosis in patients with ESCC.¹² These reports suggest that CARD9 may influence ESCC by activating NF- κ B. However, there is no report about the relationship between CARD9 and ESCC. Thus, we examined whether CARD9 can be used as a prognostic factor and further analyzed the function of CARD9 in ESCC.

2 | MATERIALS AND METHODS

2.1 | Patients and clinical specimens

We retrospectively investigated patients who underwent esophagectomy (with two- or three-field lymph node dissection, if necessary) at Chiba University Hospital between 2001 and 2011. Patient medical records and survival status were retrospectively reviewed in January 2017.

Patients were eligible for this study if they: (i) were pathologically diagnosed with ESCC; (ii) were between 20 and 85 years of age; (iii) did not receive any preoperative treatment as chemotherapy; (iv) did not have any other types of cancer; and (v) achieved pathological

TABLE 1 Patient characteristics (n = 93) according to UICC (7th edition)

Age median (min-max), y		67 (40-82)
Gender (male : female)		79:14
Pathological T factor	T1a	14
	T1b	31
	T2	7
	T3	36
	T4a	5
Pathological N factor	N0	46
	N1	29
	N2	15
	N3	3
pStage	IA/IB	33
	IIA/IIIB	25
	IIIA/IIIB/IIIC	35

Abbreviation: UICC, Union for International Cancer Control.

RO curative resection. The 11 patients who received postoperative adjuvant chemotherapy were included.

Data of 93 patients were examined after excluding patients who did not meet these criteria retrospectively. We obtained specimens from these patients and used them to evaluate CARD9 expression by immunohistochemistry. Informed consent was obtained from all patients for research purposes.

The tumor node metastasis (TNM) system (Union for International Cancer Control [UICC], 7th edition) was used to classify ESCC stages in these patients.¹³ Patient characteristics (age, gender, and pathological evaluation) are shown in Table 1.

2.2 | Esophageal squamous cell carcinoma cell culture and reagents

We used the human ESCC cell lines TE8 and T.Tn. TE8 cells were kindly provided by Dr T. Nishihira (Tohoku University, Sendai, Japan). T.Tn cells were provided by the Japanese Cancer Research Resources Bank.

Human ESCC cell lines were cultured in DMEM/nutrient mixture F-12 Ham (DMEM/F-12) (Sigma-Aldrich, St Louis, MO, USA) with 10% FBS in a humidified incubator at 37°C in an atmosphere with 5% CO₂.

Penicillin and streptomycin were obtained from North China Pharmaceutical Group Corp. (Shijiazhuang, China). We obtained 0.05% trypsin-EDTA and FBS from Invitrogen Life Technologies (Waltham, MA, USA).

2.3 | Immunohistochemistry

Expression of CARD9 was assessed by immunohistochemistry using the peroxidase-antiperoxidase complex method. Anti-human

CARD9 polyclonal antibody (1:100; ab115578; Abcam, Cambridge, UK) was used as the primary antibody, and anti-mouse/rabbit antibody (EnVision/HRP, anti-mouse/rabbit K5001; DAKO Japan, Tokyo, Japan) was used as the secondary antibody.

2.4 | Evaluation of CARD9 expression

Patients were divided into two groups. Staining intensity was judged as no staining (0 points), weak (1 point), moderate (2 points), or strong (3 points). Proportion of the stained cancer area was classified as <50% (1 point), 50%-80% (2 points), or >80% (3 points). Overall score was calculated by adding the staining intensity score to the proportion score. An overall score of two points or less was defined as low expression, and that of three points or more was defined as high expression. The diagnosis of CARD9 expression was confirmed by agreement with two pathologists.

2.5 | Transfection of small interfering RNAs

Small interfering RNAs (siRNAs) (27-mer siRNA duplexes; CARD9; OriGene Technologies, Rockville, MD, USA) and negative control siRNA (Universal scrambled negative control siRNA duplex; OriGene Technologies) were transfected into the TE8 and T.Tn cell lines using the Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol.

2.6 | Quantitative reverse-transcription PCR and next-generation sequencing

Following the transfection of cells with siCARD9 or negative control siRNA for 48 hours, total RNA from the harvested cells was converted into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Venlo, the Netherlands). Real-time PCR was carried out in triplicate using a QuantiTect SYBR Green PCR Kit (Qiagen) and a MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's protocol. Primers used for CARD9 (assay name: Hs_CARD9_1_SG) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (assay name: Hs_GAPDH_1_SG) as an internal control were obtained from Qiagen (Venlo, Netherlands). Total RNA obtained was analyzed by next-generation sequencing (NGS) (Eurofins Genomics, Tokyo, Japan).

2.7 | Cell proliferation assay

Following transfection of cells with siCARD9 or negative control siRNA for 48 hours, the proliferation assay was carried out. The inhibitory influence of siCARD9 on cell proliferation was evaluated by measuring cell viability using Cell Counting Kit-8 (CCK-8;

Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates at 5×10^3 cells/well. After 72 hours, cell viability was determined using CCK-8. Absorbance was determined at 450 nm using a microplate reader.

2.8 | Cell migration assay

Cell migration assay was carried out using a micropore membrane filter with 8 μ m pores as an insert (BD Biosciences, East Rutherford, NJ, USA). A total of 5×10^4 cells with DMEM/F-12 without FBS were seeded into the 24-well insert and, at the same time, siCARD9 or negative control siRNA was added. The lower chamber was filled with DMEM/F-12 containing 10% FBS as the attractant. After 24 hours, the cells on the upper side of the membrane were removed with cotton swabs, and the membranes were fixed and stained using Diff-Quik reagent (International Reagents, Hyogo, Japan). Cell migration was quantified by counting the number of migrated cells in a microscopic high-powered field per filter.

2.9 | Statistical analyses

Correlation between CARD9 expression levels and clinicopathological features was evaluated using Fisher's exact test. Survival rates (overall survival [OS] and disease-free survival [DFS]) were assessed using the Kaplan-Meier method and the log-rank test. Variables subjected to univariate analysis included age at diagnosis (≥ 65 and < 65 years), gender (male and female), T (T1 and T2/T3/T4), N (N0 and N1/N2/N3), stage (IA/IB and IIA/IIB/IIIA/IIIB/IIIC) and CARD9 expression (high and low). Variables subjected to multivariate analysis included age at diagnosis, gender, stage and CARD9 expression, and excluded T and N as the confounding factors of stage. Significance of these factors as prognostic factors for OS and DFS was evaluated using Cox proportional hazards model and Wald confidence intervals were shown. Correlation between two variables and numerical values was assessed using Wilcoxon signed rank test or *t* test. JMP pro version 14 software (SAS Institute Inc.) was used for all statistical analyses. $P < .050$ was considered significant.

3 | RESULTS

3.1 | Relationship between CARD9 expression and clinicopathological features

Among the 93 cases studied, 33 (35.5%) had tumors with low CARD9 expression, and 60 (64.5%) had tumors with high CARD9 expression. Examples of staining profiles of high- and low-CARD9 expression in ESCC and normal CARD9 expression in esophageal tissue are shown in Figure 1. CARD9 was strongly expressed in both cytoplasm and nucleus in the cancer cells of

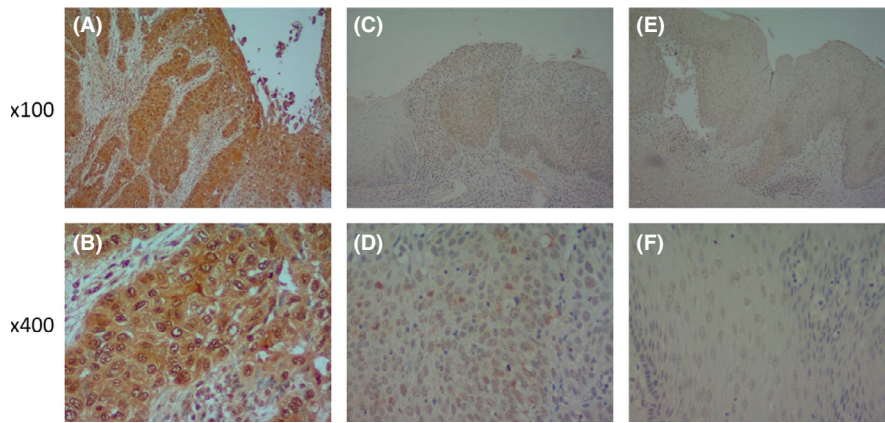


FIGURE 1 Immunohistochemical analysis of caspase recruitment domain family member 9 (CARD9) expression in esophageal squamous cell carcinoma. Images of high CARD9 expression (A and B), low CARD9 expression (C and D) and normal esophageal tissue (E and F) are shown (magnification $\times 100$ in the upper section, $\times 400$ in the lower section)

the high-expression group, and in normal esophageal squamous epithelium CARD9 was slightly expressed in the nucleus especially near the basal layer.

High CARD9 expression is significantly correlated with advanced tumor depth ($P < .001$), positive lymph node metastasis ($P = .005$), and advanced stage ($P = .001$). Age and gender did not show statistically significant associations with high CARD9 expression (Table 2). Postoperative adjuvant chemotherapy was carried out in four patients in the high CARD9 expression group and in seven patients in the low CARD9 expression group.

3.2 | Relationship between CARD9 expression and clinical prognosis

Analysis using Kaplan-Meier method and log-rank test showed that OS and DFS were significantly poor in the high CARD9 expression group (OS: $P = .027$, DFS: $P = .005$). Surprisingly, this

TABLE 2 Relationship between CARD9 expression and clinicopathological characteristics

Characteristics		CARD9 expression		P value
		Low	High	
Age (y)	≥ 65	22	32	.274
	< 65	11	28	
Gender	Male	30	49	.364
	Female	3	11	
Tumor depth	T1	24	21	$< .001$
	T2/T3/T4a	9	39	
LN status	N0	23	23	.005
	N1/N2/N3	10	37	
Stage	IA/IB	19	14	.001
	IIA/IIB/IIIA/IIIB/IIIC	14	46	

Abbreviations: CARD9, caspase recruitment domain family member 9; LN, lymph node.

tendency was shown only in males (OS, male: $P = .014$, female: $P = .944$) (Figure 2).

Univariate analysis showed that advanced tumor depth, advanced stage, and high CARD9 expression were significant prognostic factors for OS. Multivariate analysis showed that the hazard ratio (HR) of high CARD9 expression was 2.039 (95% confidence interval [CI] = 0.941-4.417, $P = .071$) (Table 3).

For DFS, univariate analysis showed that advanced tumor depth, positive lymph node metastasis, advanced stage, and high CARD9 expression were significant prognostic factors. Multivariate analysis showed that high CARD9 expression was an independent prognostic factor for DFS in these patients with ESCC (high CARD9 expression: HR = 2.538; 95% CI = 1.013-6.356, $P = .047$) (Table 4).

3.3 | Effect of CARD9 knockdown on proliferation and migration in ESCC cell lines

Expression of CARD9 was evaluated in ESCC cell lines TE8 and T.Tn. After siCARD9 transfection into these cell lines, levels of CARD9 mRNA decreased significantly (Figure 3A, TE8: $P = .002$, T.Tn: $P = .003$).

In the proliferation assay, the number of cells was significantly decreased by CARD9 knockdown in TE8 and T.Tn cell lines (Figure 3B, TE8: $P < .001$, T.Tn: $P < .001$).

In the migration assay, the number of cells penetrating the membrane was significantly decreased by CARD9 knockdown in both TE8 and T.Tn cell lines (Figure 3C, TE8: $P = .001$, T.Tn: $P = .010$).

3.4 | Next-generation sequencing analysis

We comprehensively analyzed the effect of CARD9 on RNA expression in TE8 and T.Tn ESCC cell lines. Results are shown in Table 5. CARD9 knockdown upregulated RNA expression of the genes encoding maternally expressed 3 (MEG3), IQ motif containing N (IQCN), hemopexin (HPX), cytidine/uridine monophosphate kinase 2 (CMPK2), radical S-adenosyl methionine domain containing 2 (RSAD2), dual specificity

FIGURE 2 Comparisons of overall survival (OS) (A) and disease-free survival (DFS) (B) of high- and low-caspase recruitment domain family member 9 (CARD9) expression groups of patients with esophageal squamous cell carcinoma (ESCC). OS of high- and low-CARD9 expression groups in ESCC sorted by gender (C)

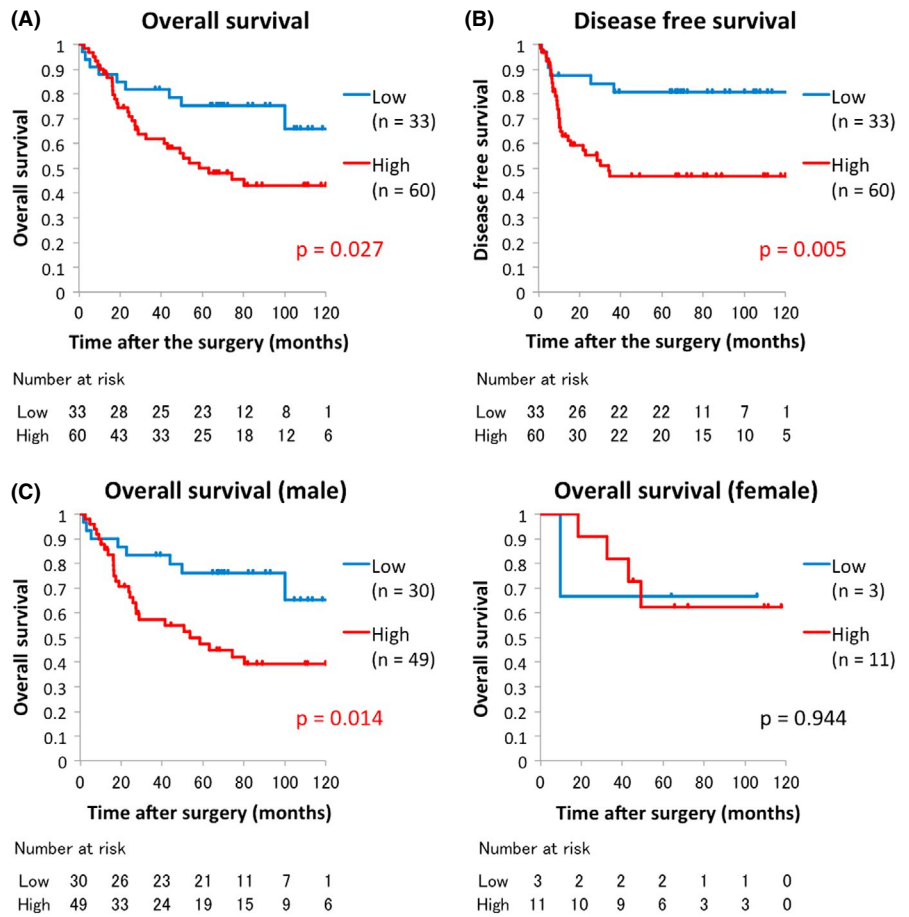


TABLE 3 Univariate and multivariate analyses of clinicopathological factors and overall survival in ESCC

Factor	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Age ≥65 y	1.036	0.555-1.933	.912	1.055	0.563-1.978	.867
Male	1.408	0.551-3.596	.475	1.780	0.689-4.597	.234
T2/T3/T4a	2.937	1.505-5.730	.002			
N1/N2/N3	1.565	0.834-2.937	.163			
Stage IIA/IIB/IIIA/IIIB/IIIC	2.251	1.096-4.624	.027	1.968	0.936-4.140	.074
High CARD9	2.267	1.077-4.770	.031	2.039	0.941-4.417	.071

Abbreviations: CARD9, caspase recruitment domain family member 9; ESCC, esophageal squamous cell carcinoma.

phosphatase 13 (*DUSP13*), and beta-1,3-galactosyltransferase 5 (*B3GALT5*). However, RNA expression of the genes encoding cluster of differentiation 14 (*CD14*), calcium voltage-gated channel auxiliary subunit beta 4 (*CACNB4*), and cysteine dioxygenase type 1 (*CDO1*) was inhibited by *CARD9* knockdown.

4 | DISCUSSION

The present study showed that *CARD9*, which plays an important role during the immune response in humans, influences malignancy in ESCC. Examination of the clinical specimens showed that *CARD9*

expression was scarce in normal tissue, but was occasionally higher in cancerous tissue. Expression of *CARD9* in ESCC strongly correlated with cancer malignancy, with prognosis being significantly poor in the high-expression group. This makes *CARD9* a significant prognostic factor for ESCC.

In addition, surprisingly, a remarkable difference was seen in OS between males of high- and low-*CARD9* expression groups, whereas no significant difference was observed in females. However, the number of females was so small that this point is the limitation of the present study and a larger group in further study is necessary. It is important to elucidate the reason behind this gender difference. Until now, only one other report has described the effect of *CARD9*

TABLE 4 Univariate and multivariate analyses of clinicopathological factors and disease-free survival in ESCC

Factor	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Age ≥65 y	0.625	0.321-1.215	.166	0.607	0.311-1.187	.145
Male	1.627	0.574-4.612	.360	2.454	0.852-7.070	.096
T2/T3/T4a	5.173	2.329-11.490	<.001			
N1/N2/N3	3.323	1.590-6.944	.001			
Stage IIA/IIB/IIIA/IIIB/IIIC	3.911	1.617-9.460	.003	3.412	1.365-8.526	.009
High CARD9	3.306	1.367-7.995	.008	2.538	1.013-6.356	.047

Abbreviations: CARD9, caspase recruitment domain family member 9; ESCC, esophageal squamous cell carcinoma.

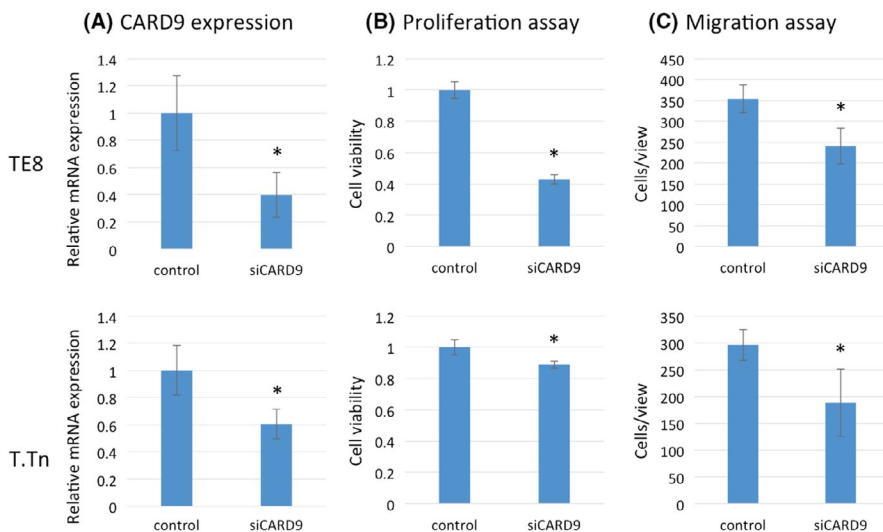


FIGURE 3 Effects of caspase recruitment domain family member 9 (CARD9) knockdown on esophageal squamous cell carcinoma (ESCC) cell proliferation and migration. ESCC cell lines (TE8 and T.Tn) were transfected with control siRNA or siCARD9. Relative CARD9 mRNA expression in ESCC cell lines after transfection of control siRNA and siCARD9 (A), and results of the proliferation assay (B) and migration assay (C) are shown. * $P < .050$

TABLE 5 Next-generation sequencing analysis of the effect of siCARD9 on RNA expression in ESCC cell lines

Gene	logFC (log2 fold change)	P value	FDR
MEG3	4.49	.00001	0.54
IQCN	5.68	.00002	0.54
HPX	5.64	.00003	0.54
CMPK2	3.51	.00004	0.54
RSAD2	3.35	.00006	0.655
CD14	-5.4	.00010	0.791
CACNB4	-5.3	.00014	0.887
CDO1	-5.27	.00015	0.887
DUSP13	3.56	.00016	0.887
B3GALT5	5.16	.00016	0.887
CARD9	-2.62	.00138	1

Abbreviations: B3GALT5, beta-1,3-galactosyltransferase 5; CACNB4, calcium voltage-gated channel auxiliary subunit beta 4; CARD9, caspase recruitment domain family member 9; CD14, cluster of differentiation 14; CDO1, cysteine dioxygenase type 1; CMPK2, cytidine/uridine monophosphate kinase 2; DUSP13, dual specificity phosphatase 13; ESCC, esophageal squamous cell carcinoma; FDR, false discovery rate; HPX, hemopexin; IQCN, IQ motif containing N; MEG3, maternally expressed 3; RSAD2, radical S-adenosyl methionine domain containing 2.

on carcinogenesis as gender specific.⁷ However, the details remain unclear and further research is desired.

To elucidate the role of CARD9 in carcinogenesis in vitro, siRNA was used to knock down CARD9 in human ESCC cell lines. As a result, cell proliferation and migration were suppressed, which strongly correlated with tumor metastasis and invasion. Thus, CARD9 seems to correlate positively with cancer malignancy. Our data supported the hypothesis that CARD9 is a promising biomarker of ESCC prognosis and suggests that CARD9 could be a potential therapeutic target. However, a molecular targeted antibody seems to be difficult to use for CARD9 because CARD9 is expressed intracellularly. Therefore, searching for molecules associated with CARD9 is very important for further examining its action and also for finding a better treatment method for ESCC.

Next-generation sequencing examination of ESCC cells with knocked down CARD9 detected some candidate genes related to CARD9 expression. Especially, MEG3 seems to be a promising long non-coding RNA (lncRNA) which had induced expression in ESCC cells transfected with siCARD9. Therefore, CARD9 could have a role in the suppression of MEG3 expression in ESCC. Recently, many reports on the association between MEG3 and cancer have been published. MEG3 seems to be a potential biomarker of cancer diagnosis and prognosis.¹⁴ MEG3 might also have tumor-suppressing

effects in ESCC. Compared to normal tissues, *MEG3* is downregulated in ESCC. Methylation of aberrant promoter of *MEG3* controls its expression.¹⁵ Treatment with a DNA-demethylating agent significantly increased *MEG3* expression in ESCC, and enforced expression of *MEG3*-activated p53. Moreover, ectopic expression of *MEG3* in ESCC cells inhibited cell proliferation, promoted apoptosis, and suppressed metastasis.¹⁶ *MEG3* also induces cell apoptosis through endoplasmic reticulum stress in ESCC.¹⁷ It is widely accepted that cancer develops from chronic inflammatory fields and that microbial infection frequently causes chronic inflammation.¹⁸ Additionally, *CARD9* induces inflammation, which is associated with DNA methylation.¹⁹ From these reports, it can be inferred that *CARD9* may function through methylation-mediated silencing of *MEG3* in ESCC.

In conclusion, the results of the present study suggest that by promoting chronic inflammation, *CARD9* plays a major role in the development of ESCC. It is expected that *CARD9* will become a promising prognostic biomarker for ESCC in the future, and it will be a potential target for ESCC treatment.

5 | STATEMENT OF ETHICS

This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Graduate School of Medicine, Chiba University, Chiba, Japan. Informed consent was obtained from all patients for research purposes.

DISCLOSURE

Conflicts of Interest: Authors declare no conflicts of interest for this article.

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