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Identification of *NCCRP1* as an epigenetically regulated tumor suppressor and biomarker for malignant phenotypes of squamous cell carcinoma of the esophagus

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Abstract. The poor prognosis and increasing incidence of esophageal squamous cell carcinoma (ESCC) highlight the need for identification of novel ESCC-associated molecular events to improve the diagnosis, and treatment of this disease. Non-specific cytotoxic cell receptor protein 1 (*NCCRP1*) was reported to be abundantly expressed in human squamous epithelium and to be involved in cell proliferation; however, the role of *NCCRP1* in ESCC remains unclear. To elucidate the oncological roles of *NCCRP1* in ESCC, *NCCRP1* expression, DNA methylation, and copy numbers were analyzed in ESCC cell lines. Nine ESCC cell lines demonstrated different *NCCRP1* mRNA expression levels and all exhibited hypermethylation of the *NCCRP1* promoter, but no copy number loss. Additionally, *NCCRP1* expression was determined in 213 surgically resected esophageal tissue samples. *NCCRP1* mRNA expression levels were reduced in ESCC tissues compared with corresponding non-cancerous adjacent tissues in 204 (95.8%) patients. Patients in the low *NCCRP1* expression group tended to have a higher recurrence rate and a shorter overall survival time compared with those in the high *NCCRP1* expression group. Additionally, multivariate analysis revealed that low *NCCRP1* expression was an independent prognostic factor (hazard ratio, 1.75; 95% confidence interval, 1.08-2.87; P=0.022). The findings of the current study indicate that *NCCRP1* acts as a putative tumor suppressor that is inactivated through promoter hypermethylation, and serves as a promising biomarker to predict postoperative prognosis in ESCC.

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Key words: esophageal cancer, *NCCRP1*, methylation, prognosis, biomarker

Introduction

Esophageal cancer remains a significant cause of cancer-related death and its incidence rate has shown a drastic increase of more than 6-fold worldwide (1,2). Esophageal squamous cell carcinoma (ESCC) is a dominant histological type of esophageal malignances (3,4). The poor prognosis and increasing incidence of ESCC highlight the need for improved detection, prediction, monitoring, and treatment methods (2,5). Existing histopathological terms, such as the pathologic TNM classification, are insufficient to accurately predict individual differences in outcome and inform personalized treatment (2,6). Genetic and epigenetic alterations, such as aberrant gene expression, copy number alterations, and DNA methylation, are associated with the development of ESCC, as well as other malignancies, and evidence for the potential prognostic role of genomic and epigenetic profiles has been accumulating (7,8). Since molecular signatures can have clinical application in risk stratification for prediction of treatment response, metastatic potential, recurrence, and survival, researchers should continue their efforts to identify novel ESCC-related molecular events (9,10).

Non-specific cytotoxic cell receptor protein 1 (*NCCRP1*) was initially cloned from fish species and predicted to be a type II/III membrane protein (11). NCCRP1 was believed to be a receptor expressed in non-specific cytotoxic cells that was responsible for their cytolytic functions (12). Later, Kallio *et al* investigated the human gene and found that NCCRP1 is expressed intracellularly and is a paralog of the F-box superfamily of proteins, which are components of the E3 ubiquitin ligase complexes and regulate the cell cycle (13). More importantly, *NCCRP1* mRNA was found to be abundantly expressed in human tissues containing squamous epithelium and silencing of *NCCRP1* caused a significant decrease in the growth of HeLa cells (13). However, the role of *NCCRP1* in ESCC is unknown.

In the present study, we focused on *NCCRP1* as a candidate ESCC-related gene for the following reasons: (1) NCCRP1 is abundant in the squamous epithelium; (2) NCCRP1 is involved in cell proliferation; (3) the *NCCRP1* gene harbors a CpG island in the promoter region (suggesting the possibility of methylation); (4) NCCRP1 is a paralog of the F-box superfamily of

Table I. Sequences of primers.

Primer	Experiment	Type	Sequence (5'-3')	Product size	Annealing temperature
NCCRP1	qRT-PCR	Forward	AAAGCTCCAGCAGAACCAAA	104 bp	60˚C
		Reverse	TAATGGCTGGTTGTTCGTCA		
	Bisulfite sequencing	Forward	TTTAGTTAATTTTAGTTTTGTGAAAT	282 bp	64˚C
		Reverse	CCACTCCTCCAACAACAACTAC		
GAPDH	qRT-PCR	Forward	GAAGGTGAAGGTCGGAGTC	226 bp	60˚C
		Probe	CAAGCTTCCCGTTCTCAGCC		
		Reverse	GAAGATGGTGATGGGATTTC		

NCCRP1, non-specific cytotoxic cell receptor protein 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

proteins that regulate the cell cycle (14-16); and, finally, (5) there are no published data related to *NCCRP1* expression in ESCC. The purpose of the present study was to evaluate the expression, regulatory mechanisms, and clinical significance of *NCCRP1* in ESCC.

Materials and methods

Ethics. This study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. Written informed consent for the use of clinical samples and data, as required by the institutional review board at Nagoya University, Japan, was obtained from all patients.

Sample collection. Nine ESCC cell lines (TE1, TE2, TE3, NUEC1, NUEC2, NUEC3, TT, TTn, and WSSC) and a control non-tumorigenic epithelial cell line (FHs74) were obtained from the American Type Culture Collection (Manassas, VA, USA), Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), or were established in our institute (17). Cells were stored at -80˚C using cell preservative solution (Cell Banker; Mitsubishi Chemical Medience Corporation, Tokyo, Japan) and cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum in an atmosphere containing 5% CO_2 at 37˚C. A total of 213 primary ESCC tissues and adjacent normal tissues were acquired from patients who underwent radical esophageal resection at Nagoya University Hospital between October 2001 and January 2016. All tissue samples were diagnosed histologically as ESCC, frozen immediately after resection, and stored at -80˚C. Specimens were classified histologically using the seventh edition of the UICC staging system for esophageal cancer. Patients were questioned to determine their levels of alcohol consumption, and excessive alcohol consumption was defined as >210 g/week for ≥3 years (18). Since 2006, neoadjuvant chemotherapy (fluorouracil combined with platinum-based drugs) was administered to patients with clinical stage II/III ESCC unless contraindicated by the patient's condition or patient refusal (19,20). Postoperative follow-up examinations included physical examination, measurement of serum tumor markers every 3 months, and enhanced computed tomography of the chest and abdominal cavity every 6 months. Adjuvant chemotherapy was administered to selected patients according to the patient's condition and the physician's discretion.

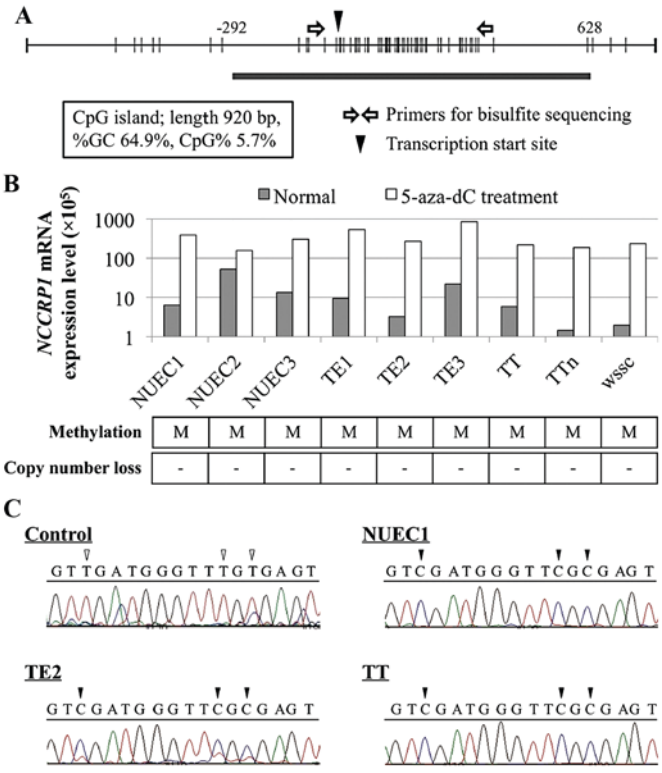

Figure 1. Expression, methylation, and copy number analysis in ESCC cell lines. (A) A CpG island (indicated by the underline) was identified around the *NCCRP1* transcription initiation site. (B) *NCCRP1* mRNA levels in nine ESCC cell lines before and after 5-aza-dC treatment. Methylation and copy number information for *NCCRP1* in the cell lines are shown below the graph. (C) Representative results of bisulfite sequence analysis. All CpG sites in the control were converted to TG, whereas those of NUEC1, TE2 and TT were CG. ESCC, esophageal squamous cell carcinoma; *NCCRP1*, Non-specific cytotoxic cell receptor protein 1; M, methylated.

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). The levels of *NCCRP1* mRNA were determined using qRT-PCR. Total RNA (10 μg) isolated from ESCC cell lines and 213 primary ESCCs and adjacent normal tissues was used as the template for cDNA synthesis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA

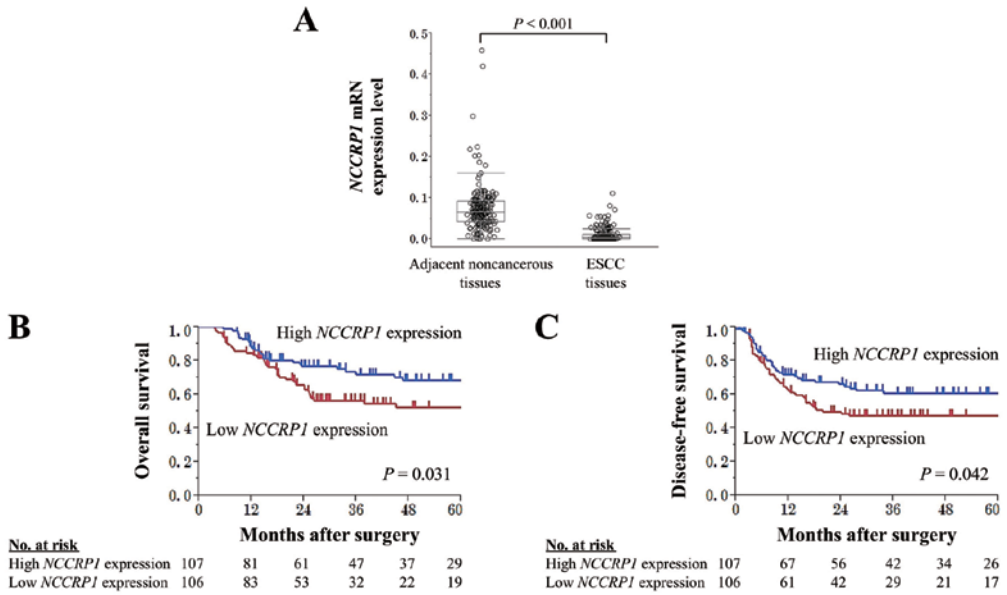

Figure 2. Expression of *NCCRP1* in clinical specimens. (A) Mean levels of *NCCRP1* mRNA expression were lower in ESCC tissues compared with corresponding normal adjacent tissues. (B) Overall and (C) disease-free survival of 213 patients with ESCC. ESCC, esophageal squamous cell carcinoma; *NCCRP1*, Non-specific cytotoxic cell receptor protein 1.

levels (TaqMan, GAPDH control reagents: Applied Biosystems, Foster City, CA, USA) were quantified to normalize expression levels (9). qRT-PCR was performed using the SYBR Green PCR Core Reagents Kit (Applied Biosystems) as follows: One cycle at 95˚C for 10 min; 40 cycles at 95˚C for 5 sec, and 60˚C for 60 sec. All samples were tested in triplicate, and samples without template were included in each PCR plate as negative controls. Real-time detection of SYBR Green fluorescence was conducted using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems). The expression level of each sample is shown as the value of the *NCCRP1* amplicon divided by that of *GAPDH* (21). Sequences of specific primers are listed in Table I.

Methylation analysis of NCCRP1 gene. Nucleotide sequence analysis was conducted to determine the presence of CpG islands around the promoter region of *NCCRP1*. CpG islands were defined as follows: ≥200-bp region with GC content >50% and CpG: Expected CpG ≥0.6 identified using Methyl Primer Express Software (Applied Biosystems). Genomic DNA isolated from the cell lines was treated with bisulfite for bisulfite sequence analysis. Bisulfite DNA from nine ESCC cell lines and control cell (FHs 74) was amplified with specific primers (Table I) and sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a 3730x l DNA Analyzer (Applied Biosystems) at Eurofins Genomics Co Ltd, Tokyo, Japan. To assess the relationship between promoter hypermethylation and *NCCRP1* transcription, GC cells ($1.5x10^6$ cells) were treated with 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich) to inhibit DNA methylation and then cultured for 6 days with medium changes on days 1, 3, and 5 (22). RNA was extracted and qRT-PCR was performed as described above.

Copy number analysis. *NCCRP1* copy number of nine ESCC cell lines was determined using TaqMan Copy Number Assays (Applied Biosystems). Twenty nanograms of genomic DNA was amplified using specific primer pairs according to the manufacturer's instructions (assay ID: Hs02638838_cn, within exon 6). Data were analyzed using CopyCaller Software (Life Technologies, Carlsbad, CA) (23). Copy number loss was defined as copy number value equal to 1 determined in the analyzed region of the *NCCRP1* locus.

Statistical analysis. Numeric variables between the two groups were compared using the Mann-Whitney U test. The χ^2 test was used to analyze the association between the expression status of *NCCRP1* and clinicopathological parameters. Overall and disease-free survival rates were calculated using the Kaplan-Meier method, and differences in survival curves were analyzed using the log-rank test. We performed multivariable regression analysis to detect prognostic factors using the Cox proportional hazards model, and variables with a P-value <0.05 were entered into the final model. All statistical analysis was performed using JMP 10 software (SAS Institute Inc., Cary, NC, USA). A P-value <0.05 was considered statistically significant.

Results

Expression, methylation, and copy number analysis of cell lines. *NCCRP1* harbors a CpG island flanking the transcription initiation site (length 920 bp, GC 64.9%, CpG 5.7%; Fig. 1A). *NCCRP1* mRNA expression levels differed among nine ESCC cell lines (Fig. 1B). Bisulfite sequence analysis revealed that CpG sites in *NCCRP1* DNA in all ESCC cells were CG (complete methylation) and that the corresponding positions in a control cell line FHs74=were TG (absence of methylation) (Fig. 1C). When we compared the levels of *NCCRP1* mRNA in ESCC cell lines before and after demethylation, reactivation of *NCCRP1* transcription was detected in all ESCC cells (Fig. 1B). Moreover, there was no detectable loss of copy number in ESCC cell lines (Fig. 1B).

Table II. Association between the expression of *NCCRP1* mRNA and clinicopathological parameters of 213 patients with squamous cell carcinoma of the esophagus.

Parameters	Low *NCCRP1* expression (n)	High *NCCRP1* expression (n)	P-value
Age (years)			
<65	47	46	
≥65	59	61	0.843
Sex			
Male	87	80	
Female	19	27	0.194
Preoperative symptoms			
Absent	24	29	
Present	82	78	0.451
Brinkman index			
<1,000	66	75	
≥1,000	40	32	0.227
Excessive alcohol consumption			
Absent	30	29	
Present	76	78	0.845
Carcinoembryonic antigen (ng/ml)			
≤5	94	96	
>5	12	11	0.807
SCC (ng/ml)			
≤1.5	65	68	
>1.5	41	39	0.737
Tumor size (cm)			
<5.0	63	62	
≥5.0	43	45	0.825
Tumor location			
Ce, Mt	65	68	
Lt, Ae	41	39	0.737
UICC pT factor			
pT1-2	39	42	
T3-4	67	65	0.712
Differentiation			
Moderate to well	90	94	
Poor	16	13	0.531
Lymphatic involvement			
Absent	30	28	
Present	76	79	0.727
Vessel invasion			
Absent	62	68	
Present	44	39	0.449
Intraepithelial spread			
Absent	79	82	
Present	27	25	0.720
Intramural metastasis			
Absent	100	97	
Present	6	10	0.305
Lymph node metastasis			
Absent	37	44	
Present	69	63	0.350

χ^2 test. Statistical significance (P<0.05). SCC, squamous cell carcinoma-related antigen; UICC, Union for International Cancer Control.

Table III. Prognostic factors for overall survival of 213 patients with squamous cell carcinoma of the esophagus.

		Univariate			Multivariable		
Variable	n	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (≥65)	120	1.28	0.80-2.09	0.308			
Sex (male)	167	1.30	0.74-2.43	0.370			
Preoperative symptoms	160	2.05	1.12-4.13	0.018	1.87	0.93-4.04	0.081
Brinkman index (≥1,000)	72	1.17	0.71-2.02	0.540			
Excessive alcohol consumption	154	0.89	0.54-1.54	0.678			
CEA (>5 ng/ml)	23	1.58	0.79-2.89	0.187			
SCC (>1.5 ng/ml)	80	1.36	0.84-2.19	0.206			
Tumor size (≥5.0 cm)	88	1.30	0.81-2.07	0.281			
Tumor location (Lt/Ae)	80	0.83	0.50-1.35	0.460			
UICC T factor (T3-4)	132	1.92	1.15-3.34	0.011	1.09	0.59-2.08	0.794
Tumor differentiation (poor)	29	1.47	0.77-2.61	0.226			
Lymphatic involvement	155	3.52	1.79-7.98	<0.001	2.87	1.37-6.80	0.004[a]
Vessel invasion	83	1.47	0.91-2.35	0.111			
Intraepithelial spread	52	1.37	0.82-2.24	0.228			
Intramural metastasis	16	2.00	0.92-3.83	0.076			
Lymph node metastasis	132	2.51	1.47-4.53	<0.001	1.68	0.95-3.15	0.077
Low *NCCRP1* expression	106	1.69	1.05-2.75	0.031	1.75	1.08-2.87	0.022[a]

[a]Statistically significant in multivariate analysis (P<0.05). CI, confidence interval; CEA, carcinoembryonic antigen; SCC, squamous cell carcinoma-related antigen; UICC, Union for International Cancer Control. Univariate analysis was performed using the log-rank test. Multivariable analysis was performed using the Cox proportional hazards model.

Clinical implications of NCCRP1 mRNA levels in surgically resected esophageal tissues. The median age of the 213 patients was 66 years (range, 44-84 years). The male:female ratio was 167:16. According to the UICC staging system (seventh edition), 42, 54, 107, and 10 patients were in pathological stages I, II, III, and IV, respectively. The median duration of patient follow-up was 35.2 months (range, 4.8-173 months) or until death. In 204 (95.8%) patients, *NCCRP1* mRNA expression levels were lower in ESCC tissues compared with the corresponding non-cancerous adjacent tissues. The mean expression level of *NCCRP1* mRNA was significantly reduced in ESCC tissues compared with that in adjacent normal tissues (Fig. 2A).

Patients were assigned to one of two groups according to their median *NCCRP1* mRNA expression level in ESCC tissues (high *NCCRP1* expression group, n=107; low *NCCRP1* expression group, n=106). No significant association was found between *NCCRP1* expression groups and clinicopathological parameters including patient sex and tumor size, location, and depth (Table II). Patients in the low *NCCRP1* expression group tended to have a shorter overall survival (OS) time than those in the high *NCCRP1* expression group (5-year OS rates were 52 and 69% for the low and high expression groups, respectively; P=0.031; Fig. 2B). In multivariate analysis for overall survival, low *NCCRP1* expression was identified as an independent prognostic factor (hazard ratio, 1.75; 95% confidence interval, 1.08-2.87; P=0.022; Table III). Disease-free survival (DFS) was also significantly poorer in the low *NCCRP1* expression group than in the high *NCCRP1* expression group (3-year DFS rates were 47 and 61% for the low and high *NCCRP1* expression groups, respectively; P=0.042; Fig. 2C). The frequency of overall recurrence after radical esophagectomy in the low *NCCRP1* expression group was higher than that of the high *NCCRP1* expression group (49 and 35%, respectively, P=0.032; Fig. 3A). No appreciable trends were found in metastasis site as the initial recurrence in comparisons between low and high *NCCRP1* expression groups (Fig. 3A).

We conducted a subgroup analysis according to administration of neoadjuvant chemotherapy (fluorouracil combined with platinum-based drugs) to further explore the significance of *NCCRP1* expression in ESCC. The prognostic impact of *NCCRP1* expression was similar between patients with and without neoadjuvant chemotherapy (Fig. 3B).

Discussion

Previous molecular studies have provided evidence that ESCC arises not only from the combined effects of environmental factors such as cigarette smoking or excessive alcohol consumption and susceptible genetic variants, but also from the accumulation of genetic and epigenetic alterations that play crucial roles in the process of cellular immortalization and tumorigenesis (6,7,24). Understanding of the molecular mechanisms and alterations behind the initiation and progression of esophageal tumorigenesis is essential for disease monitoring and identification of novel therapeutic and clinical targets for

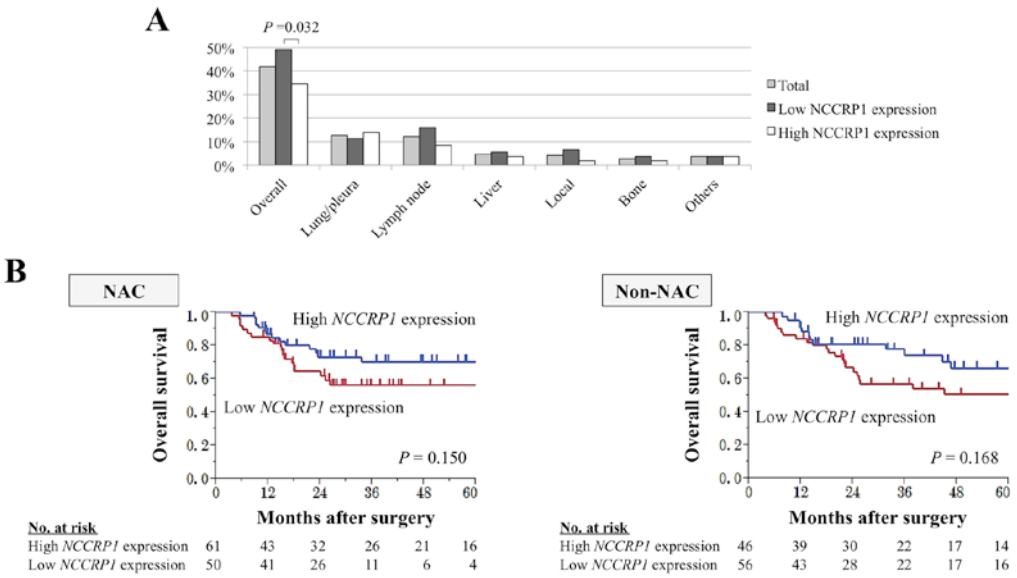

Figure 3. Clinical significance of *NCCRP1* expression. (A) Site of initial recurrence in each group according to *NCCRP1* expression. (B) Overall survival rates in subgroups according to *NCCRP1* expression and the effect of neoadjuvant chemotherapy (NAC). *NCCRP1*, Non-specific cytotoxic cell receptor protein 1.

ESCC (2,25). To date, multiple genetic and epigenetic changes in oncogenes and tumor suppressor genes (TSGs), cell cycle regulators, cell adhesion molecules, and DNA repair genes have been implicated in esophageal carcinogenesis (7,26,27). Nevertheless, the molecular pathogenesis of ESCC is still incompletely understood and it is vitally important to decipher the underlying mechanisms of carcinogenesis. We hypothesized that *NCCRP1* is a candidate ESCC-related gene.

The *NCCRP1* gene is located on chromosome 19q13.2 and encodes a 31-kDa protein composed of 275 amino acid residues (11,13). There are no previous reports on oncological roles of *NCCRP1*. In this study, we investigated the expression, methylation status, DNA copy number, and functions of *NCCRP1* in ESCC. Our results suggest that *NCCRP1* functions as a TSG that might be responsible, at least in part, for ESCC carcinogenesis since most ESCCs examined showed reduced *NCCRP1* mRNA expression compared with matched non-cancerous tissues. We also evaluated the association of *NCCRP1* expression with clinical characteristics of ESCC. Patients with low *NCCRP1* expression were likely to have a poor prognosis, implying a tumor suppressive role of *NCCRP1* in ESCC progression and suggesting that the expression status of *NCCRP1* in ESCC tissues might be a novel biomarker to predict postoperative outcomes. Of note, the expression of *NCCRP1* had no significant association with typical risk factors for ESCC prognosis, such as tumor depth and lymph node metastasis. This finding may highlight the utility of *NCCRP1* for stratifying patients at risk of adverse prognosis independent of the TNM staging system. Since findings of the JCOG9907 phase III study comparing the survival benefit of pre- or postoperative cisplatin plus fluorouracil in clinical stage II/III ESCC demonstrated the superiority of neoadjuvant chemotherapy, neoadjuvant cisplatin plus fluorouracil followed by esophagectomy has been the standard treatment for patients with ESCC in Japan (19,20). In this study, we found that the prognostic impact of *NCCRP1* expression was similar between patients who received neoadjuvant chemotherapy and those who did not. This result emphasized the clinical utility of *NCCRP1* expression to predict postoperative prognosis regardless of whether the patient received neoadjuvant chemotherapy.

Promoter hypermethylation leads to transcriptional silencing of TSGs in various malignancies (28,29). With respect to regulatory mechanisms, all examined ESCC cell lines harbored *NCCRP1* promoter hypermethylation. Furthermore, *NCCRP1* transcription increased in cells treated with a DNA methylation inhibitor. To the best of our knowledge, this is the first report of hypermethylation of *NCCRP1*. However, none of the ESCC cell lines had copy number loss at the *NCCRP1* locus. These findings indicate that promoter hypermethylation is a pivotal mechanism that inhibits *NCCRP1* transcription in ESCC. As tumor-specific aberrant DNA methylation can be detected more stably than mRNA expression levels (30), it has become recognized as a promising tool for liquid biopsy and assessment of locoregional recurrence at surgical margin imprints (31,32). Detection of *NCCRP1* methylation in the circulating blood, in addition to ESCC tissues, would enhance the diagnostic utility of *NCCRP1*.

As future perspectives, our findings can be translated into several clinical applications as follows: i) the expression and methylation status of *NCCRP1* in preoperative biopsy tissues obtained during endoscopic surveillance may identify patients requiring intensive perioperative treatment; ii) the expression levels of *NCCRP1* in surgical specimens may predict recurrence and subsequent adverse prognosis, which will likely aid efforts to design appropriate postoperative therapeutic and surveillance strategies; and iii) demethylating agents targeting *NCCRP1* may serve as therapeutics. However, this study has some limitations. Further studies including pathway analysis in esophageal carcinogenesis are needed to clarify the molecular mechanisms underlying the biological activities of *NCCRP1* in ESCC. Also, this study was limited by the relatively small sample size and lack of external validation of the reproducibility of the expression assays and their standardization across laboratories. Finally, this study is limited by its lack of direct

functional analysis of *NCCRP1*. Better understanding of the tumor suppressive functions of *NCCRP1* would be elucidated by forced expression of *NCCRP1*.

Nevertheless, taken together our findings support the conclusion that *NCCRP1* acts as a putative tumor suppressor gene that is inactivated by promoter hypermethylation and might serve as a promising biomarker to predict postoperative prognosis in ESCC.

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