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ORIGINAL ARTICLE – CANCER RESEARCH

Pathobiological role of cleft palate transmembrane protein 1 family proteins in oral squamous cell carcinoma

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Abstract

Purpose Cleft palate transmembrane protein 1 (Clptm1) and its paralog protein, Cisplatin resistance-related protein 9 (CRR9) constitute a highly conserved protein family, from *Caenorhabditis elegans* to *Homo sapiens*. In the present study, we examined the clinicopathological and biological significance of Clptm1 and CRR9 expression in oral squamous cell carcinoma (OSCC).

Methods Ninety-eight OSCC tissue specimens were immunohistochemically stained with specific antibodies to Clptm1 and CRR9. The immunoreactivity of Clptm1 and CRR9 was then correlated with clinicopathological factors, including the prognosis of patients. siRNA-mediated gene silencing of *CRR9* followed by cell proliferation, Matrigel invasion, anoikis assay, and gelatin zymography were performed using cultured OSCC cells. Subsequently, immunohistochemical examination including double staining was performed to determine the correlation between CRR9 and Bcl-xL expression in OSCC cells.

Results Non-tumorous oral squamous cells exhibited vague, weak, or little cytoplasmic staining with anti-Clptm1 and CRR9 antibodies. By contrast, robust Clptm1 and CRR9 immunoreactivity was found at the cancer invasion front in 55 and 54 of the 98 OSCC tissue specimens, respectively. Notably, CRR9 immunoreactivity was associated with more than 5 mm of depth of invasion, poor prognosis of the patients, and smoking habits ($P < 0.05$). siRNA-mediated gene silencing of *CRR9* did not alter the cell proliferation but decreased Matrigel invasion and impaired anoikis resistance in cultured Ca9-22 and SAS cells. CRR9 and anti-apoptotic Bcl-xL expression levels were correlated in pT1 OSCC tissue specimens.

Conclusions Clptm1 and CRR9 were overexpressed in many OSCC tissues. In particular, CRR9 expression may promote tumor development and have a significant poor prognostic value in OSCC, possibly through conferring invasion ability and resistance to apoptotic stimuli possibly related to Bcl-xL expression. CRR9 could be a novel molecular target for patients with OSCC.

Keywords Oral squamous cell carcinoma · Clptm1 · CRR9 · Outcome · Anoikis

Background

The incidence of oral squamous cell carcinoma (OSCC) has increased in recent years, and despite progress in chemotherapy and radiotherapy, the prognosis of advanced OSCC is still poor (Warnakulasuriya 2009, 2010; Sieviläinen et al. 2018). This is contrary to improved prognosis of patients with breast cancer, lymphoma, lung cancer, and gastrointestinal cancers, which has been greatly improved by recent molecular target and antibody-based therapies. Thus, there is a need to determine candidate target molecules in OSCC to develop new therapies.

Cleft palate transmembrane protein 1 (Clptm1) and its paralog protein, Cisplatin resistance-related protein 9

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(CRR9), otherwise known as Cleft-Lip and Palate Transmembrane Protein-1-Like (Clptm1L), constitute a highly conserved protein family in *Caenorhabditis elegans* (*C. elegans*). Mammalian transmembrane protein designated N14 was first identified as a murine thymic epithelial cell molecule, which has a role in intrathymic T-cell development (Takeuchi et al. 1997). Subsequently, a human gene, which was disrupted by a balanced chromosomal translocation in a family with cleft palate, was isolated by positional cloning (Yoshiura et al. 1998). They dependently designated the gene as *cleft palate transmembrane protein 1* (*Clptm1*), which is now recognized as a human homolog of *N14* (https://www.uniprot.org/uniprot/Q8VBZ3). Very recently, Clptm1 has been shown to regulate forward trafficking of $GABA_A$ receptors and inhibitory transmission in neural cells (Ge et al. 2018). However, the role of Clptm1 in non-neural or non-lymphocyte cells is largely unclear.

CRR9 was first isolated by screening of the cisplatin (CDDP) resistance-related gene (Yamamoto et al. 2001). CRR9 has a cancer-promoting property in lung cancer (James et al. 2014). Notably, anti-CRR9 antibody robustly inhibited growth of lung cancer xenografts (Puskás et al. 2016). A recent meta-analysis, using an online tool termed the Kaplan–Meier Plotter (http://www.kmplot.com), also indicated that CRR9 expression is significantly related to poor outcome of patients with breast and ovary cancers. Despite this progress, the exact biological properties of CRR9 are still obscure; thus, further studies are needed to elucidate the property of CRR9 in carcinogenesis.

We started the present experiment by determining the expression status of Clptm1 and CRR9 proteins in OSCC with a focus on prognosis of the patients. Subsequently, we investigated the pathobiological role of CRR9 in cultured OSCC cells. The present findings indicate that both Clptm1 and CRR9 are overexpressed in many OSCCs. In particular, overexpression of CRR9 may be related to OSCC progression through increasing invasion and conferring anchor-dependent apoptosis resistance, with significant poor prognosis values for patients with OSCC. CRR9 could be a target molecule for therapy in patients with OSCC.

Methods

Patients

In a retrospective study, we collected data on all surgically treated patients who were primarily diagnosed with OSCC. The need for written informed consent was waived by the Institutional Review Board of the Gifu University Graduate School of Medicine. Instead, the Institutional Review Board requested us to inform the patients that they could refuse the use of their tissue specimens for this present study if they did not want to participate. The present study was conducted in accordance with the ethical standards of the Helsinki Declaration in 1975, after approval of the Institutional Review Board of the Gifu University Graduate School of Medicine (specific approval number: 28–524).

Antibodies and immunohistochemical staining

The murine monoclonal antibody to Clptm1 (clone G7) and rabbit conventional antibody to CRR9 (cat no. NBP1-84378) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Novus Biologicals (Littleton, CO, USA), respectively. Murine monoclonal antibody to Bcl-xL (clone 4C12A6) was purchased from ProteinTech Group (Chicago, IL, USA).

Archived pathological tissue specimens from 98 cases of OSCC were used in this study. All tissue specimens were obtained surgically, fixed in 10% buffered formalin, and embedded in paraffin. The tissues were immunostained with antibodies using the ImmPRESS™ polymerized reporter enzyme staining system (Vector Laboratories, Inc., Burlingame, CA, USA) as previously reported (Takeuchi et al. 2000). We also performed double immunohistochemical staining using a biotin-free polymer detection kit (MACH 2, Biocare Medical, Walnut Creek, CA, USA) according to the manufacturer's protocol.

Evaluation of immunohistochemical staining and statistical analysis

We evaluated the immunohistochemical staining results as a percentage of immunoreactivity in OSCC cells. The fraction of positive cells stained at the cancer invasion front was scored after examining three high-power fields (40 ×) per tissue section for each case. The staining was considered as negative if less than 10% of invasive cancer cells exhibited immunoreactivity, and as positive if over 10% did.

Curves for overall survival were drawn using the Kaplan–Meier method, and differences in the survival rates were compared using the log-rank test. The relationship between the clinicopathological parameter was examined using the Chi square test. A P value of <0.05 was considered statistically significant.

Cell culture

The OSCC cancer cell lines SAS and Ca9-22 were obtained from RIKEN cell bank (Tsukuba, Japan) and Japan Health Science Research Resources Bank (JCRB; Osaka, Japan), respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated fetal

bovine serum (FBS). Cells were passaged in our laboratory for no more than 6 months after resuscitation.

Immunocytostaining

The detailed procedure was described previously (Egawa et al. 2018). Cells were fixed with 4% (m/v) paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 10% goat serum. Subsequently, cells were incubated with 1 µg/ml rabbit anti-CRR9 antibody or rabbit control antibody at room temperature for 1 h. After washing with PBS, cells were incubated with 1:200 diluted Alexa Fluor 488-conjugated anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA). After staining with DAPI (4′,6-diamidino-2-phenylindole) to visualize the nuclei, images were acquired using a confocal laser scanning microscope (Leica TCS SP8, Germany).

For surface membrane staining, intact cells were stained with anti-CRR9 or control antibody followed by staining with Alexa Fluor 488-conjugated antibodies and analyzed with a Guava EasyCyte cell analyzer (Hayward, CA, USA).

Reverse transcription, PCR, and quantitative real-time reverse transcription PCR

Synthesis of cDNA from total RNA and subsequent PCR was performed with a Reverse Transcription Polymerase Chain Reaction (RT-PCR) Kit (TaKaRa, Shiga, Japan) as previously described (Bunai et al. 2018). PCR products were analyzed by polyacrylamide gel electrophoresis.

Real-time PCR was performed with a SYBR Green Reaction Kit according to the manufacturer's instructions (Roche Diagnostics, GmbH, Mannheim, Germany) on a LightCycler (Roche Diagnostics).

The following primers were used for PCR: Clptm1, forward 5′-TGAGGGCCTTGTAAGTGAGC-3′; Clptm1, reverse 5′-CACAAGGGCTGGTACTCCTG-3′; CRR9, forward 5′-TGCATTACCTGCCCATCCT-3′; reverse 5′-CGC CCCAGTGAGACCTTG-3′; GAPDH, forward 5′-GAA GGTGAAGGTCGGAGTC-3′; reverse 5′-GAAGATGGT GATGGGATTTC-3′. The expression of each target gene was analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) using the LightCycler system. ΔCT values of genes were normalized to that of *GAPDH* for each triplicate set. Standard deviations were then computed for the triplicate sets, and the three target genes and fold changes were presented. The value for the groups ($n=3$) was calculated as the fold change relative to the mean value for the control siRNA-treated group (control set to 1.0). In addition, Student's t test was performed to determine significant differences. $P<0.05$ was considered statistically significant.

Small interfering RNA (siRNA)-mediated gene silencing

The detailed procedure for siRNA silencing of a target gene has been described previously (Takeuchi et al. 2012). In this study, we utilized Ambion Silencer Select pre-designed siRNAs, s37500 and s375001 (Austin, TX, USA). A green fluorescent protein (GFP)-siRNA duplex with the target sequence 5′-CGGCAAGCUGACCCUGAAGUUCAU-3′ was used as a non-silencing control. siRNAs were transfected into cells using Lipofectamine™ RNAiMAX following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Cells were used for subsequent studies 48 h after transfection.

Cell proliferation and Matrigel invasion assay

Cell proliferation was evaluated by counting the number of viable cells as previously described (Takeuchi et al. 2011). Briefly, 1×10^4 cells were cultured on standard 35 mm tissue culture dishes (BD Biosciences, San Jose, CA, USA) in triplicate. After 24, 48, and 72 h, live cells were counted.

Matrigel invasion activity was evaluated using 24-well BD BioCoat Matrigel Invasion Chamber Plates (BD Biosciences) according to the manufacturer's protocol. The procedure was described in detail previously (Takeuchi et al. 2012). Briefly, 1×10^4 cells were placed in the upper compartment of an invasion chamber. After 72 h of incubation with DMEM containing 10% (lower chamber) or 2% (upper chamber) FBS, the cells on the lower surface of the filter were counted.

These assays were carried out in triplicate. Statistical analysis was performed by Student's t test for unpaired observation. Findings with $P<0.05$ were considered significant.

Zymography analysis

Functional activity of MMPs was evaluated by gelatin zymography, as previously described by Heussen and Dowdle (1980). Briefly, cells were incubated with serum-free DMEM for 24 h. Supernatant of culture medium was collected and mixed with non-reducing SDS sample buffer. After SDS–PAGE on 10% polyacrylamide gels containing 1 mg/ml gelatin, SDS was removed from the gels by incubating in 2.5% Triton X-100 for 1 h at 24 °C. Subsequently, the gels were incubated in a buffer containing 50 mM Tris–HCl, 5 mM CaCl2, pH 7.6, for 24 h at 37 °C, and stained with Coomassie blue R 250 (0.25%). Proteolytic activities of MMPs were detected as clear bands against the blue background of the stained gelatin.

Anoikis assay and detection of apoptosis

Cells were detached from the tissue culture flasks using 0.25% trypsin. Once detached, cells were cultured in DMEM containing 10% FBS on a six-well plate (1×10^6/well) coated with Ultra Low Attachment Surface, which prevents cell attachment (Corning Life Sciences, Teterboro, NJ, USA). After 72 h in suspension, cells were collected.

The number of cells undergoing apoptosis was quantified with a fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) (PromoCell GmbH, Heidelberg, Germany) followed by analysis. Briefly, 1×10^4 cells in different culture conditions were harvested, washed, resuspended in binding buffer, mixed with Annexin V-FITC and PI, and analyzed by flow cytometry. Annexin V-positive and PI-negative cells were considered to be in the early apoptotic phase.

Results

Clptm1 and CRR9 expressions in OSCC tissue specimens in relation to prognosis

Representative results from the immunohistochemical staining are shown in Fig. 1. Weak or little Clptm1 and CRR9 immunoreactivities were found in non-tumorous oral mucosa epithelial cells (Fig. 1a, b, respectively). Clptm1

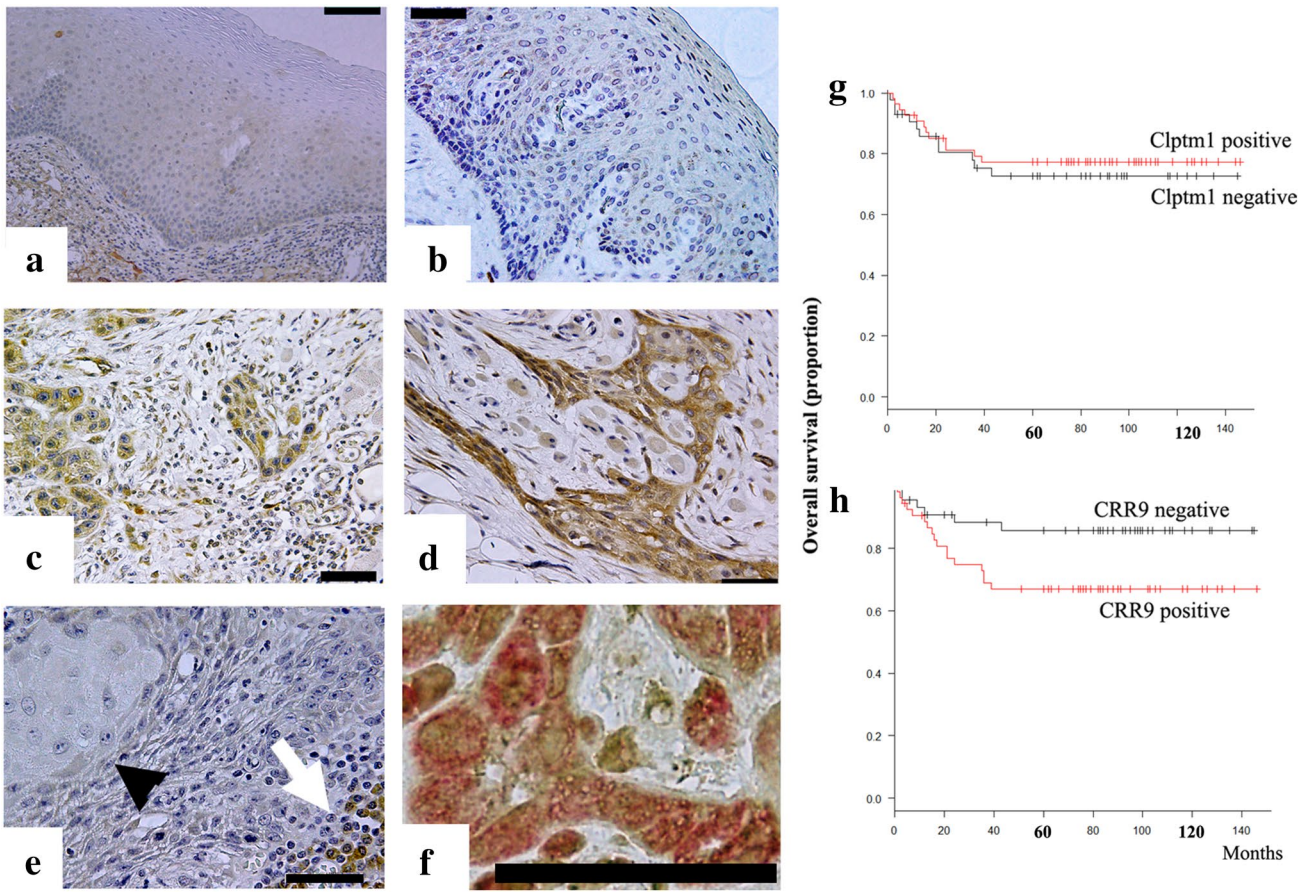

Fig. 1 Representative immunohistochemical staining and overall survival curves according to Clptm1 and CRR9 immunoreactivity at the invasion front of OSCC. **a–e** Weak Clptm1 or CRR9 immunoreactivity was found in non-tumorous oral epithelial cells (**a, b**, respectively). In contrast, Clptm1 or CRR9 immunoreactivity was found in many invasive OSCC cells. Representative positive stainings are shown in c and d, respectively. In CRR9 negative tissue specimens, note the internal positive staining in plasma cells (cancer and plasma cells are indicated by black arrow head and white arrow, respectively). Bar indicates 100 µm (**a, e**) and 50 µm (**b–d**). **f** CRR9 and Bcl-xL were co-expressed at the cancer invasion front of pT1 OSCC tissue specimens. Double immunohistochemical staining showed that red color CRR9 immunoreactivity and brown color Bcl-xL immunoreactivity were co-localized in OSCC cells at the cancer invasion front in the pT1 stage. Bar indicates 50 µm. **g, h** The Kaplan–Meier method and differences in the survival rates were compared using the log-rank test for univariate survival analysis. Clptm1 expression was not related to prognosis of patients (**g**). By contrast, overall survival rate of patients in the CRR9-positive group was significantly lower than that of patients in the CRR9-negative group [$P = 0.0426$ (< 0.05)] (**h**)

immunoreactivity was found in 55 of the 98 invasive cancer cell samples (Fig. 1c), whereas CRR9 immunoreactivity was found in 54 of the 98 invasive squamous cell carcinomas at the invasion front (Fig. 1d). In negative staining with anti-CRR9, we noted an internal positive control in plasma cells (Fig. 1e). Expression of CRR9 was reported in plasma cells (Trezise et al. 2018).

Interestingly, expression of Clptm1 and CRR9 appeared to indicate a different prognosis in OSCC, despite their close homology (supplementary Table 1). No significant correlation was observed between Clptm1 immunoreactivity and clinicopathological factors examined including the prognosis of patients (Fig. 1f). By contrast, the overall survival of patients in the CRR9-positive group was worse than that in patients of the CRR9-negative group (Fig. 1g, $P=0.043$). Interestingly, CRR9 immunoreactivity was associated with a depth of invasion of more than 5 mm ($P=0.042$) (Table 1). CRR9 immunoreactivity was related to smoking habits ($P=0.011$) (Table 1).

Relationship between CRR9 and Bcl-xL expression in OSCC tissue specimens

CRR9 has an apoptotic role through regulation of Bcl-xL expression, which is well-characterized as an anti-apoptotic protein, in lung cancer (James et al. 2014). Therefore, we aimed to elucidate the correlation between CRR9 and an anti-apoptosis protein, Bcl-xL, expression in OSCC tissue specimens. As shown in Table 2, CRR9 and Bcl-xL expression levels were significantly correlated in pT1 OSCC tissue specimens (Fisher's exact test, $P=0.0407$). Subsequent double immunohistochemical staining also demonstrated co-expression of CRR9 and Bcl-xL in OSCC cells. Representative data are shown in Fig. 2f.

Expression of CRR9 in cultured OSCC cells

Immunohistochemical staining indicated that CRR9 was overexpressed in many OSCC cells with a significant poor prognosis value. Subsequently, we examined CRR9 expression in cultured Ca9-22 and SAS cells. Immunofluorescence staining showed that CRR9 was expressed in both Ca9-22 and SAS OSCC cells. Notably, immunofluorescence staining of live cells revealed that Ca9-22 and SAS cells expressed CRR9 at the cell surface membrane. Representative staining is shown in Fig. 2.

siRNA-mediated down-regulation of CRR9 decreased Matrigel invasion activity and MMP-2 expression and in contrast, increased the anchor-dependent apoptosis sensitizing activity of cultured OSCC cells.

We next examined whether CRR9 expression affected the proliferation, invasion activity, and apoptosis resistance of Ca9-22 and SAS OSCC cells. Successful siRNA-mediated down-regulation of CRR9 at both mRNA and protein levels was achieved in Ca9-22 and SAS cells using CRR9 targeting siRNAs (Fig. 3a, b). As demonstrated in Fig. 3c, down-regulation of CRR9 expression did not significantly affect the proliferation of Ca9-22 or SAS cells. By contrast, down-regulation of CRR9 expression resulted in significant reduction of invasion of Ca9-22 or SAS cells (Fig. 3d) ($P<0.01$). Results of gelatin zymography assays indicated that siRNA-mediated down-regulation of CRR9 decreased MMP-2 expression in Ca9-22 and SAS cells (Fig. 3e). The MMP-2 band in CRR9 down-regulated Ca9-22 and SAS cells is weak when compared to that of the control cells.

We also examined whether CRR9 expression affects the sensitivity of anoikis, anchorage-dependent programmed cell death, of Ca9-22 or SAS cells. The Annexin V binding assay showed that decreased CRR9 expression significantly eliminated apoptosis-resistant of Ca9-22 or SAS cells in anchorage-dependent culture conditions (Fig. 4; Supplementary Figs. 1, 2). We also obtained similar results from two other independent Annexin V binding assays (Supplementary Figs. 1, 2).

These results indicated that CRR9 did not affect cell growth but significantly increased invasion activity, possibly through the MMP-2 pathway and anoikis resistance.

Discussion

In the present study, we aimed to unveil the pathobiological property of Clptm1 and CRR9 in OSCC. We started the experiment to examine the expression of Clptm1 and CRR9 protein with a focus on clinicopathological data. CRR9, but not Clptm1, appeared to be correlated to worse prognosis of patients with OSCC. Therefore, we further asked if CRR9 contributed to progression of OSCC cells in vitro. Notably, siRNA-mediated silencing of CRR9 reduced Matrigel invasion activity and apoptosis resistance to anoikis in both Ca9-22- and SAS-cultured OSCC cells.

Both Clptm1 and CRR9 were overexpressed in approximately half of the OSCC cells at the cancer invasive front. Clptm1 expression was not related to any of the clinicopathological factors examined including prognosis of patients with OSCC cells. By contrast, CRR9 expression was significantly related to smoking habits, which is a well-established risk factor associated with premalignant and malignant conditions of the oral cavity (Anantharaman et al. 2011). Moreover, CRR9 expression is significantly related to depth of invasion and poor prognosis of patients.

As shown in Supplementary Table 1, both human Clptm1 and CRR9 may each have a distinct homolog in *C. elegans*. Clptm1 is believed to be localized in the cytoplasm and is not a surface membrane protein (Ge et al. 2018),

Table 1 Summary of the clinicopathological characteristics of Clptm1 or CRR9 expression in OSCC

Characteristics	*n*	CLPTM1 expression		*P* value	CRR9 expression		*P* value
		Negative	Positive		Negative	Positive	
Age							
< 60	24	7	17		9	15	
≥ 60	74	36	38	0.105	35	39	0.482
Gender							
Male	50	20	30		21	29	
Female	48	23	25	0.542	23	25	0.685
Anatomical site							
Tongue	40	11	29		13	27	
Gingiva	35	22	13		22	13	
Buccal mucosa	16	7	9		7	9	
Floor of mouth	5	1	4		1	4	
Palate	2	2	0		1	1	
T-classification							
T1	28	10	18		14	14	
T2	45	19	26		14	31	
T3	13	9	4		9	4	
T4a/b	12	5	7		7	5	
Pathological stage							
Stage 1/2	50	19	31		23	27	
Stage 3/4	48	24	24	0.309	21	27	0.842
N-classification							
N0	60	23	37		31	29	
N1	17	10	7		7	10	
N2a	1	1	0		0	1	
N2b	9	5	4		4	5	
N2c	2	2	0		0	2	
N3a	0	0	0		0	0	
N3b	9	2	7		2	7	
Status of LN metastasis							
pN−	60	23	37		31	29	
pN+	38	20	18	0.211	13	25	0.1
Invasive type							
Cohesive	47	19	28		23	24	
Infiltrative	51	24	27	0.546	21	30	0.543
Lymph-vascular invasion							
Ly(−)	44	18	26		22	22	
Ly(+)	54	25	29	0.684	22	32	0.417
v(−)	72	31	41		34	38	
v(+)	26	12	14	0.621	10	16	0.496
Perineural invasion							
p(−)	88	39	49		42	46	
p(+)	10	4	6	1	2	8	0.178
Nodal extracapsular invasion							
(−)	38	19	19		15	23	
(+)	11	4	7	0.506	2	9	0.287
Smoking							
No	63	29	34		34	29	
Yes	34	13	21	0.523	9	25	**0.0108**
Alcohol use							
No	60	27	33		29	31	
Yes	25	11	14	1	10	15	0.633

Table 1 (continued)

Characteristics	*n*	CLPTM1 expression		*P* value	CRR9 expression		*P* value
		Negative	Positive		Negative	Positive	
Vital status							
Survival	75	32	43		38	37	
Death	23	11	12	0.606	6	17	**0.0426***
Depth of cancer (DOI) (mm)							
< 5	40	17	23	0.839	23	17	**0.0418***
≥ 5	58	26	32		21	37	

*Differences in the survival rates were compared using the log-rank test. Other relationships between the clinicopathological parameter, i.e., status of nodal metastasis, were examined using Chi square test. A *P* value of < 0.05 was considered statistically significant. Note the significant differences highlighted in bold. Overall survival of patients in the CRR9-positive group was worse than that in patients of the CRR9-negative group. Interestingly, CRR9 immunoreactivity was associated with a depth of invasion of more than 5 mm. CRR9 immunoreactivity was also related to smoking habits

Table 2 CRR9 and anti-apoptotic Bcl-xL expression levels were correlated in pT1 OSCC tissue specimens

pT1 OSCC *n* = 28 *P* = 0.0407	CRR9 expression Negative	CRR9 expression Positive
Bcl-xL expression		
Negative	14	9
Positive	0	5

while CRR9 is reported to also be expressed at the surface membrane (Puskás et al. 2016; Trezise et al. 2018), as observed in the present study. Combined together with the present clinicopathological findings, it is likely that CRR9 and Clptm1 may have distinctive pathobiological roles in OSCC, despite their close amino acid homology (Supplementary Table 1).

The present finding indicated that CRR9 conferred invasion ability and apoptosis resistance to anoikis, cell detachment-induced apoptosis, in OSCC cells. It is believed that not only invasion ability but also anoikis resistance leads to more aggressive behavior in OSCC (see Review in Bunek et al. 2011). We speculate that overexpression of CRR9 at the cancer invasion front may facilitate further cancer invasion and apoptosis escape when cancer cells lose cell adhesion to the extracellular matrix. In line with this speculation, the present zymography showed that siRNA-mediated gene silencing of CRR9 reduced MMP-2 expression in OSCC cells.

Accordingly, a significant correlation between the expression of CRR9 and Bcl-xL proteins in pT1 OSCC tissue specimens is noteworthy. CRR9 is believed to interact with the AKT, phosphoinositide 3-kinase, and Bcl-xL molecular pathway (James et al. 2014; Puskás et al. 2016). In line with the results of the present study, Bcl-xL is regulated by CRR9 in lung cancer cells (James et al. 2014). Bcl-xL is also

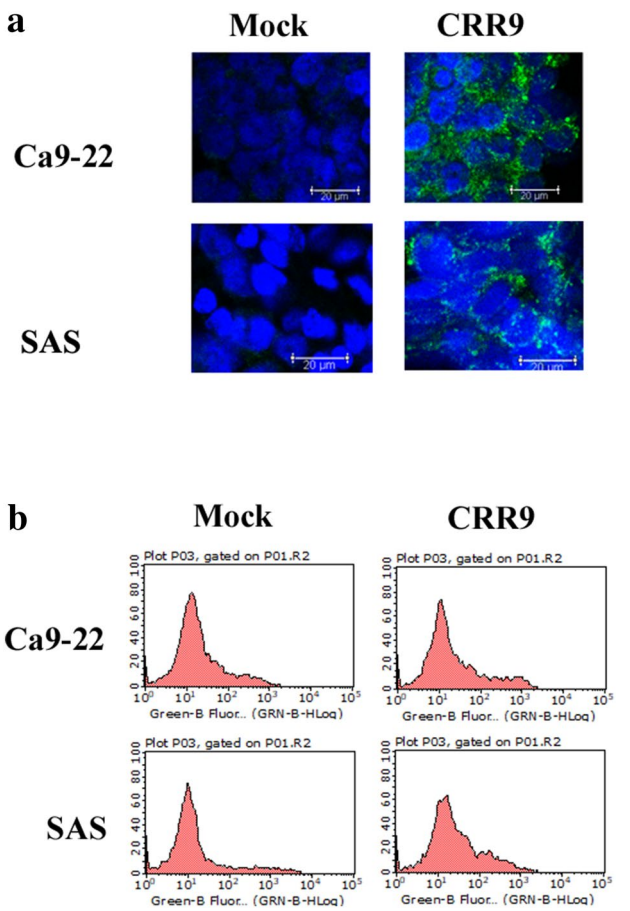

Fig. 2 Expression of CRR9 in cultured OSCC cells. Ca9-22 and SAS OSCC cells were stained with control rabbit antibody (indicated by Mock) or specific anti-CRR9 rabbit antibody (indicated by CRR9). We examined CRR9 immunoreactivity using methanol-fixed cells (**a**) and live cells (**b**). **a** Immunofluorescence staining revealed that CRR9 was expressed in Ca9-22 and SAS OSCC cells. Bar indicates 20 μm. **b** Immunocytostaining using intact OSCC cells demonstrated that CRR9 was expressed at the cell surface membrane of Ca9-22 and SAS cells

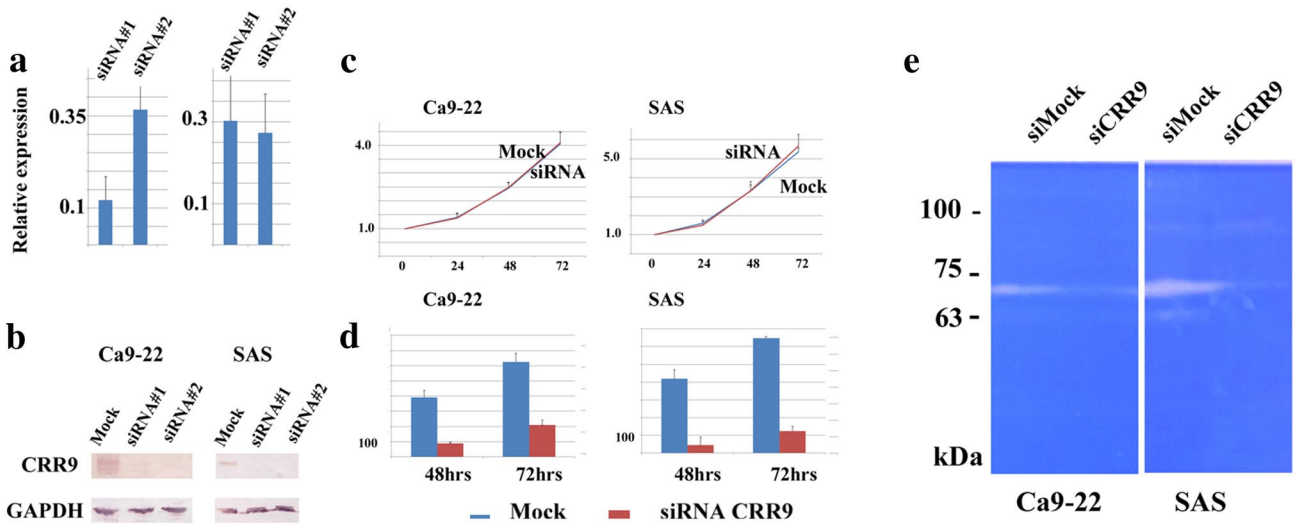

Fig. 3 Silencing of CRR9 eliminated Matrigel invasion activity and MMP-2 expression in Ca9-22 and SAS OSCC cells without affecting cell proliferation. **a, b** Both the siRNAs, s37500 and s375001 (indicated as siRNA#1 and #2, respectively), successfully down-regulated CRR9 expression at the mRNA and protein levels. The relative expression rate based on that of the control siRNA-treated group was 0.12 ± 0.06 and 0.37 ± 0.075 in siRNA#1- and #2-treated Ca9-22 cells, respectively, and 0.30 ± 0.11 and 0.27 ± 0.1 in siRNA#1- and #2-treated SAS cells, respectively. Note that there was little or no CRR9 protein band for siRNA-treated cells. GAPDH bands indicate equal protein loading in each lane. **c–e** Down-regulation of CRR9 expression did not alter the cell proliferation (**c**). By contrast, down-regulation of CRR9 resulted in down-regulation of Matrigel invasion activity in Ca9-22 and SAS cells (**d**). Representative gelatin zymograms of culture supernatants of siRNA-mediated *CRR9* silencing and control siRNA-treated Ca9-22 and SAS cells (**e**). Note the weak MMP-2 active band in CRR9 down-regulated cells, compared to control cells. In this figure, we used s37500 siRNA (Ambion, Austin, TX, USA) to silence the *CRR9*. We obtained similar results using s37500 and s375001 siRNA to silence the *CRR9*

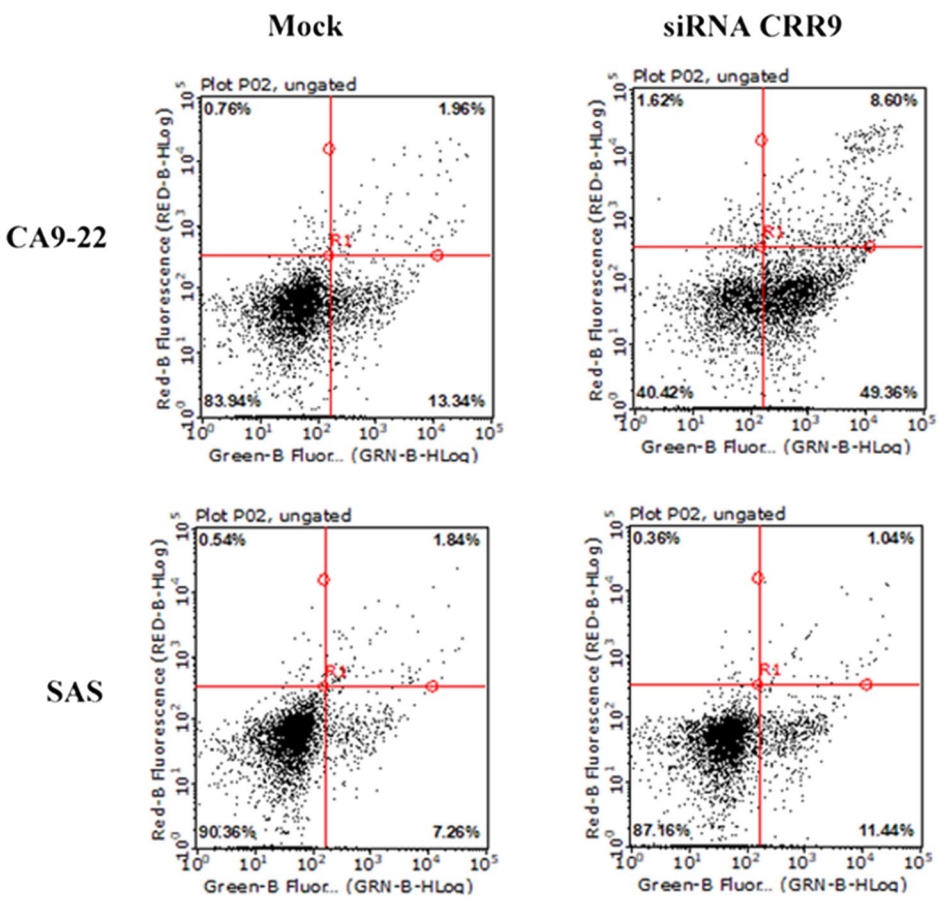

Fig. 4 Silencing of CRR9 eliminated resistance to anchor-dependent apoptosis, anoikis, in Ca9-22 and SAS OSCC cells. Ca9-22 and SAS cells were detached from the tissue culture flasks and cultured without cell attachment to induce anoiki. The number of cells undergoing apoptosis was quantified with a FITC-conjugated Annexin V and PI followed by analysis. Annexin V-positive and PI-negative cells (lower-right quadrant) were considered to be in the early apoptotic phase. Representative data using s37500 siRNA are shown. We obtained similar results using s37500 and s375001 siRNAs. The other results from two independent assays are shown in Supplementary Figs. 1 and 2. Note that silencing of CRR9 significantly increased anchor-dependent apoptosis in both Ca9-22 ($P < 0.01$, Student's *t* test) and SAS cells ($P < 0.05$, Student's *t* test)

reported to be overexpressed in OSCC cells (Zhang et al. 2014). However, further examination is needed to elucidate the detailed pathobiological property of CRR9 expression that is responsible for the poor patient outcome.

In summary, the present study indicated that overexpression of CRR9 promotes progression of OSCC and is possibly attributed to apoptosis resistance to anoikis, making CRR9 a potentially novel target molecule in OSCC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The present study was conducted in accordance with the ethical standards of the Helsinki Declaration in 1975, after approval of the Institutional Review Board of the Gifu University Graduate School of Medicine (specific approval number: 28–524).

Informed consent The need for written informed consent was waived by the Institutional Review Board of the Gifu University Graduate School of Medicine. Instead, the Institutional Review Board requested us to inform the patients that they could refuse the use of their tissue specimens for this present study, if they did not want to participate.

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