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Expression of tumor-associated antigen RCAS1 and its possible involvement in immune evasion in oral squamous cell carcinoma

T. Toyoshima[1], S. Nakamura[2], W. Kumamaru[1], E. Kawamura[1], H. Ishibashi[1], J-N. Hayashida[2], M. Moriyama[2], Y. Ohyama[1], M. Sasaki[1], K. Shirasuna[1]

Sections of [1]Oral and Maxillofacial Surgery, [2]Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, Higashi-ku, Fukuoka, Japan

BACKGROUND: RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) is known to induce apoptosis in its receptor-positive cells. The authors investigated RCAS1 expression in oral squamous cell carcinoma (SCC) and its association with the apoptosis of tumor-infiltrating lymphocytes (TILs).

METHODS: In 130 patients with oral SCC, the expression of RCAS1 in tumor cells was immunohistochemically examined and the apoptosis of TILs was examined by Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) staining.

RESULTS: RCAS1 was detected both on the cytoplasm and the membrane of tumor cells in 41 of 130 cases (31.5%). Focusing on the expression at the invasive front interacting with host immune cells, RCAS1 was detected in 22 of 130 cases (16.9%). The percentage of TUNEL-positive TILs in cases with RCAS1-positive SCCs was significantly higher than in cases with RCAS1-negative SCCs ($P < 0.0001$).

CONCLUSIONS: RCAS1 can be expressed on oral SCC cells and may be involved in the tumor escape from the host immune system by inducing the apoptosis of TILs.
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Keywords: apoptosis; immunohistochemistry; RCAS1; SCC; SiSo cells; tumor-associated antigen; TUNEL staining

Introduction

In squamous cell carcinomas (SCCs), one of the major cancers in humans, tumor-infiltrating lymphocytes (TILs) are quite commonly observed (1) and specific immunotherapy with tumor-rejection antigens is thus also expected to become a useful new treatment modality for patients with SCCs. Several antigens expressed in SCCs, including SART-1 (2, 3), SART-2 (4), SART-3 (5), cyclophilin B (6), ART-4 (7) and $p56^{lck}$ (8), have recently been identified to be a source of tumor-rejection peptides recognized by cytotoxic T lymphocytes (CTLs), and some of the non-apeptides have already been used in clinical trials for the specific immunotherapy of patients with SCCs as a cancer vaccine (9). However, the results obtained so far have not yet met the initial expectations. One of the major problems is that tumor cells may evade host immune surveillance. The postulated mechanisms for this action include defective processing and the presentation of antigens, the expression of co-stimulatory or adhesion molecules, or the production of immune-suppressor agents by tumor cells (10, 11). Tumor cells may also evade immune attack by expressing a CD95 (APO/Fas) ligand or other molecules that induce apoptosis in activated T cells (12, 13). The precise mechanisms involved in this process, however, remain undefined.

RCAS1 (receptor-binding cancer antigen expressed on SiSo cells), a novel tumor-associated antigen expressed on cancer cells, has recently been identified (14). This antigen is expressed in various cancers and acts as a ligand for a putative receptor present in various human cell lines and peripheral lymphocytes, such as T, B, and NK cells, and thus leads them to apoptosis (15). The receptor expression is enhanced by the activation of the lymphocytes, and RCAS1 inhibits the *in vitro* growth of such activated cells and induces their apoptotic cell death. Tumor cells may thus evade immune surveillance by expressing of RCAS1. RCAS1 expression has also been reported to be related to both tumor cell progression and invasion in uterine squamous neoplasias (16), and also was described to be associated with a poor prognosis in uterine adenocarcinomas (17). The prognostic significance in RCAS1 expression has

Correspondence: Seiji Nakamura, DDS PhD, Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 6381, Fax: +81 92 642 6386. E-mail: seiji@dent.kyushu-u.ac.jp
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also been reported in other cancers (18, 19), however, other reports have provided conflicting results without showing any such significant relationship (20, 21). To our knowledge, there have so far been no reports on the RCAS1 expression in oral SCC. This study was thus designed to examine the RCAS1 expression in oral SCC and to determine its prognostic significance.

Materials and methods

Patients and tissue specimens

One hundred and thirty patients with primary oral SCC, who were referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital, were studied. All patients consented prior to the procedure. SCC tissue specimens, including 49 non-metastatic and 81 metastatic specimens, were obtained from the patients by biopsies prior to either chemotherapy or radiotherapy. The diagnosis was confirmed by histopathologic examinations with H&E staining, and the grades of differentiation, the modes of tumor invasion, and the degrees of lymphocytic responses were also determined according to the criteria of WHO (22), Yamamoto and Kohama (23), and Willen et al. (24), respectively. The patients without metastasis were followed for at least 36 months, postoperatively. In addition, the normal oral epithelia from 35 patients with benign oral tumors and epithelial dysplasia from 10 patients with oral leukoplakia were also included in this study.

Immunohistochemical staining

For the immunohistochemical analysis, the streptoavidin-biotin methodology was used (25). Four micrometer-thick sections were cut from 4% paraformaldehyde-fixed, paraffin-embedded materials, mounted on glass slides, and then were air dried. The sections were deparaffinized in xylene, rehydrated in ethanol, immersed in methanol containing 0.3% hydrogen peroxidase for 10 min to block the endogenous peroxidase activity, and then were incubated with 10% normal rat serum for 30 min to eliminate any non-specific binding. Thereafter, the sections were incubated with primary monoclonal antibodies for 60 min, then with biotinylated rat antimouse antibodies for 10 min, followed by staining with avidin-biotinylated peroxidase complex (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). The primary antibodies used were 22-1-1 (anti-RCAS1, mouse IgM, 1:1000 dilution; Medical and Biological Laboratories, Nagoya, Japan) and A9 (anti-CD3, mouse IgG2a, 1:1000 dilution, kindly provided by Dr S. M. Fu, University of Virginia, Charlottesville, VA, USA). All procedures were performed at room temperature. Negative controls were treated in the same way, but the primary antibodies were replaced by normal mouse IgG or IgM. Especially, the expression of RCAS1 in SCC cells was examined at the invasion front and then was quantified by counting the percentage of reactive cells, as reported previously (16, 17, 25). The stained sections with $< 5\%$ reactive cells were considered to be negative, and those with more than 5% reactive cells were defined as positive. The cut-off points were established at 25% and 50% in line with the findings published in other reports (16, 17), and 5% to 25% (weak/focal), 25–50% (moderate), and more than 50% (strong) reactive cells defined as $+$, $++$, and $+++$, respectively.

Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) staining

For the detection of apoptotic TILs, TUNEL staining was performed by using the ApopTag Apoptosis Detection Kit (Serologicals, Norcross, GA, USA). Four micrometer-thick sections were prepared in the same way for immunohistochemical staining and then were immersed in methanol containing 0.3% hydrogen peroxidase for 10 min to block the endogenous peroxidase activity. The sections were treated with a mixture of terminal deoxynucleotidyltransferase and digoxigenin-labeled dUTP, and then with peroxidase-conjugated anti-digoxigenin antibody. Nuclear staining of the apoptotic cells was performed using 3', 3'-diaminobenzidine tetrahydrochloride dihydrate. Positive controls were established using the sections of paraffin-embedded rat mammary gland tissue obtained at the fourth day after weaning. Negative controls were prepared by omitting the enzyme incubation step. The quantification of apoptotic TILs was performed by counting the positive cells along with the total number of TILs in 4-mm^2 sections from three different areas. To count the total number of TILs, serial sections that had been immunohistochemically stained with anti-CD3 antibody were used, as the majority of TILs are CD3-positive (25). The percentage of apoptotic TILs was then calculated as the number of apoptotic TILs/the total number of CD3-positive TILs.

Statistical analyses

The statistical significance of the differences between the groups was determined by Student's *t*-test, the Mann–Whitney *U*-test, or the chi-square test. *P*-values of < 0.05 were considered to be significant.

Results

The RCAS1 was frequently detected in the prickle and granular layers of the normal oral epithelia from 27 of 35 total cases (77.1%), but the staining intensity was relatively weak (Fig. 1a). In oral epithelial dysplasias, RCAS1 was hardly detected in any layers and it was only detected in the prickle and granular layers from two of 10 total cases (20.0%; Fig. 1b). In contrast, RCAS1 was strongly detected in 41 of the 130 cases (31.5%) of oral SCC. RCAS1 was diffusely expressed both in the cytoplasm and on the membrane of SCC cells. In nine of 34 (26.5%) moderately-differentiated and 32 of 90 (35.6%) well-differentiated SCCs, RCAS1 was expressed in the tumor cells around so-called cancer pearls (Fig. 2). Interestingly, the majority of SCC cells were found to express RCAS1 even in areas depicting invasion, in 22 of the 130 cases (16.9%; Fig. 3). The expression of RCAS1 could thus be classified into two patterns.

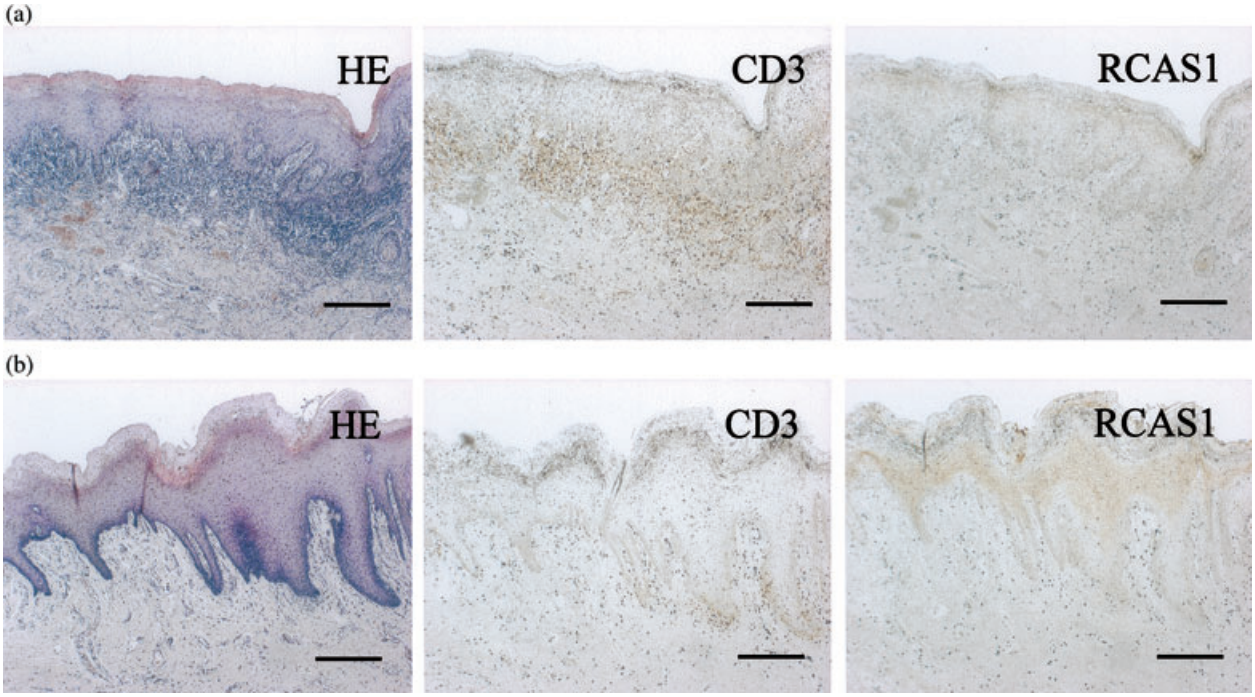

Figure 1 Immunohistochemical staining of RCAS1 in the normal oral epithelium from (a) a patient with a benign oral tumor and (b) oral epithelial dysplasia from a patient with oral leukoplakia. Serial sections from 35 normal oral epithelia and 10 oral epithelial dysplasias were stained with anti-CD3 and anti-RCAS1 antibodies, and each representative case is shown here. RCAS1 was weakly detected in the prickle and granular layers of the normal oral epithelium, while it was not found in any layers of oral epithelial dysplasia (original magnification ×10, bar 30 μm).

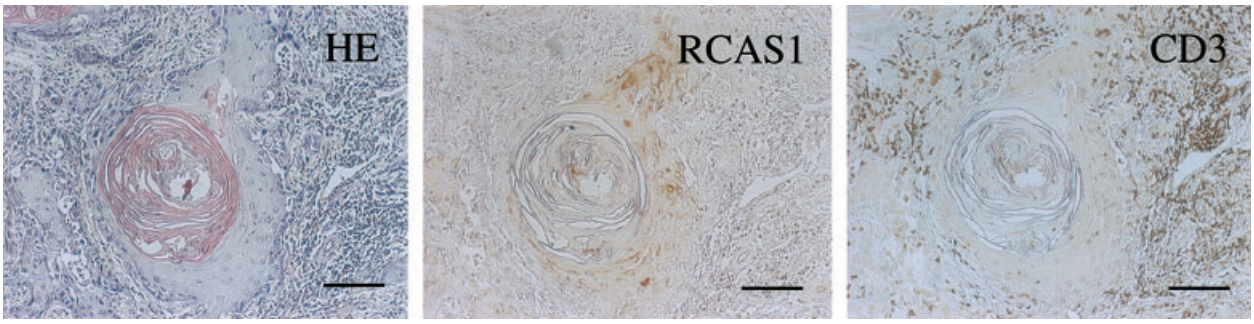

Figure 2 Immunohistochemical staining of RCAS1 in oral squamous cell carcinoma (SCC) from a patient with well-differentiated SCC. Serial sections from 130 oral SCCs were stained with anti-CD3 and anti-RCAS1 antibodies, and a representative case is shown here. RCAS1 was detected in the tumor cells around the cancer pearls. This type of the expression was observed in nine of 34 (26.5%) moderately differentiated and 32 of 90 (35.6%) well-differentiated SCCs (original magnification ×40, bar 15 μm).

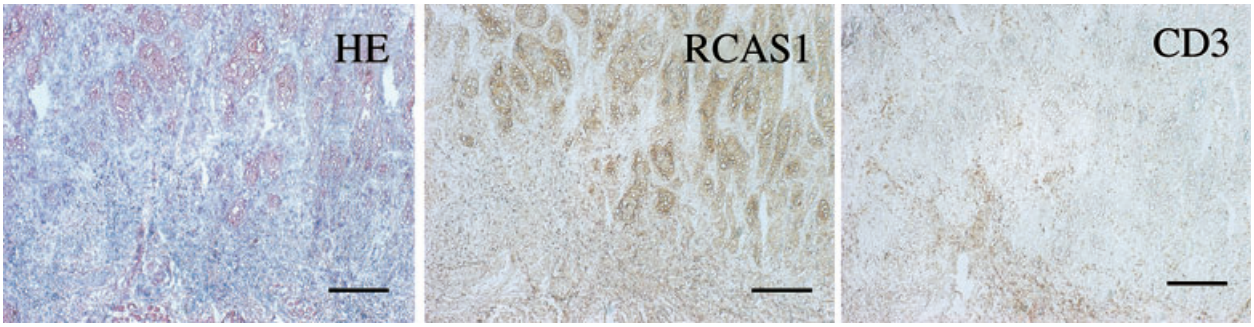

Figure 3 Immunohistochemical staining of RCAS1 in oral SCC from a patient with moderately-differentiated SCC. Serial sections from 130 oral SCCs were stained with anti-CD3 and anti-RCAS1 antibodies, and a representative case is shown here. RCAS1 was detected in the majority of tumor cells even in areas depicting invasion. This type of the expression was observed in 22 of the 130 SCCs (16.9%). (original magnification ×10, bar 30 μm).

Table 1 Association between RCAS1 expression and clinicopathological variables in all patients with oral SCC

Variables	*Category*	*Number of cases*	*Degree of RCASI expression*[a]				*Significance*[b]
			–	+	++	+++	
Age (years)	<65	58	40[c]	7	7	4	NS
	>65	72	49	12	6	5	
Sex	Male	72	45	12	8	7	NS
	Female	58	44	7	5	2	
T factor	1	25	17	4	2	2	NS
	2	52	33	9	8	2	
	3	24	19	3	1	1	
	4	29	20	3	2	4	
N factor	+	81	53	12	10	6	NS
	–	49	36	7	3	3	
Stage	1	24	18	3	2	1	NS
	2	46	30	8	6	2	
	3	24	18	2	4	0	
	4	36	23	6	1	6	
Differentiation[d]	Well	93	61	15	10	7	NS
	Moderate	31	22	4	3	2	
	Poor	6	6	0	0	0	
Mode of tumor invasion[e]	1	16	14	2	0	0	NS
	2	15	8	2	4	1	
	3	62	41	10	6	5	
	4	37	26	5	3	3	
Cellular response[f]	1	29	18	4	3	4	NS
	2	51	36	6	8	1	
	3	42	30	8	1	3	
	4	8	5	1	1	1	
Apoptosis of TILs[g]	+	82	60	10	7	5	$P < 0.05$
	–	48	29	9	6	4	

[a]RCAS1 was detected by immunohistochemical staining with the 22-1-1 antibody, and the degree of expression was estimated as described in the Materials and methods.
[b]The statistical significance of the association was determined by the chi-square test.
[c]The number of cases is indicated.
[d]The histological grades of differentiation were determined according to the criteria of WHO (22).
[e]The histological modes of tumor invasion were determined according to the criteria of Yamamoto and Kohama (23).
[f]The histological degrees of cellular response were determined according to the criteria of Willen et al. (24).
[g]Apoptosis was detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining, and the quantification of TUNEL-positive TILs was performed as described in the Materials and methods.
NS, not significant; TILs, tumor-infiltrating lymphocytes.

As RCAS1 has been reported to induce growth arrest and apoptotic cell death in RCAS1 receptor-positive lymphocytes (15), we focused on the expression of RCAS1 at the invasive front interacting with host immune cells, and any association with clinical and histological variables was also examined (Table 1). At the invasive front, RCAS1 was – in 108, + in 10, ++ in seven, and +++ in five of the 130 cases. The expression of RCAS1 was not significantly associated with any clinical characteristics including age, sex, tumor size, lymph node metastasis, and stage, and histological characteristics including the grade of differentiation, the mode of tumor invasion, and the degree of cellular responses, except for the frequencies of apoptotic TILs. Furthermore, the disease-specific 5-year survival rate was not significantly different between the RCAS1-positive (+, ++, and +++) and RCAS1-negative (–) SCC cases (Fig. 4).

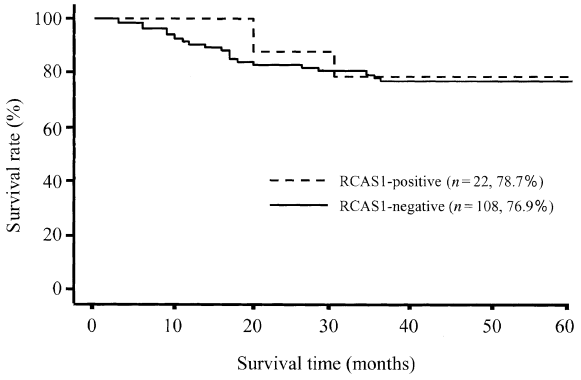

Figure 4 A Kaplan–Meier analysis of the disease-specific survival of patients with RCAS1-positive (+, ++, and +++) and RCAS1-negative (–) oral SCCs. The survival rate was not significantly different between these two groups.

As mentioned above, apoptosis of TILs was frequently observed around the RCAS1-positive SCC cells at the invasive front (Figs 5 and 6). The presence of apoptotic TILs was significantly associated with that of RCAS1-positive SCC cells ($P < 0.05$, chi-square test; Table 1). The percentages of apoptotic TILs were 42.6% ± 16.5% and 30.2% ± 19.7% in cases with RCAS1-positive and RCAS1-negative SCCs, respectively, and these percentages were significantly

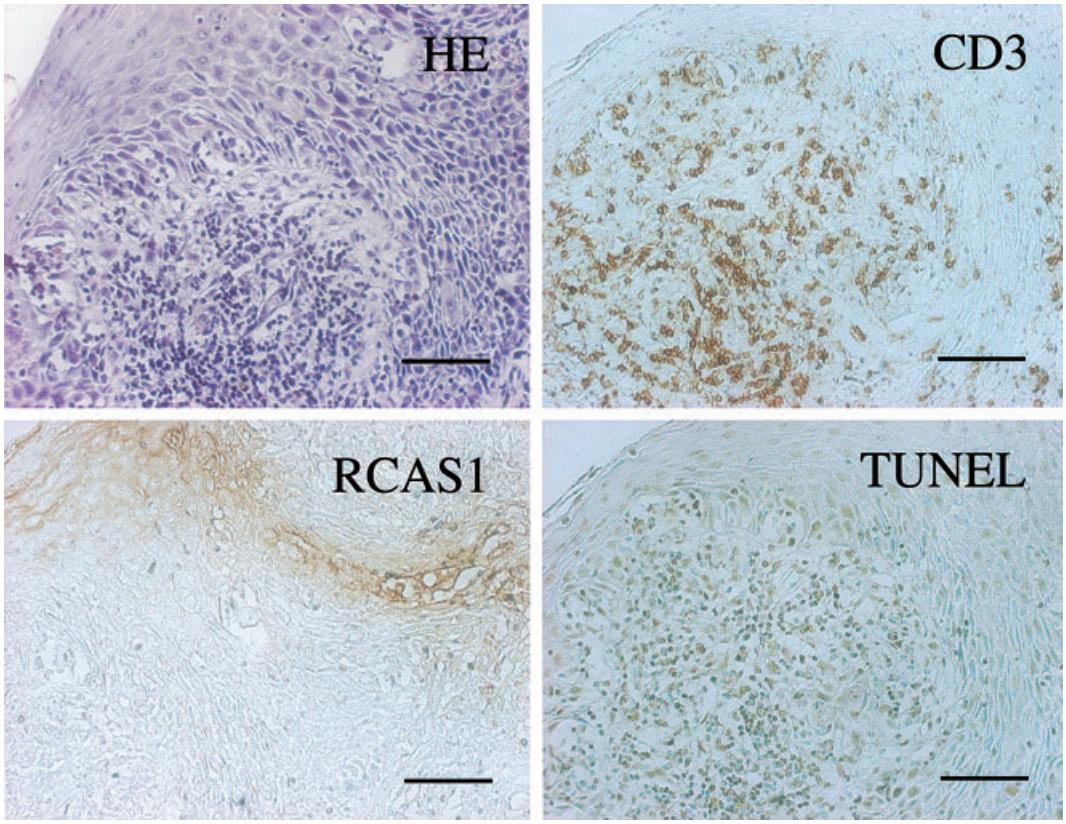

Figure 5 The presence of apoptotic tumor-infiltrating lymphocytes (TILs) around RCAS1-positive oral SCC. Immunohistochemical staining with anti-CD3 and anti-RCAS1 antibodies and Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining were performed in serial sections from 130 oral SCCs, a representative case of RCAS1-positive tumor cells is shown here. TUNEL-positive TILs are frequently seen around RCAS1-positive tumor cells (original magnification ×40, bar 15 μm).

different ($P < 0.0001$, Student's *t*-test and Mann–Whitney *U*-test; Fig. 7).

Discussion

The RCAS1 was originally identified to be a tumor-associated antigen that responds to a mouse monoclonal antibody 22-1-1 against a human uterine adenocarcinoma cell line, SiSo (14). This antigen was strongly expressed in uterine and ovarian carcinomas (16, 17), and also in non-gynecologic cancers, including pancreatic (18), lung (19), gastric (21) and colon (26), and so on, however, has not been detected in either normal ovary tissue or the tissue of any other organs (14, 16). In the present study, RCAS1 expression was investigated in the normal epithelia and squamous neoplasms of the oral cavity, using immunohistochemical staining with the 22-1-1 antibody. In general, neoplasias arising in the oral cavity are considered to progress from dysplasia to invasive carcinoma. RCAS1 was weakly detected in the prickle and granular layers of the normal epithelia, but it was not found in any layers of epithelial dysplasias. In contrast, SCCs from approximately 30% of the cases strongly expressed RCAS1. These findings suggest that the RCAS1 expression might therefore be related to tumor cell progression and invasion in the oral squamous cell epithelia. Furthermore, the expression of RCAS1 in tumor cells could be classified into two patterns. One is its expression in the majority of tumor cells, while the other is its limited expression in tumor cells around cancer pearls that tend to be seen in well- and moderately differentiated SCCs.

Interestingly, the isolation of a cDNA encoding RCAS1 and the following studies have revealed a possible function of this antigen (15). RCAS1 is a type II membrane protein able to form homo-oligomers, and can also be secreted from tumor cells. A receptor against the RCAS1 antigen exists has also been found to exist in immune cells such as T, B, and NK cells. This receptor expression was enhanced by the activation of the lymphocytes, and when these activated cells were cultured with RCAS1 peptides *in vitro*, their growth was suppressed, thus eventually leading to cell death by apoptosis. These findings suggest that tumor cells might thereby avoid the host immune system by expressing RCAS1.

In the present study, we thus focused on the expression of RCAS1 at the invasive front which interacted with the host immune cells. As expected, the apoptotic death of TILs was frequently observed around tumor cells expressing RCAS1. To our knowledge, this is the first report suggesting that RCAS1 in tumor cells might induce the apoptosis of TILs *in vivo*. The present study thus supports the hypothesis that the expression of

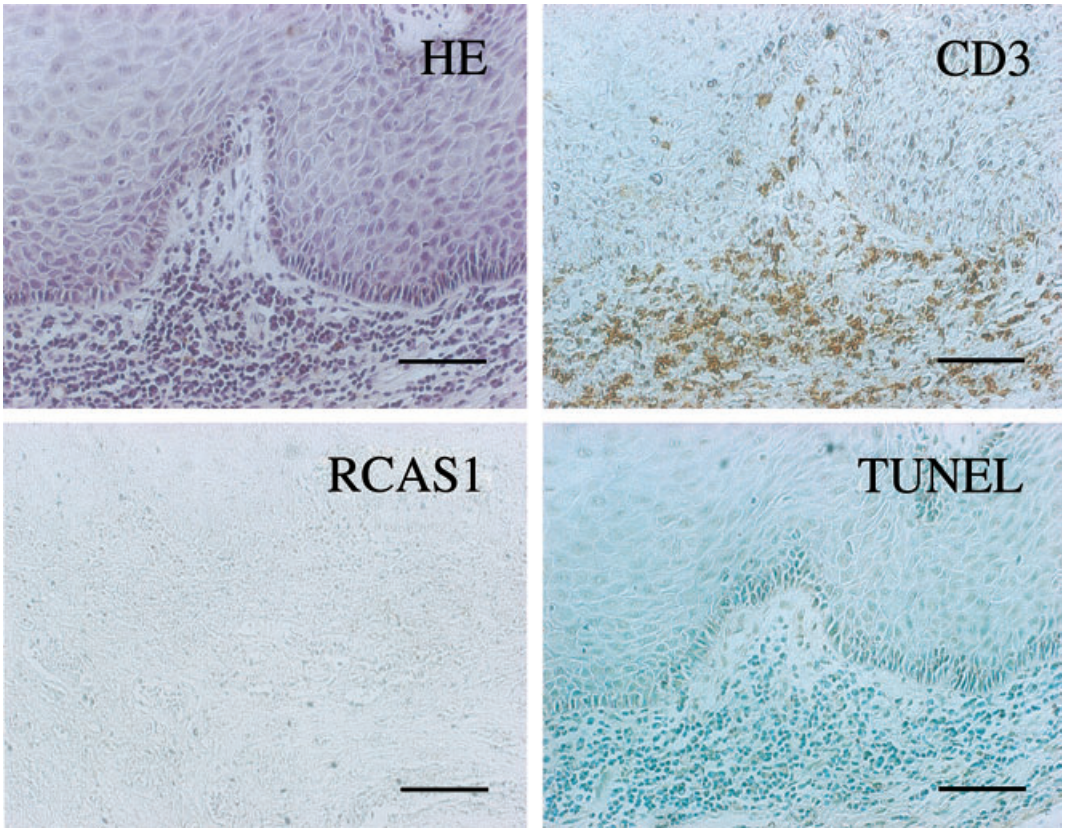

Figure 6 The absence of apoptotic TILs around RCAS1-negative oral SCC. Immunohistochemical staining with anti-CD3 and anti-RCAS1 antibodies and TUNEL staining were performed on serial sections from 130 oral SCCs, a representative case of RCAS1-negative tumor cells is shown here. TUNEL-positive TILs are hardly seen around the RCAS1-negative tumor cells (original magnification ×40, bar 15 μm).

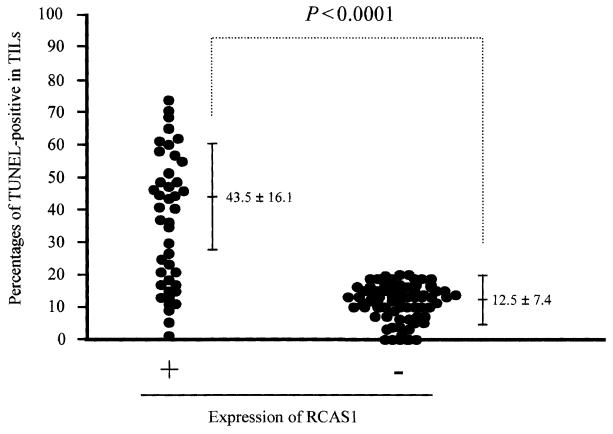

Figure 7 The association of the RCAS1 expression in oral SCCs with a high percentage of apoptoic TILs. The percentages of TUNEL-positive in TILs were significantly higher in the patients with RCAS1-positive tumor cells than in those with RCAS1-negative tumor cells ($P < 0.0001$, Student's *t*-test and Mann–Whitney *U*-test). Values are the mean ± SD.

RCAS1 may be one of the escape mechanisms for tumor cells to avoid the host immune system. Contrary to our expectations, the expression of RCAS1 was not significantly associated with the degree of lymphocytic infiltration. This discrepancy in our results may have been because of differences from patient to patient in either the immunogenecity of their tumor cells or in the immunoreactivity of the host, as the host immune surveillance is influenced by a variety of such factors.

The RCAS1 expression was closely related to a poor prognosis in patients with various cancers (18, 19), although conflicting results have been described in other reports. We therefore investigated the prognostic significance of the RCAS1 expression in patients with oral SCC. In the present study, the RCAS1 expression was not significantly related to the 5-year survival rate and was thus indicated not to be a useful predictor of a poor prognosis in patients with oral SCC. In oral SCCs, surgical treatments can be adapted in most of patients and the 5-year survival rate is almost 80% and thus higher than the rate in other cancers (27). Furthermore, it is generally accepted that the prognosis is closely associated with the invasive and metastatic potential of tumor cells, but not with the host immune system as is quite commonly observed (23, 28, 29). As a result, these discrepancies in our findings may be because of differences in host immune surveillance, tumor malignancies such as growth, invasive and metastatic potentials, or the type of treatments used in the examined cancers.

In addition, it is interesting to note that the RCAS1 expression was observed in the prickle and granular

layers of normal oral epithelia and also in the cancer pearls occurring in oral SCCs. RCAS1 has been reported to possibly play a crucial role in controlling erythropoiesis by modulating the apoptosis of erythroid progenitor cells (30). A similar process may also occur during the differentiation or keratinization of oral epithelial cells. Further studies identifying RCAS1 receptors on oral epithelial cells will greatly help to elucidate the involvement of RCAS1 in epithelial maturation.

In conclusion, the current study indicated that the RCAS1 expression might be related to tumor cell progression and invasion in the oral squamous cell epithelia, while also playing a role in tumor's ability to avoid the host immune system by inducing the apoptosis of TILs.

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