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The biological role of the unique molecule RCAS1: a bioactive marker that induces connective tissue remodeling and lymphocyte apoptosis

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TABLE OF CONTENTS

1. ABSTRACT

RCAS1 is a receptor-binding cancer antigen which is expressed on human uterine cervical adenocarcinoma cell line (SiSo). Finding a correlation between the expression of this gene and the overall survival of patients with 14 different types of cancer points to the clinical significance of this gene. Moreover, the expression RCAS1 correlates with other clinicopathological parameters including the histological type of cancer, its differentiation, tumor size, clinical stage, the depth of invasion, lymphovascular space involvement, lymph node metastasis, and positive peritoneal cytological results. RCAS1 can induce apoptosis in peripheral lymphocytes *in vitro* as well as in an increased number of apoptotic tumor-infiltrating lymphocytes. RCAS1 is also believed to contribute to the escape of tumor cells from immune surveillance. RCAS1 is secreted via ectodomain shedding, and its expression is related to changes in the characteristics of the extracellular matrix and to a reduced number of vimentin-positive tumor stromal cells, findings that suggest that RCAS1 may induce connective tissue remodeling. The concentration of RCAS1 in serum or pleural effusions has been found to be significantly higher in patients with several different types of cancer as comapred to normal controls. Together, the available data shows that RCAS1 may have value as a biomarker for monitoring therapeutic efficacy. Further exploration of the biological function of RCAS1 should help in the development of new therapeutic strategies against human malignancies.

2. INTRODUCTION

Despite advances in treatment over the past decades, the long-term survival rate of patients with advanced cancer remains poor. A growing understanding of cancer cell biology has led to the identification of a number of potential therapeutic targets. One such target may be the receptor-binding cancer antigen expressed on SiSo cells (RCAS1), which can be detected by the 22-1-1 monoclonal antibody (MoAb) and was first reported to be a tumor marker for gynecological cancer (1). 22-1-1 MoAb was generated by the fusion of mouse myeloma cells with spleen cells derived from mice immunized with the human uterine-cervical adenocarcinoma cell line SiSo (2). Subsequent immunohistochemical studies revealed that RCAS1 expression was an independent prognostic factor for patients with 12 malignancies derived not only from the uterine cervix and the endometrium, but also from the brain, lung, pleural mesothelium, esophagus, stomach, bile duct, gallbladder, pancreas, colon, and gastrointestinal mesenchyme (3-14).

Estrogen receptor-binding fragment-associated antigen 9 (EBAG9), which was isolated by the use of the CpG-genomic binding site cloning method, was reported to be identical to RCAS1 (15). It has been suggested that EBAG9 may play a specific role in the carcinogenesis of breast cancer as the gene EBAG9 was amplified in 21% of

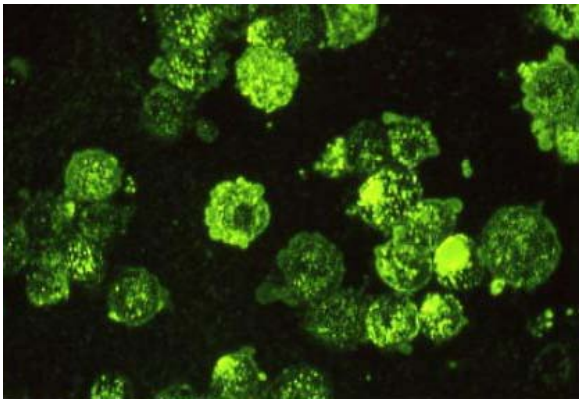

Figure 1. Cell staining with 22-1-1 MoAb. SiSo cells showed strongly positive expression of RCAS1 both in the cytoplasm and on the cell membrane.

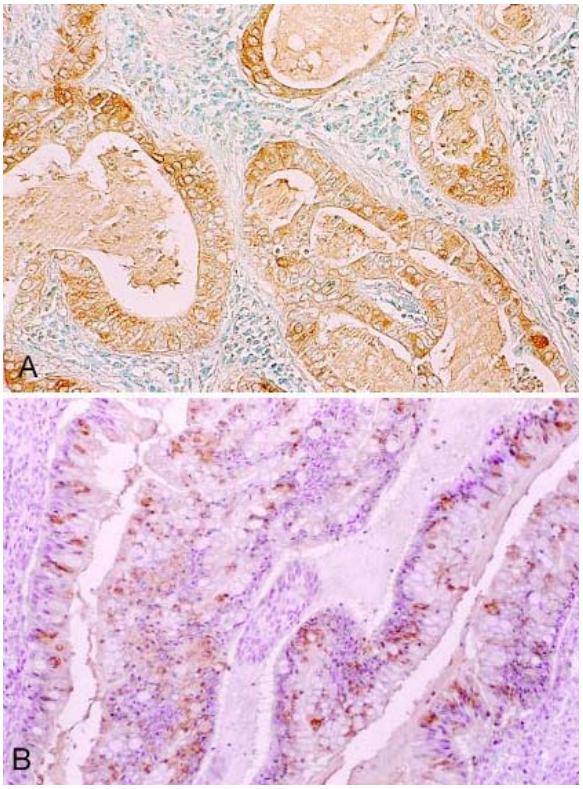

Figure 2. (A) Adenocarcinoma of the uterine cervix. RCAS1 was detected both in the cytoplasm and on the membrane of cancer cells, as well as in tumor cells in the glandular lumen (original magnification x100). (B) Ovarian mucinous cystadenocarcinoma stained strongly for RCAS1 (original magnification x100).

129 cases of primary breast cancer (16). An immunohistochemical study with antibody to EBAG9 showed that this antigen was associated with a statistically significantly poor prognosis of patients with prostate or renal cell cancer (17, 18).

Since RCAS1 can induce apoptosis in putative receptor-expressing cells, including peripheral lymphocytes, the involvement of RCAS1 in tumor progression has been extensively studied. Moreover, RCAS1 expression in non-cancerous tissues has been investigated (19, 20). Here we will review the clinical significance and function of RCAS1 and show, with the aid of new experimental data, its valuable potential in the development of new therapeutic strategies against human cancer.

3. RCAS1 EXPRESSION IN CANCEROUS TISSUES

3.1. Gynecological cancer

3.1.1. Uterine and ovarian cancers

An early report demonstrated that 22-1-1 MoAb reacted with tumor cell lines derived from only uterine cervical and ovarian adenocarcinoma (1). The use of 22-1-1 MoAb showed positive staining of SiSo cells for RCAS1 both in the cytoplasm and on the cell membrane (Figure 1). The expression of RCAS1 was significantly greater in cancerous tissues than in normal tissues obtained from the uterus and ovaries. The following positive immunohistochemical results for RCAS1 in uterine and ovarian cancers were found: 87% of uterine cervical squamous cell carcinomas (50 out of 57 cases), 87% of uterine cervical adenocarcinomas (56 out of 64 cases), 66% of uterine endometrial adenocarcinomas (68 out of 103 cases), and 58% of ovarian carcinomas (10 out of 17 cases). RCAS1 was detected both in the cytoplasm and on the membrane of the tumor cells (21). RCAS1 was also observed in some adenocarcinoma cells in the glandular lumen, which indicated RCAS1 secretion (Figure 2A). It is intriguing that mucus-producing cells, such as those in ovarian mucinous cystadenocarcinoma, stained strongly for RCAS1 (Figure 2B). It was consequently suggested that RCAS1 may have a possible role as a marker for mucus-secreting tumors, and it may therefore be advantageous to study the expression of RCAS1 in non-gynecological neoplasms that originate from mucus-secreting glandular cells.

3.1.2. Malignant transformation

RCAS1 expression was also investigated in the progression from pre-cancerous lesions to cancer of the uterine cervix (22) and endometrium (23). In uterine cervical neoplasia, RCAS1 was not detected in dysplastic lesions (0 out of 47 cases). However, 20% of carcinoma *in situ* cases (4 out of 20 cases) and 16% of microinvasive carcinoma cases (2 out of 12 cases) stained positively for RCAS1. Moreover, areas in uterine cancers with histological microinvasion stained more strongly than did carcinoma *in situ* lesions. Even greater RCAS1 expression (82%; 57 out of 69 cases) was found in invasive squamous cell carcinomas. However, 26% (12 out of 46) of normal endometrial specimens, 32% (13 out of 40) of hyperplastic endometrial specimens, and 68% (83 out of 121) of endometrial adenocarcinoma specimens had positive staining for RCAS1. This data indicates that RCAS1 expression was significantly higher in adenocarcinoma in the endometrium than in a normal or hyperplastic endometrium. Significantly greater RCAS1 expression was also observed in grade 3 tumors than in grade 1 or 2 tumors. Together these findings suggest that RCAS1 expression may be associated with malignant transformation in the uterine cervix and endometrium.

3.1.3. RCAS1 expression and clinicopathological variables

With regard to the association between RCAS1 expression and clinicopathological variables in patients with invasive uterine cervical cancer, expression of RCAS1 was significantly correlated with the invasion of the lymphovascular space, lymph node metastasis at two or more sites, and tumor volume (24). In uterine endometrial cancer, its expression was significantly correlated with age at the time of surgery, as well as tumor stage, the extent of myometrial invasion, and positive peritoneal cytological results (4). These findings indicate that the evaluation of RCAS1 expression can provide crucial information about the clinical behavior of uterine cervical and endometrial cancers that may help in the management of patients with these diseases. In ovarian cancer, EBAG9 expression, evaluated via immunohistochemistry, was observed both at the surface and in the cytoplasm of carcinoma cells in 51% of cases (46 out of 90 cases) (25). Although no significant relationship between EBAG9 immunoreactivity and overall survival of ovarian cancer patients was determined, significantly greater EBAG9 expression was associated with serous histology and advanced disease. In addition, a highly significant correlation between EBAG9 and estrogen receptor immunoreactivity was noted.

3.2. Non-gynecological cancer

In non-gynecological cancer, many studies have evaluated the association between RCAS1 expression and tumor progression. RCAS1 was detected via immunohistochemistry in 98% of gastric carcinomas (61 out of 62 cases) (26). However, it was also expressed in non-cancerous gastric epithelial cells, including cells in gastric adenoma, gastric ulcer, and normal gastric epithelia, but the pattern of expression in benign cells was strikingly different from that in malignant cells. In normal gastric mucosa, gastric ulcer, and gastric adenoma, RCAS1 localized only in the perinuclear region of the mucosal epithelial cells, whereas tumor cells in most gastric cancers showed a diffuse localization pattern in the cytoplasm and cell membranes. RCAS1 mRNA levels in gastric adenocarcinoma tissues were significantly higher than those in non-neoplastic tissues, as shown by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Moreover, this analysis, together with immunohistochemistry, demonstrated that RCAS1 was intensely expressed in advanced stages of colorectal cancer, but only weakly expressed in normal colon tissues (27).

RCAS1 expression was reportedly related to several clinicopathological variables as follows: histological differentiation in thyroid (28), lung (6), gastric (26), hepatocellular (29), and breast (30) cancer; tumor size in gastric cancer (31); clinical stage in esophageal (32, 33), gallbladder (11), and pancreatic (12) cancer; depth of invasion in thyroid (28), esophageal (8), gastric (31), and gallbladder (11) cancer; lymphovascular space involvement in gallbladder cancer (11); and lymph node metastasis in esophageal (8), gastric (9, 31), gallbladder (11), pancreatic (12), and colorectal (13) cancer. RCAS1 is thus a clinical prognostic factor for patients with these malignancies, so the expression and distribution of RCAS1 are suggested to be involved in the malignant transformation and tumor progression in human cancers derived not only from gynecological tissues, but also from these other non-gynecological organs.

4. BIOLOGICAL FUNCTIONS OF RCAS1

4.1. Biochemical features

Biochemical characteristics of RCAS1 include sensitivity to trypsin but resistance to treatment with hyaluronidase, tunicamycin, *O*-glycanase, *N*-acetyl-D-galactosaminidase, and neuraminidase (1). RCAS1 cDNA, isolated by the use of an expression cloning methodology (34), was found to contain a 5′-untranslated region of 242 nucleotides, a coding region of 639 nucleotides (213 amino acids), and a 3′-untranslated region of 179 nucleotides. RCAS1 has an N-terminal transmembrane segment (8-27 amino acids) and a coiled-coil structure in its C-terminal portion (179-206 amino acids), which indicates that RCAS1 is a type-II membrane protein that can form oligomers via the coiled-coil structure.

RCAS1 was found to be secreted in the supernatant of SiSo cell cultures and was also detected in the vaginal discharge of uterine cervical cancer patients (1). This soluble RCAS1 protein induced apoptosis in putative receptor-expressing cells, including various human cell lines and normal peripheral lymphocytes such as T, B, and natural killer cells (34). An investigation with an RCAS1-glutathione *S*-transferase (GST) fusion protein showed that a truncated RCAS1 protein lacking the C-terminal coiled-coil structure did not bind to receptor-positive cells. This finding indicates that the formation of a homologous RCAS1 complex may be necessary for the maintenance of binding activity through the coiled-coil region. Nakashima *et al.* first reported that the RCAS1-GST fusion protein could induce apoptosis in activated human T lymphocytes *in vitro* (34), and Chinese researchers subsequently obtained the same result (35). In addition, the RCAS1-GST fusion protein reportedly induced tyrosine phosphorylation of several cytoplasmic proteins in K562 chronic myelogenous leukemia cells, which express the putative RCAS1 receptor, within 5 minutes of incubation. Such data indicates the existence of several signal transduction pathways that induce apoptosis after RCAS1 stimulation (34). The number of apoptotic K562 cells significantly increased 24 hours after the initiation of incubation with RCAS1. Because benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD fmk) strongly inhibited RCAS1-induced apoptosis, it was suggested that RCAS1 may induce activation of caspases. Western blot analyses were therefore performed to evaluate the activation of mitogen-activated protein kinase (MAPK) during RCAS1-induced apoptosis in K562 cells. Flow cytometry showed that biotinylated soluble RCAS1, which was purified from SiSo cell culture supernatant, bound to K562 cells (Figure 3A). After 72 hours of incubation with soluble RCAS1 at a concentration of 10 U/ml, apoptosis was detected in 85% of K562 cells by means of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay (Figure 3B). The induction of apoptosis depended on the RCAS1 concentration level and incubation

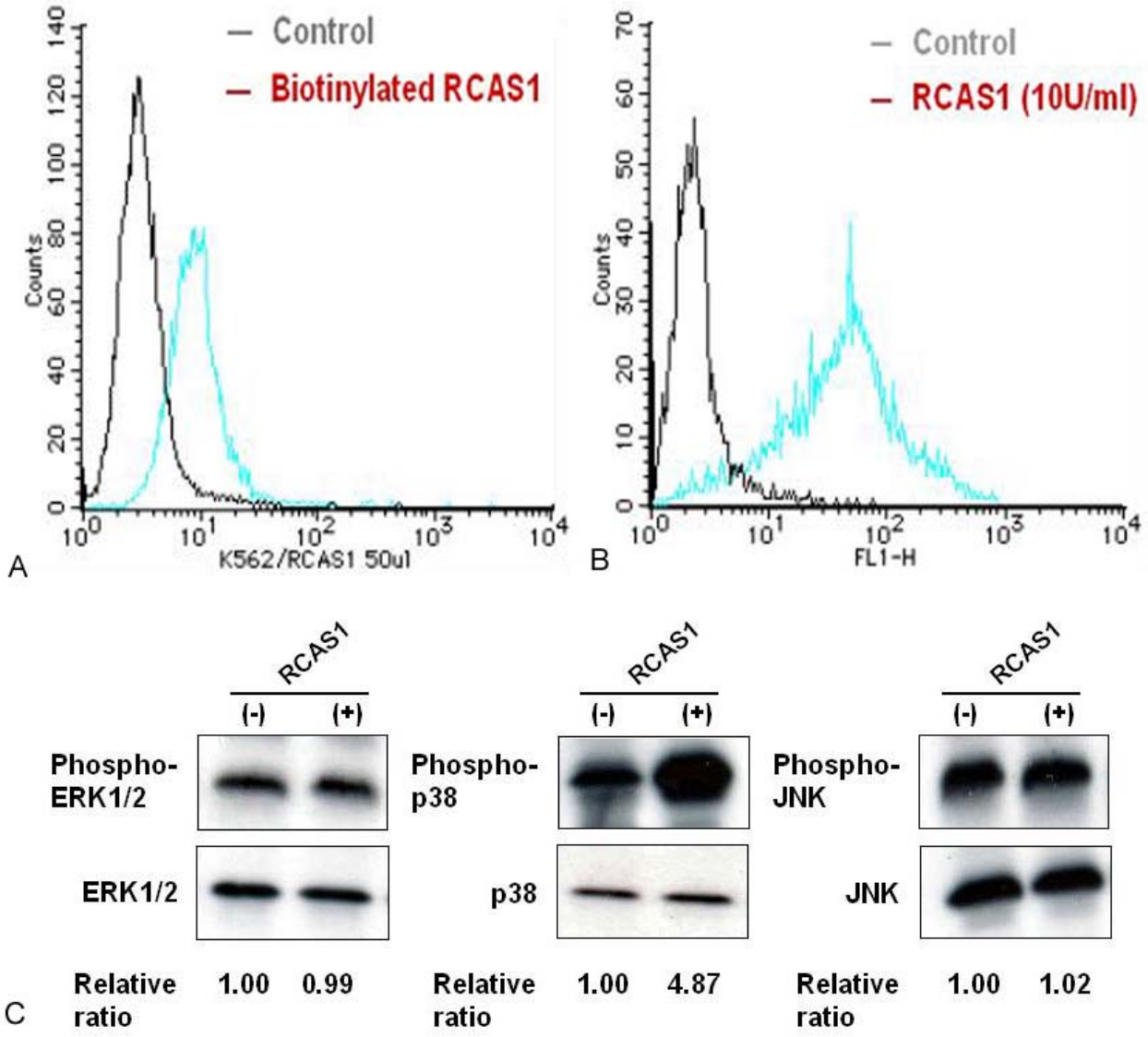

Figure 3. Activation of MAPK in RCAS1-induced apoptosis in K562 cells. (A) Expression of the putative RCAS1 receptor on K562 cells. RCAS1, prepared from SiSo cell culture supernatant by salting out with ammonium sulfate, was labeled with biotin. K562 cells were incubated with the biotinylated RCAS1 followed by incubation with avidin-fluorescein isothiocyanate. Flow cytometric analysis was then performed to assess putative RCAS1 receptor expression. (B) The TUNEL assay showed that apoptosis was induced in K562 cells after 72 hours of incubation with soluble RCAS1. (C) Phosphorylation of p38 increased after incubation with RCAS1, but the phosphorylation levels of ERK1/2 and JNK were unchanged.

time (data not shown). In this situation, phosphorylation of p38 MAPK (p 38) increased, but the levels of phosphorylation of both extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) were unchanged (Figure 3C).

4.2. RCAS1, apoptosis, and erythropoiesis

RCAS1 was reportedly involved in the regulation of apoptosis in erythroid progenitor cells (36). Erythroid colony-forming cells express high levels of RCAS1 receptor. When soluble RCAS1 was added to culture supernatant, these cells underwent apoptosis, including collapse of the mitochondrial transmembrane potential and activation of caspases 8 and 3. It is interesting that the addition of the anti-Fas blocking antibody failed to reduce this RCAS1-induced apoptosis, indicating that the effects of RCAS1 were independent of Fas activation. Histochemical staining also revealed the expression of RCAS1 in the cytoplasm of bone marrow macrophages. Stimulation of these macrophages *in vitro* with lipopolysaccharide markedly enhanced RCAS1 expression, and the lipopolysaccharide-stimulated macrophages induced apoptosis in erythroid progenitor cells through RCAS1 production (37). Thus RCAS1, which is mainly

produced by macrophages in hematopoietic tissue, was suggested to have a crucial role in controlling erythropoiesis.

4.3. Mechanisms of RCAS1 secretion as related to cancer

Because RCAS1 is secreted into a culture supernatant of SiSo cells, the mechanism of RCAS1 secretion was investigated with this cell line (24). SiSo cells were treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) that activates protein kinase C. RCAS1 expression disappeared from cell membranes after TPA treatment, but the amount of secreted RCAS1 in the culture supernatant increased. RCAS1 expression was restored, however, after the addition of GM6001, a protease inhibitor. RCAS1 is secreted via a type of proteolytic processing called ectodomain shedding, which is thought to involve a protein kinase C-dependent pathway (38). Ectodomain shedding of growth factors, growth factor receptors, cell adhesion molecules, and extracellular matrix proteins has been observed (39). Shedding of membrane proteins changes their fate, location, and mode of action, thus affecting their biological activities. This shedding process is therefore an important regulatory step in the function of membrane proteins involved in cell-cell communication during development, cell differentiation, and tissue maintenance.

Although the mechanisms of the regulation of RCAS1 expression and secretion remain unclear, the several possible explanations may be related to the aggressive characteristics of cancer. First, RCAS1, determined to be identical to EBAG9, was localized to chromosome 8q23 (15), and the RCAS1/EBAG9 gene region was over-represented in 21% (27 out of 129 cases) of primary breast cancers (16). It was therefore suggested that RCAS1/EBAG9 may play a specific role in breast cancer carcinogenesis. Second, ectodomain shedding is induced by the addition of growth factors and the activation of MAPK (40, 41). Moreover, activated mitogenic signaling pathways may be involved in the aggressive behavior of human cancer (42). In cancer cells with aggressive potential, therefore, excess RCAS1 may participate in the accelerated turnover of RCAS1 via ectodomain shedding. Third, although a few reports have described RCAS1 expression in normal uterine endometrium and gastric mucosa (19, 26), one study comparing serum RCAS1 concentrations in cancer patients and healthy subjects found significantly higher concentrations in the patients (43). Therefore, different mechanisms of RCAS1 expression and shedding may exist in both cancer tissue and normal tissue.

4.4. Relation of RCAS1 to Tn antigens

One report suggested that 22-1-1 MoAb recognizes the tumor-associated *O*-linked glycan *N*-acetyl-D-galactosamine (Tn, GalNAc), whose surface expression is modulated by RCAS1 (44). However, we demonstrated, via immunohistochemistry and flow cytometry, discrepant expression patterns for RCAS1 and Tn antigens (24). In addition, reactivity of the antigenic epitope, which is detected by 22-1-1 MoAb, did not change after treatment with *O*-glycanase or *N*-acetyl-D-galactosaminidase (1). Another report claimed that RCAS1, which is a putative antigen recognized by 22-1-1 MoAb, could not induce apoptosis in K562 cells, as shown when K562 cells were incubated with concentrated supernatant from MCF-7 cell cultures, which strongly expressed RCAS1 (45). To evaluate this claim, we performed an enzyme-linked immunosorbent assay (ELISA) with supernatants from SiSo and MCF-7 cell cultures (46). The antigen recognized by 22-1-1 MoAb was barely detected in the MCF-7 cell culture supernatant but was abundant in the SiSo culture supernatant. A different study reported that not all RCAS1-expressing cells expressed RCAS1 on the cell surface or secreted RCAS1 (47). On the basis of this data, therefore, we could not conclude that 22-1-1 MoAb does not recognize RCAS1, and we believe that further investigation of 22-1-1 MoAb reactivity and RCAS1 biological function is warranted.

4.5. RCAS1 in non-human species

RCAS1/EBAG9 cDNA has been isolated from mice and dogs (48, 49). Both murine and canine RCAS1 cDNAs have an open reading frame of 642 base pair nucleotides encoding a protein of 213 deduced amino acids. The predicted amino acid sequences of murine RCAS1 and canine RCAS1 showed 98% and 96% homologies with those of human RCAS1, respectively. Moreover, both murine RCAS1 and canine RCAS1 have an N-terminal transmembrane segment and a coiled-coil structure in the C-terminal portion, which are highly conserved in human RCAS1. It is intriguing that the incubation of NIH3T3 mouse fibroblastic cells with recombinant murine EBAG9 protein resulted in the suppression of cell growth (48). In an immunohistochemical study, canine RCAS1 was not expressed in normal mammary glands but was expressed in 100% (15 out of 15 cases) of the malignant mammary tumors examined (50). In most malignant mammary tumors, RCAS1 was localized in the cytoplasm, without polarity of expression, and *in situ* DNA fragmentation in a cluster of differentiation (CD) 3-positive tumor-infiltrating lymphocytes (TILs) was observed near the RCAS1-expressing tumors.

4.6. Interaction of RCAS1 and Snapin

EBAG9 also reportedly interacted with Snapin, which is probably a modulator of synaptotagmin-associated regulated exocytosis (51). Because EBAG9 reduced the phosphorylation of Snapin, the association of Snapin with the synaptosome-associated proteins of 25 and 23 kDa (SNAP25 and SNAP23) was decreased. Enhancing the interaction between EBAG9 and Snapin inhibited secretion of neuropeptide Y from PC12 cells *in vitro*, whereas evoked neurotransmitter release from hippocampal neurons remained unaltered.

5. CLINICAL SIGNIFICANCE OF RCAS1 EXPRESSION AND SECRETION *IN VIVO*

The previous sections described the biological functions of RCAS1. In this section, we will review the results of reported studies on the clinical significance of RCAS1 expression and secretion that have relied on

samples of blood, pleural effusions, and tissues for their analyses.

5.1. Laboratory values of RCAS1 concentrations

RCAS1 that has been detected on cell membranes (1) is a type-II membrane protein with an N-terminal transmembrane segment (34). RCAS1 is secreted via proteolytic processing (24) and was found in the vaginal discharge of uterine cervical cancer patients (1). Via an ELISA, the serum concentration of RCAS1 was measured in samples collected from both healthy blood donors and patients with uterine cervical or endometrial cancer (46). In the healthy subjects, the serum RCAS1 concentration was 4.37 ± 0.25 U/ml (mean ± SEM). Corresponding RCAS1 values in stage I/II and stage III/IV cervical cancer patients were 9.41 ± 1.83 U/ml and 8.38 ± 1.67 U/ml, respectively. For patients with endometrial cancer, RCAS1 values were 7.04 ± 1.05 U/ml for stage I/II patients and 8.54 ± 2.69 U/ml for stage III/IV patients. RCAS1 values were therefore significantly higher in uterine cancer patients than in healthy blood donors. Serum RCAS1 values classified according to histological subtype were 14.16 ± 3.74 U/ml for cervical adenocarcinoma and 7.31 ± 1.15 U/ml for cervical squamous cell carcinoma, the values for adenocarcinoma thus being significantly higher. RCAS1 concentrations were also measured in ovarian tumor patients (52). Patients with benign ovarian tumors had an RCAS1 concentration of 5.66 ± 0.70 U/ml; patients with cancerous ovarian tumors, 11.63 ± 1.74 U/ml. The RCAS1 value was significantly higher for cancer patients than for either healthy blood donors or patients with benign tumors. Interestingly, patients with the mucinous histological subtype of both benign and malignant tumors had high serum RCAS1 levels, but the levels were significantly higher in the cancer patients than in the patients with benign tumors of the mucinous or endometrioid histological subtype.

Moreover, the RCAS1 value was statistically associated with the response to treatment in patients with uterine and ovarian cancer. *In vitro* growth of K562 cells was evaluated by using the tetrazolium salt (WST-1) assay (46, 52). Serum from these cancer patients significantly inhibited cell growth in comparison to serum from healthy blood donors. However, this suppressive effect on cell growth was partially negated after immunoprecipitation with 22-1-1 MoAb to remove RCAS1. All of this data has indicated that RCAS1 may be a biomarker for uterine and ovarian cancer by virtue of its ability to predict the results of medical treatment and inhibit the cell growth of its putative receptor-expressing cells.

The high concentration of RCAS1 in blood samples from patients with non-gynecological carcinoma was also described. The serum RCAS1 level was higher in patients with gastrointestinal tract cancers than in a control group and was significantly higher in patients with lymph node involvement than in lymph node-negative patients (43). In addition, serum RCAS1 concentrations in patients with pancreatic adenocarcinoma were significantly higher than those in patients with chronic pancreatitis, acute pancreatitis, or autoimmune pancreatitis (53).

5.2. RCAS1 as a tumor marker in diagnosis and treatment

The sensitivity and specificity of RCAS1 in diagnosis of benign or malignant conditions were also analyzed. Results showed that in pancreatic cancer RCAS1 had a specificity similar to that of carbohydrate antigen 19-9 (CA19-9), but with a higher sensitivity (54). Another study reported that the sensitivity of RCAS1 alone (55%: 12 out of 22 cases) was higher than that of carcinoembryonic antigen (CEA) alone (27%: 6 out of 22 cases) and that the specificity of RCAS1 alone (92%: 33 out of 36 cases) was greater than that of CA19-9 alone (78%: 28 out of 36 cases) (55). The sensitivity of the combination of RCAS1 and CA19-9 (95%: 21 out of 22 cases) was superior to that of CA19-9 alone (73%: 16 out of 22 cases), RCAS1 alone (55%: 12 out of 22 cases), CEA alone (27%: 6 out of 22 cases), RCAS1 plus CEA (59%: 13 out of 22 cases), and CA19-9 plus CEA (82%: 18 out of 22 cases). These results suggest that RCAS1 is a valuable serum marker and that the combination of RCAS1 and CA19-9 is highly sensitive for the diagnosis of pancreatic cancer. Moreover, in biliary cancer, the percentage of serum samples positive for soluble RCAS1 was 63% (26 out of 41 cases), which was significantly higher than the 9% (6 out of 62 cases) found in benign biliary disease (56). Also in cholangiocellular carcinoma, higher positive serum results were obtained for RCAS1 than for CA19-9 and CEA (57). In hepatocellular carcinoma, however, serum RCAS1 levels had little clinical significance (47).

With regard to the usefulness of the serum RCAS1 value as a tumor marker, serum RCAS1 levels varied according to the course of the disease and the effect of treatment in extramammary Paget's disease and biliary carcinomas (58, 59). Therefore, RCAS1 has potential as a valuable biomarker for monitoring therapeutic efficacy in these diseases. In addition, RCAS1 levels were estimated in pleural effusions: malignant pleural effusions had significantly higher RCAS1 concentrations than did non-malignant effusions (60). By multivariate analysis, the pleural fluid RCAS1 value was an independent prognostic factor in lung cancer patients with pleural effusion (61). Together, this data shows that measurements of RCAS1 concentrations in serum and pleural effusions contributed to diagnostic accuracy and may be useful for estimating tumor progression or the effects of treatment.

5.3. RCAS1 in relation to apoptosis, immune surveillance, and tumor progression

Apoptotic cell death and cell growth inhibition induced by RCAS1 were first reported in 1999 (34). To date, more than 15 published immunohistochemical studies have attempted to assess, by evaluating RCAS1 expression and the number of TILs and apoptotic lymphocytes around tumor cells, involvement of RCAS1 in tumor cell evasion of immune surveillance. Immunohistochemical methods were used to determine the relationship between the expression of RCAS1, Fas ligand, and tumor necrosis factor alpha, and the number of apoptotic lymphocytes in primary lesions and metastatic lymph nodes in patients with uterine cervical cancer (62). The number of cells with

positive expression of RCAS1, but not of the two other molecules, correlated significantly with the number of apoptotic lymphocytes in uterine cervical cancer and metastatic lymph nodes. Not only uterine cancer, but also malignancies in other organs showed increased numbers of apoptotic lymphocytes or decreased numbers of TILs as follows: glioma (5), oral squamous cell cancer (63, 64), lung cancer (65), breast cancer (66), esophageal cancer (8), gastric cancer (9, 31), biliary tract cancer (67), and colon cancer (13). It is intriguing that *in situ* DNA fragmentation in CD3-positive TILs was observed even in canine malignant mammary tumors that expressed RCAS1 (49). The existence of RCAS1-expressing tumors indicates the possible induction of apoptotic cell death in TILs through RCAS1 expression. These observations suggest that RCAS1 likely plays an important role in tumor progression and the escape of tumor cells from immune surveillance.

Other reports established the association of RCAS1 expression with the apoptotic cell death of surrounding lymphocytes in non-cancerous tissues. Dutsch-Wicherek et al. analyzed the expression of RCAS1 and DNA fragmenting factor-45, which is a substrate for caspase-3, in nasal polyps and concluded that nasal polyps can regulate the cytotoxic immune response via alterations in RCAS1 expression (68). Minami et al. investigated the role of RCAS1 in the human immunodeficiency virus (HIV)-1 infection (69). They stimulated CD4-positive T cells, monocytes, and several cell lines with HIV-Tat protein and showed that Tat significantly increased mRNA transcription levels and the secretion of soluble RCAS1. Moreover, apoptosis induced by HIV-Tat was blocked by inhibiting RCAS1 expression with small interfering RNA (siRNA). Immunohistochemistry was also performed to determine whether RCAS1 was expressed in the Epstein-Barr virus (EBV)-associated Hodgkin's disease (HD) and whether RCAS1 participated in immune evasion (70). RCAS1-expressing malignant Hodgkin and Reed-Sternberg cells were found in 93% (14 out of 15 cases) EBV-positive HD cases, but only 40% (8 out of 20 cases) EBV-negative HD cases. Apoptotic lymphocytes surrounding these malignant cells were present in 91% (20 out of 22 cases) of RCAS1-positive cases, but not in any RCAS1-negative cases (0 out of 13 cases). These results indicate that RCAS1 functions in one mechanism of peripheral lymphocyte depletion induced by HIV and EBV infection.

We previously investigated the characteristics of connective tissue around tumor cells in uterine cervical cancer (24). We found significant associations between expression levels of RCAS1 and those of matrix metalloproteinase (MMP)-1 and laminin-5. These molecules—MMP-1, an interstitial collagenase, and laminin-5, an extracellular matrix molecule—are reportedly involved in tumor invasion (71, 72). MMP-1 promotes tumor invasion and metastasis through digestion of extracellular matrix and activation of various intracellular signaling pathways (73). Another study found that the number of cells expressing vimentin significantly decreased in relation to RCAS1 expression level. Vimentin is an intermediate filament protein, and alterations in its expression correlate with cell behavior alterations such as epithelial-mesenchymal transition (EMT) (74). EMT is a process during cell development in which epithelial cells acquire a mesenchymal and invasive phenotype, so EMT is believed to be a critical step in the progression of many types of carcinoma. However, we observed that the number of vimentin-positive stromal cells decreased inversely according to RCAS1 expression by tumor cells. While the mechanisms governing this phenomenon are unclear, these results indicate that RCAS1 may contribute to tumor progression not only via induction of lymphocyte apoptosis, but also via connective tissue remodeling of tumor stroma.

5.4. RCAS1 expression in normal uterine endometrium and gastric mucosa

Thus far, a few reports have demonstrated low levels of RCAS1 expression in normal uterine endometrium and gastric mucosa (19, 26). In uterine cervical glands, RCAS1 expression was observed in 93% (40 out of 43) of specimens examined, and it was mainly localized in superficial cervical glands. In nearby areas of squamous metaplasia, RCAS1 was strongly expressed. In uterine cervical squamous epithelium, RCAS1 expression was seen in 84% (26 out of 31) of the specimens. In the uterine corpus, RCAS1 expression was found in 87% (28 out of 32) of the specimens, mainly in the endometrial glands of the basalis layer. In addition, age and RCAS1 expression showed a significant positive correlation. In comparison, RCAS1 expression was seen in all normal gastric mucosa specimens, in the perinuclear region of epithelial cells, and most gastric cancers showed a diffuse RCAS1 distribution in tumor cell cytoplasm and cell membranes. In the female genital organs, RCAS1 expression was noted in the cytoplasm and whole cell membranes of normal cells. Estrogen induced RCAS1/EBAG9 *in vitro* (15), but RCAS1 expression was not immunohistochemically different in the proliferative, secretory, and post-menopausal states of the uterine endometrium (4, 23). Further investigation is necessary to clarify the mechanisms of control and function of RCAS1 expression in these normal tissues.

6. CONCLUSIONS AND PERSPECTIVES

Modern molecular and cellular biotechnological methods have provided new data about medical diseases that can help in the development of new therapeutic strategies against cancer. Various agents, including chemical compounds and antibodies, have been shown to inhibit tumor growth and to improve the survival and treatment response rates of cancer patients (75, 76). RNA interference, mediated by siRNA, is a highly specific mechanism for suppressing the expression of individual genes (77), and a biomolecular therapeutic agent that utilizes siRNA has provided an encouraging possibility in cancer therapy (78). Therapies that target specific molecules with unique biological functions are promising strategies in the treatment of human cancers. In this review, we described the biological role of RCAS1, which has potential value as a unique bioactive marker via induction

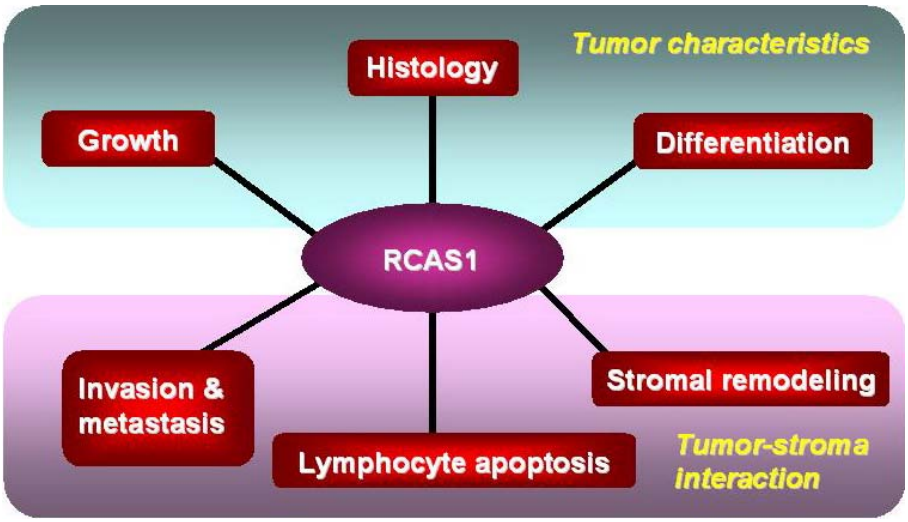

Figure 4. Schematic presentation of biological functions of RCAS1.

of lymphocyte apoptosis and connective tissue remodeling (Figure 4). Inasmuch as RCAS1 is a promising target in cancer therapy, the development of RCAS1 inhibitors should allow us to explore new therapeutic options for human malignancies.

7. ACKNOWLEDGMENTS

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Abbreviations: RCAS1: receptor-binding cancer antigen expressed on SiSo cells, MoAb: monoclonal antibody, EBAG9: estrogen receptor-binding fragment-associated antigen 9, RT-PCR: reverse transcriptase-polymerase chain reaction, GST: glutathione *S*-transferase, Z-VAD fmk: benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, MAPK: mitogen-activated protein kinase, TUNEL: terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling, p38: p38 MAPK, ERK1/2: extracellular signal-regulated kinase 1/2, JNK: c-Jun N-terminal kinase, TPA: 12-*O*-tetradecanoylphorbol 13-acetate, Tn, GalNAc: *N*-acetyl-D-galactosamine, ELISA: enzyme-linked immunosorbent assay, CD: cluster of differentiation, TIL: tumor-infiltrating lymphocyte, SNAP: synaptosome-associated protein, SNAP25: SNAP of 25 kDa, WST-1: tetrazolium salt, CA19-9: carbohydrate antigen 19-9, CEA: carcinoembryonic antigen, HIV: human immunodeficiency virus, siRNA: small interfering RNA, EBV: Epstein-Barr virus, HD: Hodgkin's disease, MMP: matrix metalloproteinase, EMT: epithelial-mesenchymal transition

Key Words: RCAS1, Bioactive marker, Apoptosis, Lymphocyte, Ectodomain shedding, Tumor progression, Tumor stroma, Review

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