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# Novel therapeutic strategies to target RCAS1, which induces apoptosis via ectodomain shedding

**Running title:** Cancer treatment by targeting RCAS1

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**Conflict of interest statement:** None declared

## 1. Summary

The expression of receptor-binding cancer antigen expressed on SiSo cells (RCAS1) is associated with aggressive characteristics and poor overall survival for 15 different human malignancies. The correlation between RCAS1 expression and several clinicopathological variables, including tumor size, clinical stage, invasion depth and lymph node metastasis highlights this molecule's clinical significance. RCAS1 is a biomarker because: (1) its concentration in serum or pleural effusion is significantly higher in cancer patients; (2) its level is associated with treatment response; and (3) high RCAS1-valued serum from cancer patients inhibits growth of RCAS1 putative receptor-expressing K562 cells. RCAS1 is secreted by ectodomain shedding and induces apoptosis in peripheral lymphocytes and natural killer (NK) cells. Although its putative receptor and mechanism of apoptosis induction remain undefined, RCAS1 is believed to help tumor cells evade immune surveillance. RCAS1 expression is also related to changes in extracellular matrix characteristics, reduction of vimentin-positive stromal cells, and increased microvessel density (MVD), all suggesting that RCAS1 may induce connective tissue remodeling. Further exploration of RCAS1 biological function will facilitate development of novel therapeutic strategies that target RCAS1.

## 2. Introduction

Although current multimodality therapies integrate surgery, radiation therapy and chemotherapy, the prognosis of patients with advanced and recurrent cancer remains poor. Increasing the understanding of the molecular pathogenesis of cancer has led to the development of novel therapeutics that target activated regulators critical for tumor cell growth, survival, invasion and angiogenesis (Weiner *et al.*, 2010; Murukesh *et al.*, 2010). For example, mutations in genes encoding epidermal growth factor (EGF) family proteins and their receptors, as well as the signal transduction pathways they govern, have an impact on tumor progression and clinical outcome (Siena *et al.*, 2009; Yotsumoto *et al.*, 2009) and have been targeted by small molecules and antibodies (Vokes and Choy, 2003). Exploiting such regulators therapeutically may provide more specific yet less toxic treatments compared to traditional cytotoxic approaches (McNeel *et al.*, 2005; Chien *et al.*, 2009).

RCAS1 might be one such therapeutic target. RCAS1 was first detected by the 22-1-1 monoclonal antibody (MoAb) and was reported to be a tumor-associated antigen in human uterine and ovarian carcinomas (Sonoda *et al.*, 1996). Subsequent immunohistochemical studies revealed that RCAS1 is a prognostic factor for 15 different types of human cancer and its expression correlates with tumor aggressiveness (Sonoda *et al.*, 2008; Giaginis *et al.*, 2009). Basic research revealed that RCAS1 induces lymphocyte apoptosis and connective tissue remodeling (Nakashima *et al.*, 1999; Sonoda *et al.*, 2005a). RCAS1 is secreted following proteolytic

processing. This proteolytic processing, also referred to as ‘ectodomain shedding’, occurs for growth factors, growth factor receptors, cell-adhesion molecules, extracellular matrix proteins and other membrane proteins such as the β-amyloid precursor protein (Izumi *et al.*, 1998). Ectodomain shedding affects the biological activity of membrane proteins by altering their localization and mode of action. In the case of membrane-anchored growth factors, ectodomain shedding can convert them into diffusible factors, which greatly influences their functions (Massague and Pandiella, 1993). Concerning RCAS1, apoptosis is mainly induced by the secreted rather than the membrane-anchored form (Sonoda *et al.*, 2010).

Based on this accumulating evidence, RCAS1 may play a pivotal role in tumor progression. Molecular targeted therapies that inhibit RCAS1 signaling pathways may provide promising new avenues for human cancer therapies. Here, the unique biological activity of RCAS1 is reviewed and its potential value as a targeting molecule for cancer treatment is discussed.

## 3. Clinical significance of RCAS1

### 3.1. RCAS1 expression and clinicopathological variables

An early report revealed that 22-1-1 MoAb against RCAS1 reacted with tumor cell lines derived from uterine and ovarian adenocarcinoma (Sonoda *et al.*, 1996). In SiSo cells, RCAS1 is positively stained both in the cytoplasm and cell membrane, while immunohistochemistry indicates that RCAS1 expression is significantly higher relative to normal tissues in cancerous tissues obtained from the uterus and ovary, and is also detectable in both the cytoplasm and cell membrane of these tumor cells. RCAS1 is also observed in some adenocarcinoma cells in the glandular lumen, which indicates RCAS1 secretion. Intriguingly, mucus-producing cells, such as ovarian mucinous cystadenocarcinoma, intensely stain for RCAS1 (Razvi *et al.*, 1999; Sonoda *et al.*, 2009), so it may be advantageous to study the expression of RCAS1 in non-gynecological neoplasms that originate from mucous-secreting cells.

To date, many studies have evaluated RCAS1 expression in non-gynecological cancers. RCAS1 was detected via immunohistochemistry in 98% of gastric carcinomas (Kubokawa *et al.*, 2001). Tumor cells in most gastric cancers show a diffuse localization of RCAS1 in the cytoplasm and cell membranes. RCAS1 mRNA levels in gastric adenocarcinoma tissues are also significantly higher than those in non-neoplastic tissues as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Moreover, RT-PCR, together with immunohistochemistry, demonstrated that RCAS1 was intensely expressed

in advanced stages of colorectal cancer (Leelawat *et al.*, 2003).

RCAS1 expression was investigated during the progression from pre-cancerous lesions to cancer of the cervix (Sonoda *et al.*, 1998) and endometrium (Sonoda *et al.*, 2000) (Table 1). In cervical neoplasia, RCAS1 was not detected in dysplastic lesions. However, 20% of carcinoma *in situ* cases and 16% of microinvasive carcinoma cases stained positively for RCAS1. Moreover, areas in uterine cancers with histological microinvasion stained more strongly for RCAS1 than did carcinoma *in situ* lesions. Even greater RCAS1 expression (82%) was found in invasive squamous cell carcinomas. On the other hand, 26% of normal endometrial specimens, 32% of hyperplastic endometrial specimens, and 68% of endometrial adenocarcinoma specimens had positive staining for RCAS1. These data indicate that RCAS1 expression was significantly higher in adenocarcinoma in the endometrium than in normal or hyperplastic endometrium. Together, these findings suggest that RCAS1 expression may be associated with malignant transformation in the cervix and endometrium.

RCAS1 expression is reportedly related to several clinicopathological variables including: histological differentiation in thyroid (Ito *et al.*, 2003), lung (Izumi *et al.*, 2001), gastric (Kubokawa *et al.*, 2001), hepatocellular (Aoki *et al.*, 2003), and breast (Rousseau *et al.*, 2002) cancer; tumor size in gastric (Nakamura *et al.*, 2004) and cervical cancer (Sonoda *et al.*, 2005a); clinical stage in esophageal (Nakakubo *et al.*, 2002; Kato *et al.*, 2005), gallbladder (Oshikiri *et al.*, 2001), pancreatic (Hiraoka *et*

*al.*, 2002), and endometrial cancer (Sonoda *et al.*, 2003); depth of invasion in thyroid (Ito *et al.*, 2003), esophageal (Tsujitani *et al.*, 2007), gastric (Nakamura *et al.*, 2004), gallbladder (Oshikiri *et al.*, 2001), and endometrial cancer (Sonoda *et al.*, 2003); lymphovascular space involvement in gallbladder (Oshikiri *et al.*, 2001) and cervical cancer (Sonoda *et al.*, 2005a); and lymph node metastasis in esophageal (Tsujitani *et al.*, 2007), gastric (Fukuda *et al.*, 2002; Nakamura *et al.*, 2004), gallbladder (Oshikiri *et al.*, 2001), pancreatic (Hiraoka *et al.*, 2002), colorectal (Okada *et al.*, 2003), and cervical cancer (Sonoda *et al.*, 2005a) (Table 2). 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. These findings indicate that evaluation of RCAS1 expression can provide crucial information about the clinical behavior of human cancers that may help manage the treatment of patients with these diseases.

### 3.2. RCAS1 as a biomarker in diagnosis and treatment

RCAS1 existing on cell membranes is a type-II membrane protein with a cytoplasmic N-terminal segment (Nakashima *et al.*, 1999). RCAS1 is secreted via proteolytic processing (Sonoda *et al.*, 2010) and is found in the vaginal discharge of cervical cancer patients (Sonoda *et al.*, 1996). Via an enzyme-linked immunosorbent assay (ELISA), the serum concentration

of RCAS1 was measured in samples collected from both healthy blood donors and patients with cervical or endometrial cancer (Sonoda *et al.*, 2006). RCAS1 values were significantly higher in uterine cancer patients than in healthy blood donors, with the values for adenocarcinoma being significantly higher than squamous cell carcinoma of the cervix. RCAS1 concentrations were also measured in ovarian tumor patients (Sonoda *et al.*, 2007a). The RCAS1 value was significantly higher for ovarian 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 ovarian tumors had high serum RCAS1 levels, but the levels were significantly higher for cancer patients than patients with benign tumors of the mucinous or endometrioid histological subtype.

A high concentration of RCAS1 in blood samples from patients with non-gynecological carcinoma was also reported by using ELISA (Table 3). The serum RCAS1 level was higher in patients with gastrointestinal tract cancers than in a control group and was significantly higher for patients having lymph node involvement compared to lymph node-negative patients (Coban *et al.*, 2006). 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 (Akashi *et al.*, 2003). Serum RCAS1 levels were significantly increased in colon cancer patients compared to healthy individuals (Leelawat *et al.*, 2003; Giaginis *et al.*, 2009b). Increased RCAS1 levels were significantly associated with

advanced Dukes' stage and high histopathological tumor grade. By univariate analysis, colon cancer patients with elevated RCAS1 levels had significantly shorter overall survival times, and multivariate analysis revealed serum RCAS1 to be an independent prognostic factor of this malignancy. Analysis of the sensitivity and specificity of RCAS1 in diagnosis of benign or malignant conditions suggested that RCAS1 is a valuable serum marker and that a combination of RCAS1 and carbohydrate antigen 19-9 (CA19-9) is highly sensitive for the diagnosis of pancreatic cancer (Yamaguchi *et al.*, 2005; Ozkan *et al.*, 2006). Moreover, in biliary cancer, the percentage of serum samples positive for soluble RCAS1 was significantly higher than in benign biliary disease (Enjoji *et al.*, 2004a). For cholangiocellular carcinoma, higher positive serum results were obtained for RCAS1 than for CA19-9 and carcinoembryonic antigen (CEA) (Watanabe *et al.*, 2003). RCAS1 levels were estimated in pleural effusions, with malignant pleural effusions having significantly higher RCAS1 concentrations compared to non-malignant effusions (Aoe *et al.*, 2004). By multivariate analysis, the pleural fluid RCAS1 value was an independent prognostic factor for lung cancer patients with pleural effusion (Aoe *et al.*, 2006).

RCAS1 value was statistically associated with the response to treatment in patients with uterine and ovarian cancer (Sonoda *et al.*, 2006; Sonoda *et al.*, 2007a). Dutsch-Wicherek *et al.* also reported that RCAS1 level increased in cancer patients whose relapse was confirmed with various types of squamous- and adeno-carcinomas (Dutsch-Wicherek and

Wicherek, 2008). With regard to the usefulness of the serum RCAS1 value as a tumor marker, serum RCAS1 levels varied according to disease course and the effect of treatment in extramammary Paget's disease and biliary carcinomas (Enjoji *et al.*, 2003; Yoshida *et al.*, 2008; Enjoji *et al.*, 2004b), again suggesting that RCAS1 might contribute to the diagnosis and estimation of tumor progression in human malignancies. Serum from uterine and ovarian cancer patients but not serum from healthy blood donors could significantly inhibit the growth of K562 chronic myelogenous leukemia cells that express the putative RCAS1 receptor, and this suppressive effect could be partially negated upon immunoprecipitation with 22-1-1 MoAb to remove RCAS1 (Sonoda *et al.*, 2006; Sonoda *et al.*, 2007a).

Taken together, these data indicate that RCAS1 may be a biomarker for human cancer by virtue of its ability to predict the results of medical treatment and inhibit the cell growth of its putative receptor-expressing cells. Therefore, measurements of RCAS1 concentrations in serum and pleural effusions can contribute to diagnostic accuracy and may be useful for estimating tumor progression or the effects of treatment. Further exploration regarding the function of RCAS1 could aid the development of novel therapeutic strategies for human malignancies that target RCAS1.

## 4. Biological function of RCAS1

### 4.1. Biochemical features

RCAS1 complementary deoxyribonucleic acid (cDNA) isolated using expression cloning methodology contains 5′- and 3′-untranslated regions of 242 and 179 nucleotides, respectively, separated by an intervening coding region of 639 nucleotides (213 amino acids) (Nakashima *et al.*, 1999). RCAS1 has an N-terminal transmembrane segment (amino acids 8-27) and a coiled-coil structure in its C-terminal portion (amino acids 179-206), which indicates that RCAS1 is a type-II membrane protein that can form oligomers via a coiled-coil structure. RCAS1 is sensitive to trypsin but resistant to treatment with hyaluronidase, tunicamycin, *O*-glycanase, *N*-acetyl-D-galactosaminidase and neuraminidase (Sonoda *et al.*, 1996). Estrogen receptor-binding fragment-associated antigen 9 (EBAG9), which is identical to RCAS1, is localized to chromosome 8q23 (Ikeda *et al.*, 2000). RCAS1/EBAG9 cDNA has been isolated from mice and dogs (Tsuchiya *et al.*, 2001; Okamura *et al.*, 2003) with each having an open reading frame of 642 nucleotides encoding a protein of 213 amino acids. The predicted amino acid sequences of murine RCAS1 and canine RCAS1 showed, respectively, 98% and 96% identity with that of human RCAS1. Moreover, both murine 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. In an immunohistochemical study, canine RCAS1 was not expressed in normal mammary glands but was expressed in all of the

malignant mammary tumors examined (Okamura *et al.*, 2004). In most canine malignant mammary tumors, RCAS1 was localized in the cytoplasm without the polarity of expression seen in human tumors.

### 4.2. RCAS1 and apoptosis

RCAS1 was found to be secreted in the supernatant of SiSo cell cultures and was also detected in the vaginal discharge of cervical cancer patients (Sonoda *et al.*, 1996). 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 (Nakashima *et al.*, 1999). An investigation using 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, indicating that formation of a homologous RCAS1 complex may be necessary to maintain 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* (Nakashima *et al.*, 1999), and Hong *et al.* subsequently obtained the same result (Hong *et al.*, 2006). In addition, the RCAS1-GST fusion protein reportedly induced tyrosine phosphorylation of several cytoplasmic proteins in K562 cells within 5 minutes of addition. The number of apoptotic K562 cells also significantly increased 24 hours after addition of RCAS1. Such data point to the existence of several signal transduction pathways that induce apoptosis following RCAS1 stimulation

(Nakashima *et al.*, 1999). We performed Western blot analyses in order to evaluate the activation of mitogen-activated protein kinase (MAPK) during RCAS1-induced apoptosis in K562 cells (Sonoda *et al.*, 2008). The induction of apoptosis depended on RCAS1 concentration and incubation time. In this situation, p38 MAPK (p 38) phosphorylation increased, but phosphorylation levels of both extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) were unchanged. Han *et al.* reported that knockdown of RCAS1 expression by RNA interference restored T cell proliferation, reduced apoptosis and partially reversed the T cell function of interferon γ (IFN)-γ secretion (Han *et al.*, 2007). Nishinakagawa *et al.* analyzed apoptosis induced by RCAS1 using a mouse fibroblast L cell line transformed with a tetracycline-induced RCAS1 gene expression system (Nishinakagawa *et al.*, 2010). In these transformed cells, RCAS1 induced cytochrome c release and activation of caspase-3, which are hallmarks of apoptosis, while decreasing cyclin D3 levels. These results agree with the finding that RCAS1-induced apoptosis in K562 cells is strongly inhibited by benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD fmk) (Nakashima *et al.*, 1999). In addition, the incubation of NIH3T3 mouse fibroblastic cells with recombinant murine EBAG9 protein resulted in the suppression of cell growth (Tsuchiya *et al.*, 2001).

To date, more than 15 published immunohistochemical studies have attempted to assess the involvement of RCAS1 in tumor cell evasion of immune surveillance by evaluating RCAS1 expression and the number of tumor-infiltrating lymphocytes (TILs) and apoptotic lymphocytes around

tumor cells (Table 4). Immunohistochemistry was used to determine the relationship between the expression of RCAS1, tumor necrosis factor α (TNF)-α, and Fas ligand, and the number of apoptotic lymphocytes in primary lesions and metastatic lymph nodes in patients with uterine cervical cancer (Sonoda *et al.*, 2005b). The number of cells with positive expression of RCAS1, but not TNF-α or Fas ligand, correlated significantly with the number of apoptotic lymphocytes in cervical cancer and metastatic lymph nodes. In addition to cervical cancer, malignancies such as glioma (Nakabayashi *et al.*, 2010), oral squamous cell cancer (Fukuda *et al.*, 2004; Toyoshima *et al.*, 2006), lung cancer (Iwasaki *et al.*, 2000), breast cancer (Suzuki *et al.*, 2001), esophageal cancer (Tsujitani *et al.*, 2007), gastric cancer (Fukuda *et al.*, 2002; Nakamura *et al.*, 2004), biliary tract cancer (Enjoji *et al.*, 2002), and colon cancer (Okada *et al.*, 2003) showed increased numbers of apoptotic lymphocytes or decreased numbers of TILs. *In situ* DNA fragmentation in cluster of differentiation (CD) 3-positive TILs was observed even in canine malignant mammary tumors expressing RCAS1 (Okamura *et al.*, 2003), suggesting the possible induction of apoptotic cell death in TILs upon RCAS1 expression. These observations support a critical role for RCAS1 in tumor progression and the escape of tumor cells from immune surveillance.

### 4.3. RCAS1 in relation to tumor progression

We previously investigated the characteristics of connective tissue around tumor cells in cervical cancer (Sonoda *et al.*, 2005a). We found

significant associations between RCAS1 expression levels and those of matrix metalloproteinase (MMP)-1 and laminin-5. MMP-1, an interstitial collagenase, and laminin-5, an extracellular matrix molecule, are reportedly involved in tumor invasion (Skyldberg *et al.*, 1999; Brinkerhoff *et al.*, 2000). MMP-1 promotes tumor invasion and metastasis by digesting extracellular matrix proteins and activating various intracellular signaling pathways (Przybylo and Radisky, 2007). Another study found that the number of cells expressing vimentin significantly decreased in relation to RCAS1 expression level (Sonoda *et al.*, 2005a; Sonoda *et al.*, 2009). Vimentin is an intermediate filament protein, and changes in its expression correlating with cell behavior alterations such as epithelial-mesenchymal transition (EMT) (Kokkinos *et al.*, 2007). EMT is a process during cell development where epithelial cells acquire a mesenchymal and invasive phenotype, therefore EMT is believed to be a critical tumor-stroma interaction in which stromal fibroblasts within the tumor environment facilitate tumor growth and progression. However, we observed that the number of vimentin-positive stromal cells decreased inversely according to RCAS1 expression by tumor cells. In *in vitro* experiments, the growth of L cells was suppressed after stimulation by soluble RCAS1, and the expression of vimentin was markedly diminished (Sonoda *et al.*, 2009). Although the biological significance of this finding is unclear, several possible explanations exist for how the tumor induces a perturbation in stromal characteristics. First, during apoptosis in response to various stimuli, vimentin is proteolyzed by caspases to generate pro-apoptotic

fragments that amplify apoptosis (Byun *et al.*, 2001). RCAS1 also induces apoptosis in putative-receptor expressing cells (Nakashima *et al.*, 1999). Thus, RCAS1 may influence the growth of stromal cells in the tumor microenvironment. Second, tumor stroma secrete several substances that inhibit tumor growth (Karlan *et al.*, 2002), such as Transforming growth factor β (TGF)-β, which suppresses proliferation of early stage ovarian carcinoma (Nilsson and Skinner, 2002). Therefore, tumor cells might induce alterations in stromal characteristics in order to down-regulate inhibitory effects from the stroma. Third, vimentin-deficient fibroblasts display a reduction in stiffness, mechanical stability, motility, and directional migration towards chemo-attractive stimuli (Eckes *et al.*, 1998). In addition, absence of vimentin is correlated with aberrant expression and distribution of surface adhesion molecules (Nieminen *et al.*, 2006). Accordingly, reduction of vimentin expression in the stroma might alter tumor-stroma communication, which may result in accelerated tumor progression.

We previously reported that RCAS1 expression significantly correlates with MVD via vascular endothelial growth factor (VEGF) expression in cervical cancer (Sonoda *et al.*, 2007b). The introduction of an RCAS1-encoding gene into COS-7 cells accelerated *in vivo* tumor growth by promoting angiogenesis, which was based on increased expression of TGF-β, TGF-β receptor I, and hypoxia-inducible factor 1α (HIF)-1α, and the phosphorylation of ERK1/2. Therefore, RCAS1 is thought to induce VEGF production through the TGF-β and MAPK signaling pathways.

Recently, Liby *et al.* reported that Akt3 regulates RCAS1 expression and VEGF secretion (Liby *et al.*, 2011). A blockade of Akt3 resulted in smaller, less vascularized tumors in a xenograft mouse model that was correlated with a reduction in VEGF expression due to the down regulation of RCAS1. Additionally, RCAS1 expression is significantly associated with the expression levels of MMP-1 and laminin-5 in cervical cancer (Sonoda *et al.*, 2005a). The regulation of extracellular matrix degradation and remodeling, which is induced by MMPs and laminin, plays a pivotal role in the control of angiogenesis (Davis and Senger, 2005). Therefore, RCAS1 may augment angiogenesis through complex mechanisms. Dutsch-Wicherek *et al.* previously reported RCAS1 expression in healthy stroma of laryngeal and pharyngeal cancer (Dutsch-Wicherek *et al.*, 2009). Interestingly, RCAS1 was detected not only in cancer cells, but also in stroma adjacent to the tumor. Moreover, the RCAS1 stromal expression was associated with a high risk of local recurrence. While the mechanisms governing these phenomenona 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.

### 4.4. Mechanisms of RCAS1 secretion

Because RCAS1 is firstly reported to exist in the vaginal discharge of cervical cancer patients (Sonoda *et al.*, 1996) and is secreted into the culture supernatant of SiSo cells (Sonoda *et al.*, 2005a), the mechanism of RCAS1 secretion was investigated (Sonoda *et al.*, 2010). RCAS1 is

secreted by ectodomain shedding that is induced by phorbol ester, pro-inflammatory cytokines, various stress-inducing stimuli, growth factors, and G-protein-coupled receptor (GPCR) ligands. Ectodomain shedding of growth factors, growth factor receptors, cell adhesion molecules, and extracellular matrix proteins have been observed (Izumi *et al.*, 1998). Shedding of membrane proteins changes their fate, localization and mode of action, which affects their biological activities and represents an important regulatory step in the function of membrane proteins involved in cell-cell communication during development, cell differentiation and tissue maintenance. We also reported that apoptosis is mainly induced by secreted but not membrane-anchored RCAS1 (Figure 1). There are cases in which the soluble but not membrane-anchored forms of proteins are biologically active. In *Drosophila*, the EGF receptor (EGFR) ligand Spitz influences a subset of developmental processes that are regulated by EGFR, but only the secreted form of Spitz triggers EGFR signaling cascades (Schweitzer *et al.*, 1995). Processing of the membrane-anchored precursor form is required for biological activity of the Notch ligand Delta in *Drosophila* (Qi *et al.*, 1999). Therefore, regulation of the conversion of membrane-anchored proteins into a soluble form would be important to modify the action of such molecules, including RCAS1. The activity of RCAS1 in cancer progression may be enhanced by ectodomain shedding, which is an important step in RCAS1 induction of apoptosis. Theoretically, the mode of action of secreted molecules could be distinct from those of membrane-anchored proteins in the following respect: membrane-anchored ligands can transmit

signals only to neighboring cells, while soluble ligands can diffuse and act at a distance. RCAS1 is also thought to be involved in the acquisition of malignant phenotypic characteristics and tumor progression through the remodeling of stromal tissue (Sonoda *et al.*, 2008). The important role of stromal tissue in supporting tumorigenic processes has been clarified (Kim *et al.*, 2005). During tumor progression, invasion and metastasis, active cross-talk occurs between tumor cells and the stroma, which is mediated mainly by direct cell-cell contact or by paracrine cytokine and growth factor signaling (Bhowmick and Moses, 2005). Shedding of soluble molecules is often enhanced in tumor cells, suggesting that signaling pathways that are activated in transformed cells may induce ectodomain shedding (Fan and Derynck, 1999). Such a potentially important role for shedding in tumor formation is further supported by a transgenic mouse model showing that mice overexpressing cleavable, but not uncleavable, TGF-α have an increased incidence of tumor development, suggesting that ectodomain shedding may be part of a positive feedback mechanism in the case of TGF-α (Sandgren *et al.*, 1990). Moreover, TNF-α and Fas ligand, which are pivotal regulators of apoptosis, also induce cell proliferation (Natoli *et al.*, 1998; Wajant *et al.*, 2003). Therefore, overexpression and ectodomain shedding of RCAS1 may contribute to tumor progression via not only stromal remodeling, but also tumor cell proliferation. The events occurring downstream of RCAS1 activation in these pathways and the metalloproteinases responsible for RCAS1 shedding are now under investigation. Taken together, these data indicate that RCAS1 is a unique

molecule that may contribute to tumor progression by its multiple functions. Further exploration regarding the regulatory mechanisms involved in the conversion of membrane-anchored RCAS1 into its soluble form should aid the development of novel therapeutic strategies that target RCAS1.

## 5. New therapeutic strategy of cancer by targeting RCAS1

Conventional chemotherapy, such as nucleic acid analogs and cell division inhibitors, is still the mainstay of cancer treatment. However, limitations of the efficacy of these anticancer agents against advanced or recurrent tumors require the development of molecular targeted medicines that are based on cancer characteristics (Mullenders and Bernards, 2009; Yotsumoto *et al.*, 2009). A biomarker is a biological molecule found in blood, other body fluids or in tissues that characterize cancer and is generally produced by either the tumor itself or other tissues in response to the presence of cancer or other associated conditions (Kulasingam *et al.*, 2010). The past two decades have witnessed an explosive growth in the amount of genomic and proteomic data, major advances in knowledge of molecular mechanisms of human diseases and rapid development of new technologies for molecular diagnostics and therapy (Phan *et al.*, 2009). This has translated into a new era of molecular medicine in which disease detection, diagnosis and treatment can be tailored to each individual's molecular profile (Ginsburg and McCarthy, 2001; Jain, 2002). This revolution is based on the availability and application of new biomarkers for predicting disease behavior, advanced technologies for rapid detection and diagnosis, new therapies for molecular and cellular targeting and computing technologies for data analysis and management (Allison, 2008).

RCAS1 is one biomarker for human malignancies (Sonoda *et al.*, 2008). Observation of biomarkers can now be used to diagnose cancer earlier, aid prognosis and predict therapeutic response (Murukesh *et al.*,

2010). Prognostic biomarkers correlate with outcomes independently of treatment, and predictive markers correlate with the impact of specific treatment on outcome (Koopman *et al.*, 2009). RCAS1 can be used by immunohistochemistry to estimate the malignant potential and prognosis of thyroid, lung, breast, esophageal, gastric, gallbladder, hepatocellular, pancreatic, colorectal, cervical and endometrial cancer. On the other hand, soluble RCAS1 can be measured by ELISA to evaluate response to treatment and assess recurrence of Paget's disease, lung, esophageal, gastric, biliary, cholangiocellular, pancreatic, colon, cervical, endometrial, and ovarian cancer. Future validation of biomarkers and their eventual incorporation into clinical practice holds promise for improved cancer treatment (Jain *et al.*, 2009). Accumulating evidence indicates that RCAS1 can be applicable for cancer treatment as a targeted molecule. With the increased knowledge of the molecular pathways by which cytotoxic drugs exert their effects, it became possible to study the role of various key enzymes and targets involved. For example, the ErbB receptor family is overexpressed in numerous human tumors and this overexpression correlates with poor prognosis and resistance to therapy. Use of ErbB-specific antibodies against receptors (Herceptin or Erbitux) or ErbB-specific small molecule inhibitors of receptor tyrosine kinase activity (Iressa or Tarceva) has shown clinical efficacy in some kinds of solid tumors (Witters *et al.*, 2008). Concerning RCAS1, several therapeutic strategies could be considered in order to suppress the expression and function of this molecule for the treatment of cancer.

One strategy could be to modulate RCAS1 expression using ribonucleic acid (RNA) interference. RNA interference, mediated by siRNA, is a highly specific technique for suppressing expression of individual genes (Elbashir *et al.*, 2001). Therefore, siRNA technology holds great promise as a therapeutic intervention for targeted gene silencing in cancer and other diseases. A small interfering RNA (siRNA) was previously constructed to target RCAS1 (Ogushi *et al.*, 2005; Han *et al.*, 2007; Sonoda *et al.*, 2007b; Liby *et al.*, 2011) and Han *et al.* introduced RCAS1-specific siRNA into MCF-7 cells (Han *et al.*, 2007). This siRNA-induced suppression of RCAS1 expression in MCF-7 cells effectively reduced T lymphocyte apoptosis and recovered T cell function, as well as IFN-γ secretion in *in vitro* experiments. We reported that knockdown of RCAS1 expression by siRNA significantly suppressed the *in vivo* growth of SiSo and HOUA cells (Sonoda *et al.*, 2007b). Liby *et al.* silenced RCAS1 by siRNA, resulting in reduction in VEGF secretion of ES2 ovarian cancer cells (Liby *et al.*, 2011). Ogushi *et al.* showed that intratumoral administration of siRNA against EBAG9 induced overt regression of tumors following implantation of murine renal cell carcinoma Renca cells (Ogushi *et al.*, 2005). Further development of siRNAs for anti-cancer therapy depends on the development of safe and effective nanocarriers for systemic administration because the major limitations for the use of siRNA as a therapeutic agent are its degradation by serum nucleases, poor cellular uptake and rapid renal clearance following systemic administration (Ozpolat *et al.*, 2010).

A second strategy for inhibiting RCAS1 function is to use MoAbs. Serum from uterine and ovarian cancer patients inhibited growth of RCAS1 putative receptor expressing cells. However, this suppressive effect could be partially negated after immunoprecipitation with 22-1-1 MoAb to remove RCAS1 (Sonoda *et al.*, 2006; Sonoda *et al.*, 2007a). MoAbs are effective in inhibiting the function of ligand and receptor molecules. Several MoAbs are now used in clinical practice and novel MoAbs have also been manufactured and tested for their efficacies in preclinical settings (Hotte *et al.*, 2008; Schwartz *et al.*, 2010). Combinations of MoAbs and other antitarget agents (*e.g.,* chemotherapeutic agents and small substance inhibitors) produce synergistic effects (Bolos *et al.*, 2010). For example, a combination of bevacizumab and erlotinib used to treat patients with refractory non-small-cell lung cancer has shown remarkable effectiveness (Hainsworth *et al.*, 2005). An anti-RCAS1 MoAb might therefore be a candidate treatment strategy. When the RCAS1 putative receptor is isolated in the future, synergistic anti-cancer activities could be expected that target both RCAS1 and its receptor.

A third strategy is to control the protease function that is involved in RCAS1 ectodomain shedding. RCAS1 is secreted by ectodomain shedding, and apoptosis is induced mainly by the soluble rather than the membrane-anchored form (Sonoda *et al.*, 2010). Therefore, inhibition of RCAS1 secretion is crucial to control its function. Sheddase inhibition prevents the cleavage of RCAS1, thereby suppressing availability of RCAS1 and its receptor activation and subsequent activity of downstream

pathways. Use of a sheddase inhibitor with lapatinib or Herceptin results in synergistic antitumor activity (Merchant *et al.*, 2008; Finn, 2010). However, since active proteases cleave a variety of substrates, including growth factors and extracellular matrix proteins, there is a greater risk of multiple and severe side effects from protease inhibitors (Hynes and Schlange, 2006). A single protease inhibitor affects EGF shedding, as well as cytokines, adhesion molecules and other growth factors. Therefore, if protease inhibitors are to have clinical application, their use should be limited to very specific disease states and very short time periods (Kataoka, 2009).

A major impediment to the effective treatment of cancer is the molecular heterogeneity of the disease, which is also reflected in an equally diverse pattern of clinical responses to therapy. To improve this situation the development of novel and highly specific targets for therapy is of utmost importance. The identification of useful biomarkers is crucial and recent advances in biomarker discovery have raised new opportunities in the emerging fields of personalized and predictive medicine. RCAS1 is a promising biomarker applicable for cancer treatment in a clinical setting. Recent advances across biology, chemistry, engineering and medicine should lead to major advances in cancer diagnosis and treatment by targeting RCAS1.

## 6. Conclusions

Given the efficacy plateau reached in cancer treatment using standard cytotoxic therapy, recent strategies have focused on molecular targets. Inhibition of critical pathways for malignancy based on improved understanding of molecular mechanisms will likely play a major role in the future of cancer treatment. In this review, I described the biological role of RCAS1, which has potential value as a unique biomarker and for its ability to induce lymphocyte apoptosis and connective tissue remodeling. Inasmuch as RCAS1 is a promising target in cancer therapy, we should investigate the clinical relevance of RCAS1 targeting strategies.

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## Figure legend

Figure 1

RCAS1 induces apoptosis via ectodomain shedding by protein kinase C δ (PKC)-δ, Ras-MAPK, and transactivation pathways.

**Table 1. RCAS1 expression in uterine cervix and endometrium**

| Pathological diagnosis | No. of cases | No. of positive cases* | Reference |
|---|---|---|---|
| Cervix | | | Sonoda K *et al.* 1998 |
| dysplasia | 47 | 0 (0) | |
| SCC** in situ | 20 | 4 (20) | |
| microinvasive SCC | 12 | 2 (16) | |
| invasive SCC | 69 | 57 (82) | |
| Endometrium | | | Sonoda K *et al.* 2000 |
| simple hyperplasia | 18 | 5 (27) | |
| complex hyperplasia | 11 | 4 (36) | |
| atypical hyperplasia | 11 | 4 (36) | |
| invasive cancer | 121 | 83 (68) | |

*Number in parentheses is the percentage of positive cases.

**SCC: squamous cell carcinoma.

**Table 2. Association between RCAS1 expression and clinicopathological variables**

| Clinicopathological variables | Type of neoplasia | Reference |
|---|---|---|
| Histological differentiation | thyroid | Ito *et al.*, 2003 |
| | lung | Izumi *et al.*, 2001 |
| | gastric | Kubokawa *et al.*, 2001 |
| | hepatocellular | Aoki *et al.*, 2003 |
| | breast | Rousseau *et al.*, 2002 |
| Size | gastric | Nakamura *et al.*, 2004 |
| | cervical | Sonoda *et al.*, 2005a |
| Clinical stage | esophageal | Nakakubo *et al.*, 2002; Kato *et al.*, 2005 |
| | gallbladder | Oshikiri *et al.*, 2001 |
| | pancreatic | Hiraoka *et al.*, 2002 |
| | endometrial | Sonoda *et al.*, 2003 |
| Depth of invasion | thyroid | Ito *et al.*, 2003 |
| | esophageal | Tsujitani *et al.*, 2007 |
| | gastric | Nakamura *et al.*, 2004 |
| | gallbladder | Oshikiri *et al.*, 2001 |
| | endometrial | Sonoda *et al.*, 2003 |
| Lymphovascular space involvement | gallbladder | Oshikiri *et al.*, 2001 |
| | cervical | Sonoda *et al.*, 2005a |
| Lymph node metastasis | esophageal | Tsujitani *et al.*, 2007 |
| | gastric | Fukuda *et al.*, 2002; Nakamura *et al.*, 2004 |
| | gallbladder | Oshikiri *et al.*, 2001 |
| | pancreatic | Hiraoka *et al.*, 2002 |
| | colorectal | Okada *et al.*, 2003 |
| | cervical | Sonoda *et al.*, 2005a |

**Table 3. Secreted RCAS1 concentration in human carcinoma**

| Type of neoplasia | Specimen | No. of cases | RCAS1 level (U/ml)* | Reference |
|---|---|---|---|---|
| lung | pleural effusion | 20 | 15.1±33.6** | Aoe *et al.*, 2004 |
| | | 59 | 36.3±114** | Aoe *et al.*, 2006 |
| biliary | serum | 41 | 71.7 | Enjoji *et al.*, 2004a |
| | | 39 | 83.1±130.5** | Enjoji *et al.*, 2004b |
| cholangiocellular | serum | 23 | 29.6±39.4** | Watanabe *et al.*, 2003 |
| pancreatic | serum | 22 | 113.0±70.8** | Yamaguchi *et al.*, 2005 |
| | | 39 | 61.1 | Ozkan *et al.*, 2006 |
| gastrointestinal tract*** | serum | 82 | 83.9±102.3** | Coban *et al.*, 2006 |
| colon | serum | 10 (stage I) | 9.2±6.2** | Leelawat *et al.*, 2003 |
| | | 18 (stage II) | 15.8±16.1** | |
| | | 32 (stage III/IV) | 20.0±18.7** | |
| cervical | serum | 41 (stage I/II) | 9.4±1.8**** | Sonoda *et al.* 2006 |
| | | 22 (stage III/IV) | 8.3±1.6**** | |
| endometrial | serum | 35 (stage I/II) | 7.0±1.0**** | Sonoda *et al.* 2006 |
| | | 15 (stage III/IV) | 8.5±2.6**** | |
| ovarian | serum | 61 | 11.6±1.7**** | Sonoda *et al.* 2007a |
| Paget's disease | serum | 1 | 22.0 | Enjoji *et al.*, 2003 |
| | | 6 | 6.1±3.4***** | Yoshida *et al.*, 2008 |

*RCAS1 level was measured by ELISA (enzyme-linked immunosorbent assay).

**Mean±SD.

*** 14 cases of esophageal, 32 cases of gastric, and 36 cases of colon cancers were included.

****Mean±SEM.

*****RCAS1 level was measured in the follow-up after treatment (mean±SD).

**Table 4. Immunohistochemical analysis of an association between RCAS1 expression and apoptotic lymphocytes in human malignancy**

| Type of neoplasia | Detection method | No. of cases | Result | Reference |
|---|---|---|---|---|
| glioma | T lymphocyte number | 57 | Statistical data* | Nakabayashi *et al.*, 2010 |
| oral | TUNEL** | 89 (RCAS1 negative tumor) | 12.5±7.4*** | Toyoshima *et al.*, 2006 |
| | | 41 (RCAS1 positive tumor) | 43.5±16.1*** | |
| breast | T lymphocyte number | 9 (RCAS1 negative tumor) | 124.1±6.5**** | Suzuki *et al.*, 2001 |
| | | 82 (RCAS1 positive tumor) | 189.5±22.1**** | |
| lung | TUNEL* | 36 (low RCAS1-expression tumor) | 4.1±3.2***** | Iwasaki *et al.*, 2000 |
| | | 30 (high RCAS1-expression tumor) | 12.2±8.9***** | |
| esophageal | T lymphocyte number | 75 | Statistical data****** | Tsujitani *et al.*, 2007 |
| gastric | Single-strand DNA | 96 (low RCAS1-expression tumor) | 7.5±6.2***** | Fukuda *et al.*, 2002 |
| | | 33 (high RCAS1-expression tumor) | 9.5±7.0***** | |
| | TUNEL* | 10 (RCAS1 negative tumor) | 0.32*** | Nakamura *et al.*, 2004 |
| | | 10 (RCAS1 positive tumor) | 0.92*** | |

| | | | | |
|---|---|---|---|---|
| colon | TUNEL* | 26 (RCAS1 negative tumor) | 7.9±1.0***** | Okada *et al.*, 2003 |
| | | 32 (RCAS1 positive tumor) | 11.2±1.0***** | |
| cervical | TUNEL* | 120 (primary lesion) | 5.0±4.1***** | Sonoda *et al.* 2005b |
| | | 55 (metastatic lymph node) | 5.1±4.1***** | |

*The number of T lymphocytes was significantly reduced in RCAS1 positive regions.

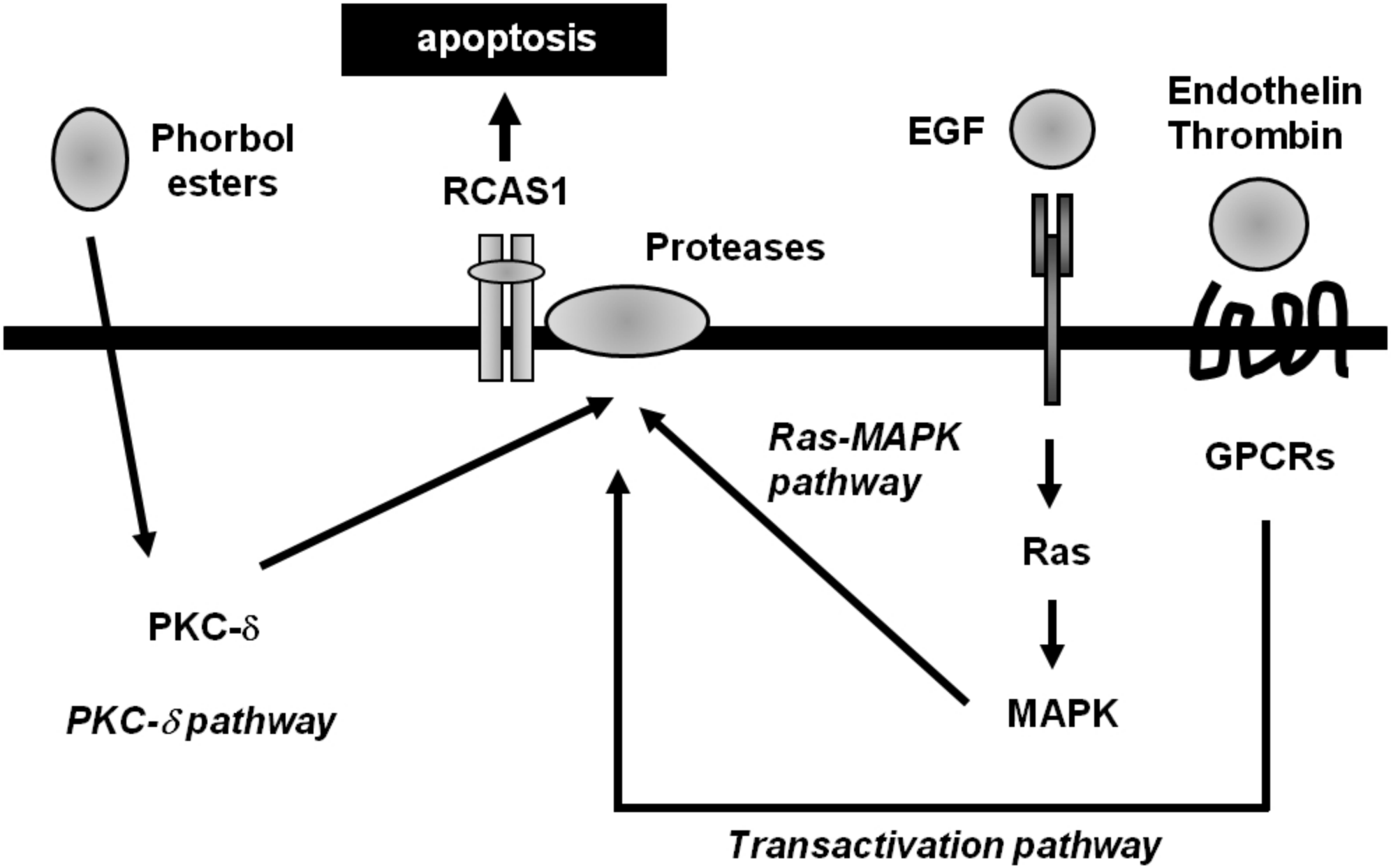
**TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

***Apoptotic index was the number of apoptotic cells in 100 lymphocytes (mean or mean±SD).

****Mean±95% confidence interval.

*****Apoptotic index was the number of apoptotic cells in 1,000 lymphocytes (mean±SD).

******Low T lymphocyte density tended to be correlated with RCAS1 expression.


apoptosis
Phorbol esters
RCAS1
Proteases
EGF
Endothelin
Thrombin
Ras-MAPK pathway
GPCRs
Ras
PKC-δ
MAPK
PKC-δ pathway
Transactivation pathway