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ORIGINAL ARTICLE

Upregulation of tumour associated antigen RCAS1 is implicated in high stages of colorectal cancer

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Background: RCAS1 (receptor binding cancer antigen expressed on SiSo cells) is a tumour associated antigen. It is involved in immune evasion by tumour cells, by binding to receptors on cells involved in the immune response, such as T cells and natural killer cells, and inducing apoptosis. High expression of RCAS1 has been demonstrated immunohistochemically in tumours of the cervix, breast, lung, and stomach; however, the expression of RCAS1 has never been investigated in colorectal cancer.
Aims: To investigate the expression of RCAS1 in colorectal cancer and identify at which stages of colorectal carcinogenesis it is expressed.
Methods: Sixty surgically resected colorectal cancer specimens obtained from Rajavithi Hospital, Bangkok, Thailand were studied. RCAS1 expression was detected immunohistochemically using monoclonal anti-RCAS1 antibody. RCAS1 mRNA expression was also investigated by reverse transcription polymerase chain reaction in the freshly isolated tissues, and serum RCAS1 was measured by enzyme linked immunosorbent assay.
Results: Staining for the RCAS1 protein was intense in high stages of colorectal cancer, but weak in normal tissues. The RCAS1 mRNA results correlated with the immunohistochemistry results. Positive serum RCAS1 concentrations were found in 10 of 18 patients with stage II disease and 12 of 32 with stage III and IV, but not in patients with stage I disease. All lymph node and liver metastases showed high expression of RCAS1 protein.
Conclusions: RCAS1 appears to be upregulated in high stages of colorectal cancer, both in the serum and the tissue. RCAS1 expression might be a useful additional criterion for staging this cancer.

See end of article for authors' affiliations

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It has been well established that human colorectal carcinogenesis is a multistep process, involving the progression from benign polyps to malignant tumours, and eventually metastases.[1–3] During these processes, many kinds of protein antigens are expressed and some are recognised as non-self by the immune system.[4] To survive in the body, tumours must develop a mechanism to evade immune surveillance.[5] A novel tumour associated antigen, RCAS1 (receptor binding cancer antigen expressed on SiSo cells), which is identified by the 22-1-1 monoclonal antibody, is highly expressed in many kinds of tumours, such as those of the lung, oesophagus, stomach, and liver.[6–14] There is evidence that RCAS1 is present as a type II transmembrane protein on the cell surface and also as a secreted soluble protein.[6] The antigen acts as a ligand for a putative receptor present on the cells of the immune system, such as natural killer cells and activated T cells. Binding of the RCAS1 ligand induced apoptosis of these receptor expressing cells, so that RCAS1 probably plays an important role in the evasion of host immune surveillance by tumour cells.[6–15] Despite the high incidence and intensive study of colorectal tumours, there are no reports on the expression of RCAS1 molecules in this disease. In addition, the mechanism of metastasis of colorectal tumours to the regional lymph nodes, which contain many types of immune cells, is still controversial.

"RCAS1 probably plays an important role in the evasion of host immune surveillance by tumour cells"

In our study, we investigated the expression of RCAS1 in human colorectal tumours at different tumour stages, including primary colorectal cancer stages I, II, III, and IV and lymph node and liver metastases, by reverse transcription polymerase chain reaction (RT–PCR) and immunohistochemical assays of fresh tissues removed from the patients. The secreted form of the RCAS1 antigen in the serum of these patients was also measured to determine its concentration in the systemic circulation.

MATERIALS AND METHODS

Tissue samples were collected from 60 patients undergoing surgical resection at Rajavithi Hospital, Bangkok, Thailand. The study protocol was prepared and approved by the ethics committees of Rajavithi Hospital. Immediately after resection, specimens were divided into two parts for RNA isolation and for immunohistological analysis. Tissues destined for RNA isolation were immediately placed into RNAlater solution (Qiagen, Hilden, Germany) and stored at −20°C. Total RNA was extracted by TRIzol reagent (Life Technologies, Gibco BRL, Burlington, Ontario, Canada), according to the manufacturer's instructions. Specimens for immunohistological analysis were fixed in 10% phosphate buffered formalin (pH 7.4), routinely processed, and embedded in paraffin wax. The histopathological diagnosis was evaluated by a reference pathologist. Tumours were staged according to the TNM staging system. Serum samples from these patients and 30 healthy controls (with permission) were also collected for the detection of RCAS1 by enzyme linked immunosorbent assay (ELISA).

Abbreviations: ELISA, enzyme linked immunosorbent assay; PBS, phosphate buffered saline; RCAS1, receptor binding cancer antigen expressed on SiSo cells; RT–PCR, reverse transcription polymerase chain reaction

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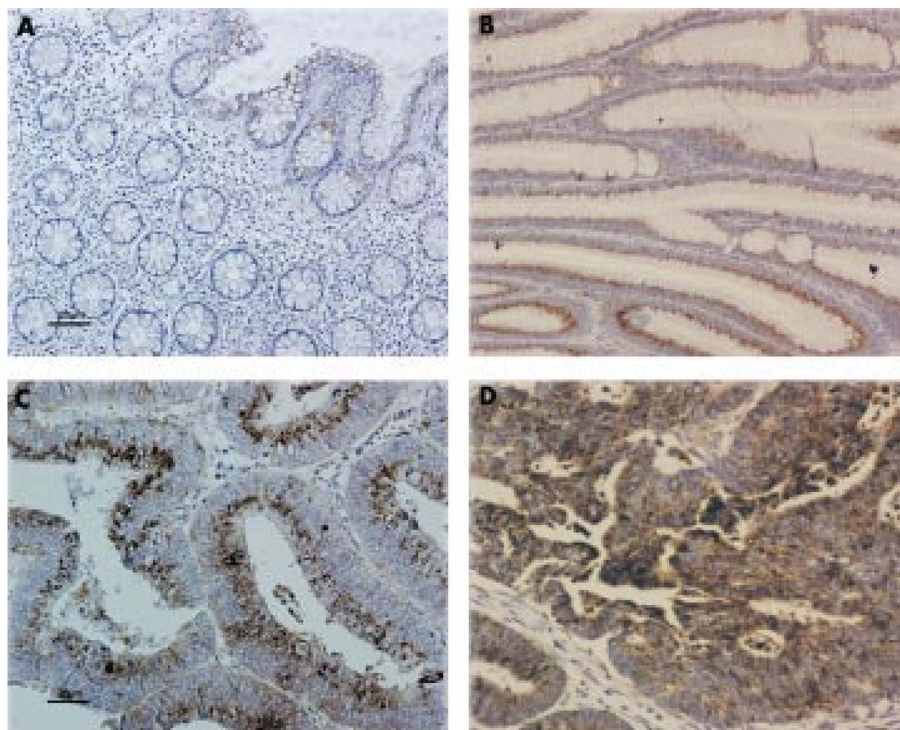

Figure 1 Representative immunohistochemical staining for RCAS1. (A) Absence of staining and (B) the supranuclear fine granular pattern. Both of these patterns were observed in the normal part of the tumour samples and in adenomatous polyps. (C, D) Cancer cells were positive for RCAS1, and showed a heterogeneous mixed staining pattern.

Immunohistochemical staining

Paraffin wax sections (4 µm thick) on glass slides were dewaxed in xylene and transferred to alcohol. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol, for 30 minutes at room temperature. The sections were boiled in 10mM citrate buffer (pH 6.0) for four minutes in a microwave oven (750 W) for antigen retrieval. Non-specific binding was blocked by incubating with 3% normal horse serum for 20 minutes. After washing in phosphate buffered saline (PBS), sections were incubated overnight at 4°C with a 1/1000 dilution of mouse monoclonal antibody (22-1-1), IgM class, which reacts with human RCAS1 (Medical and Biological Laboratories Co, Nagoya, Japan). A biotinylated rabbit antimouse IgM (Dako, Glostrup, Denmark) was applied to the sections for 30 minutes, followed by an avidin–biotin–peroxidase conjugate (ABC Elite; Vector Laboratories, Burlingame, California, USA) for 30 minutes at room temperature. The immunohistochemical reaction was developed with freshly prepared 3,3′ diaminobenzidine tetrahydrochloride solution (Histofine SAB-PO kit; Nichirei Inc, Tokyo, Japan). The slides were counterstained with haematoxylin, dehydrated through alcohol, and cleared in xylene before mounting. As a negative control, the primary antibody was replaced with mouse IgM. The slides were evaluated microscopically as follows: no staining and supranuclear fine granular staining were judged as normal, but heterogeneous mixed cytoplasmic and cell membrane staining were considered abnormal. Slides with abnormal immunohistochemical findings were then looked at under high power magnification (×400) on an Olympus BH2 microscope (field width, 0.5 mm) and scored into four categories based on the percentage of positively stained cells, as follows: negative, < 5%; weak, 1+ or 5–25%; moderate, 2+ or 25–50%; and strong, 3+ or > 50%.

RT–PCR

mRNA analysis was performed by RT–PCR. A 1 µg sample of total RNA was subjected to RT–PCR using the OneStep RT–PCR kit (Qiagen), according to the manufacturers' instructions. PCR was carried out using 40 cycles of one minute at 94°C, one minute at 60°C, and one minute at 72°C. The primer set designed for human RCAS1 consisted of the forward primer 5′-ACCTTACTGCCCTCCGTCTA-3′ and the reverse primer 5′-CTTCTTCATTAGCCGTTGTG-3′. The resulting RCAS1 RT–PCR products (fragments of 802 bp) were analysed on a 1% agarose gel.

ELISA

The concentration of the RCAS1 antigen was measured in the serum samples from the 60 patients with colorectal cancer and 30 healthy controls by a semiquantitative sandwich ELISA, according to the method described previously.[13] Briefly, after serum samples were pretreated with sialidase (Sigma, St Louis, Missouri, USA), each well of a 96 well plate (Pro-Bind Immunoassay plate; Becton Dickinson, San Diego, California, USA) was coated with monoclonal antibody 22-1-1 at a concentration of 25 µg/ml in PBS, and incubated at room temperature for one hour. After washing with 20mM Tris/HCl buffer three times, 200 µl/well of blocking buffer with 1% bovine serum albumin was added, and incubated at room temperature for one hour. The plate was washed three times and incubated serially with serum samples, biotinylated anti-human RCAS1 IgM monoclonal antibody, and streptavidin–peroxidase conjugate (a 20 000× dilution in blocking buffer). The TMB microwell peroxidase substrate system kit (KPL, Gaithersburg, Maryland, USA) was used for detection, according to the manufacturer's instructions. Antigen concentrations were calculated from absorbance values obtained with the sample and a reference standard. The reference standard was culture fluid from SiSo cells.

Statistical analysis

Statistical analysis was performed by means of the χ^2 test, Fisher's exact test, and ANOVA. A p value < 0.05% was considered significant.

RESULTS

There were three patterns of RCAS1 immunostaining seen in the specimens obtained from patients with colorectal cancer (fig 1). Absence of staining (fig 1A) and a supranuclear fine granular pattern (fig 1B) were seen predominantly in normal tissue and adenomatous polyps. In contrast, heterogeneous mixed patterns, with both the cytoplasm and the cell

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Table 1 Relation between clinicopathological features and immunohistochemical staining of primary colorectal cancer specimens

Variables	Total (n=60)	RCAS1 immune intensity		p Value
		1–2+	3+	
Ages				
<60	29	15	14	
>60	31	9	22	0.113
Sex				
Male	19	6	13	
Female	41	18	23	0.41
Tumour differentiation				
Well	32	17	15	
Moderate/poor	28	7	21	0.026*
T				
I, II	29	14	15	
III, IV	31	10	21	0.292
N				
0	28	16	12	
I, II	32	8	24	0.017*
M				
0	52	22	30	
1	8	2	6	0.457
Stage				
I, II	28	16	12	
III, IV	32	8	24	0.017*

*p<0.05; TNM staging system (T, tumour; N, lymph nodes; M, distant metastasis).

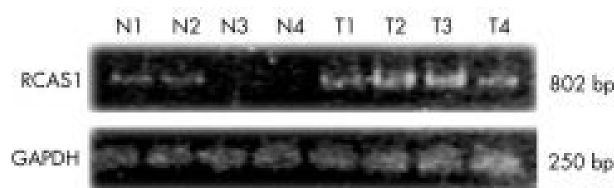

Figure 2 Reverse transcription polymerase chain reaction (RT–PCR) showing representative RCAS1 mRNA expression in colorectal cancer specimens. The upper panel shows RCAS1 PCR products from non-cancerous specimens (N1, N2, N3, and N4) and cancerous specimens (T1, T2, T3, and T4). The lower panel shows glyceraldehyde 3-phosphate dehydrogenase (GAPDH) PCR products (control).

membrane staining (fig 1C,D), were seen in the malignant part of the colorectal specimens. Immunoreactivity for RCAS1 was seen in all the cases under examination. The reaction intensity was grade 2+ (fig 1C) (25–50% reactive area) and 3+ (fig 1D) (> 50% reactive area).

Table 1 shows the demographics of the immunostaining and its association with clinicopathological features. Variables such as age, sex, and depth of invasion did not correlate with immunostaining for RCAS1 ($p > 0.05$). Tumour differentiation grading, lymph node status, and histopathological staging correlated significantly with immunostaining for RCAS1 ($p < 0.05$). Immunoreactivity for RCAS1 was higher in stage III and IV disease (77.8%) than in stage I and II disease (45.8%).

RCAS1 mRNA expression was detected by RT–PCR in tissues isolated from patients with colorectal cancer (fig 2). Figure 2 shows the RCAS1 cDNA products of representative samples taken from four patients. Expression was higher in cancerous tissue (T1–T4) than in non-cancerous tissue (N1–N4) taken from the same patients.

RCAS1 expression was examined in local lymph nodes and metastases to the liver. Eighty two lymph node metastases from 36 patients and eight liver metastases from eight patients were immunostained with the anti-RCAS1 monoclonal antibody. RCAS1 expression was upregulated in the lymph node (fig 3A) and the liver metastasis specimens (fig 3B).

Serum RCAS1 concentrations

Figure 4 and table 2 show the results of the analysis of serum RCAS1 concentrations in healthy donors and in patients with colorectal cancer. The serum RCAS1 concentrations in patients with colorectal cancer were not significantly different from the normal values ($p = 0.25$). According to Akashi *et al*,[15] the cut off value for serum RCAS1 concentrations in patients with colorectal cancer was arbitrarily defined as 22.54 U/ml (two standard deviations greater than the mean in the healthy donors). Therefore, serum RCAS1 concentrations of > 22.54 U/ml were defined as positive and those of < 22.54

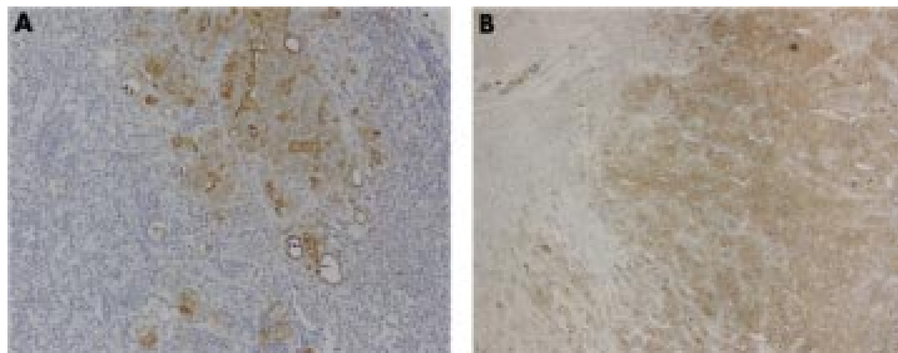

Figure 3 Representative immunohistochemical staining for RCAS1 (original magnification, ×200). (A) RCAS1 expression in lymph node metastases and in (B) liver metastases.

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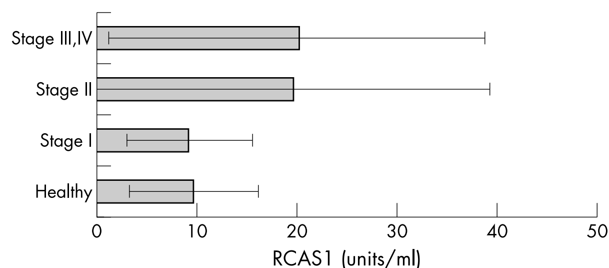

Figure 4 Serum concentrations of RCAS1 (units/ml) as assessed by enzyme linked immunosorbent assay in healthy controls and patients with stage I, II, and III/IV colorectal cancer.

U/ml were defined as negative. Using these criteria, we found that all serum RCAS1 concentrations in healthy donors (n = 30) and stage I patients (n = 10) were negative. Positive serum RCAS1 results were found in 10 of 18 patients with stage II disease and 12 of 32 patients with stage III and IV disease.

DISCUSSION

According to previous studies,[1–3] the carcinogenic process requires the accumulation of a series of genetic alterations in the DNA of tumour tissue. During this progression, abnormal tumour antigens may be expressed, and these might be able to activate the immune system. Many kinds of tumours evade immune surveillance by expressing the fas ligand antigen,[5] but previous studies have shown that the fas ligand is not involved in immune evasion in colorectal cancer.[16] Since the RCAS1 antigen was first identified as a novel tumour antigen by Nakashima *et al*,[6] this group has localised and measured the expression of RCAS1 in human cancers by immunohistochemistry.[6–15] They found that RCAS1 expression correlated with tumour progression in cervical cancer,[7 8] breast cancer,[9] oesophageal cancer,[10] bile duct cancer,[11 12] and lung cancer.[13 14]

> "As suspected, RCAS1 expression was high in all cases of colorectal cancer examined, particularly in high stage cancers"

Our study is the first to explore RCAS1 expression in human colorectal cancer, both at the mRNA level by an RT–PCR technique and at the protein level by immunohistochemistry. As suspected, RCAS1 expression was high in all cases of colorectal cancer examined, particularly in high stage cancers. The expression of RCAS1 was also detected in normal colorectal epithelium and in adenomatous polyps. RCAS1 expression gradually increased with the stage of differentiation of colorectal cancer and it significantly correlated with the stage grouping of this disease. Our results contrasted with most of the other previous reports, which have shown negative immunostaining for RCAS1 in the normal portion of other malignant tissues.[7–13] Studies in patients with cervical cancer,[7] breast cancer,[9] oesophageal squamous cell carcinoma,[10] bile duct cancer,[11 12] and lung cancer[13 14] suggest that the prognosis of RCAS1 positive cases is significantly worse than for negative cases. Thus, it is probable that RCAS1 expression in colorectal cancer could help in the determination of clinical prognosis. However, all of the patients studied here are still alive, so that we cannot determine the overall survival or make any conclusion regarding prognosis. Further prospective studies should be undertaken to compare the overall survival of patients with colorectal cancer and high RCAS1 expression with patients not expressing this antigen. In addition, we found that RCAS1 expression was upregulated in lymph node and liver metastases. To date, the mechanism used by cancer cells to evade immune surveillance in lymph nodes and liver has not been elucidated. The presence of RCAS1 in lymph node and liver metastases may help to define its role in the induction of apoptosis. Serum RCAS1 concentrations in patients with colorectal cancer were not significantly different from normal controls ($p = 0.25$). This is in contrast to pancreatic cancer, where positive serum RCAS1 was seen in most cases (80%). The serum concentration was significantly higher than that seen in patients with chronic pancreatitis, acute pancreatitis, and autoimmune pancreatitis.[15] In our study, positive serum RCAS1 was found in only 10 of 18 patients with stage II colorectal cancer and in 12 of 32 with stage III and IV disease. Differences in the biological features of the tumours and the limited number of serum specimens in our study may account for this. Further investigations on serum RCAS1 concentrations in colon cancer, in addition to other types of cancer, are required.

In conclusion, RCAS1 antigen expression increases gradually during the process of tumorigenesis. This antigen was highly expressed in high stages of colorectal cancer and also in tumour metastases. The upregulation of RCAS1 in lymph nodes indicates that RCAS1 may play a role in immune evasion of colorectal cancer.

Take home messages

- The RCAS1 protein was highly expressed in high stages of colorectal cancer, but only weakly expressed in normal tissues, and RCAS1 mRNA results correlated with the immunohistochemical results
- Positive serum RCAS1 concentrations were found in 47.5% of patients with stage II disease, in 35.5% of those with stage III and IV, but in none of the patients with stage I disease
- All lymph node and liver metastases highly expressed the RCAS1 protein
- Thus, RCAS1 appears to be upregulated in high stages of colorectal cancer so that measuring its expression might be a useful additional criterion for staging this cancer

ACKNOWLEDGEMENT

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Table 2 Serum RCAS1 concentrations in healthy controls and in patients with colorectal cancer

	Serum RCAS1 concentration, by ELISA			
	Healthy (n=30)	Stage I (n=10)	Stage II (n=18)	Stage III–IV (n=32)
Mean (U/ml)	9.70*	9.28*	15.88*	20.06*
SD	6.41	6.27	16.19	18.73
25th–75th centile	6.70–12.70	7.41–11.90	9.60–22.15	13.30–26.82

*Cut off value in colorectal cancer is 22.54 U/ml.
ELISA, enzyme linked immunoassay.

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