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# Detection of autoantibodies against Rabphilin-3A-like protein as a potential biomarker in patient's sera of colorectal cancer

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ABSTRACT

*Background:* Rabphilin-3A-like (RPH3AL) protein functions in the regulation of hormone exocytosis, and mutations in the *RPHA3L* gene have been associated with tumorigenesis in colorectal cancer (CRC). We evaluated the potential use of anti-RPH3AL autoantibodies as a marker for CRC detection.
*Methods:* Sera from 84 patients with CRC and 63 healthy controls were analysed for the presence of RPH3AL autoantibodies with a Western blotting assay.
*Results:* The frequencies of RPH3AL autoantibodies in the early stage, advanced stage and all CRC patients were 64.7%, 78.0% and 72.6%, respectively. These values are significantly higher than the frequency of RPH3AL autoantibodies in healthy controls (15.9%, $P<0.001$). Although the presence of RPH3AL autoantibodies did not correlate with clinical parameters, RPH3AL autoantibodies were found in 69.4% (34/49) of CRC patients who were negative for carcinoembryonic antigen. The value of the area under the receiver operating characteristic curve of RPH3AL autoantibody was 0.84, which suggests that screening for these autoantibodies could potentially be used for CRC diagnosis.
*Conclusion:* Circulating RPH3AL autoantibodies are prevalent in patients with CRC, and detection of these autoantibodies might provide a novel non-invasive approach for CRC diagnosis.


## 1. Introduction

Colorectal cancer (CRC) is one of the most common cancers and impacts public health worldwide [1]. The burden of health care for CRC patients is heavy [2]. The benefits of early screening for CRC include the reduction of both mortality and the cost of cancer treatment [3]. Although several types of screening tests for the early detection of premalignant lesions of the colon and rectum are available, the faecal occult blood test (FOBT) is still the most widely used non-invasive screening option [4]. Nevertheless, CRC screening rates have remained low because FOBT has poor sensitivity and is inconvenient [5]. The discovery of novel biomarkers to assist the early detection of CRC is urgently needed, as it will greatly improve the management of this malignant disease.

The detection of serological biomarkers as cancer screening tools is a superior approach to traditional CRC screening methods because biomarkers can be analysed in convenient and relatively non-invasive ways. The diagnostic values of various kinds of markers (including proteins, carbohydrate antigens, cytokines, antibodies and DNA markers) for CRC detection have been evaluated [6–11]. However, the low sensitivity of detection of most of these blood biomarkers is not adequate for clinical applications [12]. The carcinoembryonic antigen (CEA) is the first blood marker found to be associated with CRC [13], and the overall sensitivity of this marker for CRC detection varies between 43% and 69% [8].

Autoantibodies that target tumor-associated antigens (TAAs) are another type of potential biomarkers for early cancer diagnosis [14]. Aberrant cellular proteins that change in structure, expression level or cellular location appear during tumorigenesis are thought to be released into the blood and may induce a humoral immune response. Cancer surveillance mechanisms function to detect and remove precancerous lesions during the early events of carcinogenesis. Autoantibodies, the product of cancer immunity, may be generated well before the release of cellular antigens reaches a detectable level

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and can be regarded as signals that indicate the presence of the tumour [15–17].

Several hypotheses have been proposed to explain how these autoantibodies are generated during tumour progression, including host-immune reactions to TAAs, antigenic stimulation resulting in genetic mutations of tumour cells or immune dysregulation induced by the neoplastic process [15,16]; however, the detailed mechanisms of this process are still not completely understood. Nevertheless, numerous autoantibodies present in CRC have been reported by our group and others, including sFasL [9], annexin A [18], GA-733-2 [19,20], p53 [21], MUC5AC [22] and HER2-neu [23]. However, the clinical use of these autoantibodies as serological markers for CRC detection is still impracticable because the sensitivities of detection are less than 30% [12].

Rabphilin-3A-like (RPH3AL) is a protein that consists of 302 amino acids (38 kDa) and has 77.9% similarity to the Rabphilin-3A protein. RPH3AL is expressed abundantly in endocrine cells and has been reported to be involved in the regulation of hormone exocytosis through its interactions with the cytoskeleton [24,25]. Although the precise biological function of RPH3AL in CRC carcinogenesis is still unknown, mutations in the *RPHA3L* gene, which is located at the *17p13.3* locus, have been suggested to be associated with tumour development in CRC [26,27]. We have previously reported the identification of several aberrantly expressed proteins in CRC tissue using a multiplexed label-free quantitation strategy with integrates an efficient gel-assisted digestion protocol, high performance liquid chromatography–quadrupole mass spectrometry (LC–MS/MS) technologies [28]. Among these aberrantly expressed proteins, overexpression of RPH3AL protein was observed in cancerous tissue compared with the corresponding normal tissue. Based upon the evidence mentioned above, we speculated that elevated RPH3AL protein levels might elicit the induction of a humoral response and that the presence of autoantibodies against RPH3AL could serve as a marker for cancer diagnosis. To extend our previous findings, the aims of this study were to investigate whether autoantibodies against RPH3AL could be detected in the sera of patients with CRC and to explore whether the detection of these autoantibodies could have a clinical application as a diagnostic biomarker for CRC detection.

**Table 1**
Correlation between anti-RPH3AL autoantibodies and clinicopathological characteristics of CRC patients.

| Characteristics | Number tested | Anti-RPH3AL autoantibodies | | *p* Value |
|---|---|---|---|---|
| | | Patient number (%) | | |
| | | Positive | Negative | |
| Age (y) | | | | NS |
| <60 | 30 | 23 (77.4) | 7 (22.6) | |
| ≧60 | 54 | 38 (70.9) | 16 (29.1) | |
| Gender | | | | NS |
| Male | 51 | 36 (71.2) | 15 (28.8) | |
| Female | 33 | 25 (76.5) | 8 (23.5) | |
| Histological grade (differentiation) | | | | |
| Well | 10 | 9 (90.0) | 1 (10.0) | NS |
| Moderate | 66 | 47 (72.1) | 19 (27.9) | |
| Poorly | 8 | 5 (62.5) | 3 (37.5) | |
| Dukes' classification | | | | |
| Early stage (A and B) | 34 | 22 (64.7) | 12 (35.3) | NS |
| Advanced stage (C and D) | 50 | 39 (78.0) | 11 (22.0) | |
| *TNM classification* | | | | |
| Tumour status | | | | NS |
| T1, T2 | 15 | 11 (73.3) | 4 (26.7) | |
| T3, T4 | 69 | 50 (72.5) | 19 (27.5) | |
| Lymph nodes metastasis | | | | NS |
| Negative (N0) | 34 | 22 (64.7) | 12 (35.3) | |
| Positive (N1, N2, and N3) | 50 | 39 (78.0) | 11 (22.0) | |
| Metastasis status | | | | NS |
| Negative (M0) | 69 | 49 (71.0) | 20 (29.0) | |
| Positive (M1) | 15 | 12 (80.0) | 3 (20.0) | |
| Tumour site | | | | NS |
| Right-side (cecum, ascending) colon | 13 | 10 (76.9) | 3 (23.1) | |
| Left-side (transverse, descending, sigmoid) colon | 30 | 20 (66.7) | 10 (33.3) | |
| Rectum | 41 | 31 (75.6) | 10 (24.4) | |
| CEA concentration (ng/ml) | | | | |
| ≧5 | 35 | 27 (77.1) | 8 (22.9) | NS |
| <5 | 49 | 34 (69.4) | 15 (30.6) | |

## 2. Materials and methods

### 2.1. Study subjects and blood samples collection

All CRC patients were diagnosed by pathological examination of tumour biopsies, and their sera were obtained at the time of diagnosis prior to receiving chemotherapy treatment. Based on Dukes' tumour stage criteria, the numbers of patients with CRC diagnosed in the early (Dukes' stages A and B) and advanced (Dukes' stages C and D) stages were 34 and 50, respectively. Sera samples from all non-tumour controls were obtained during annual health examinations and met the following criteria: no family or personal history of CRC, no obvious evidence of other types of malignancy and no inflammatory bowel conditions or autoimmune diseases. The control group consisted of 41 males and 22 females with a mean age 61.8 (range from 35 to 83) y. There was no significant difference in sex ($P=0.588$) and age ($P=0.174$) between CRC and control group. All sera were divided into aliquots and stored at −80 °C until analysis. Clinical parameters and CEA values of the study subjects were obtained from individual medical records and are shown in Table 1. This protocol was approved by the Human Clinical Trial Committee of Chang-Gung Memorial Hospital, and all study subjects gave informed consent.

### 2.2. Cloning and expression of recombinant RPH3AL protein

The full-length cDNA (855 base pairs) of the *RPH3AL* gene was purified and amplified by reverse transcription-PCR using total RNA isolated from Colo205 cells (ATCC no. CCL-222). A *RPH3AL* gene-specific primer set (forward primer, 5′-TAGGATCCCATCTTCGGCAGCGGGAAT-3′; reverse primer, 5′-ATCTCGAGGGCTCGTCCAGTTGTGTCTTTTAC-3′) was used. The PCR product was inserted into the pET30b expression vector (Novagen, EMD Chemicals Inc., Darmstadt, Germany) to produce a fusion protein with six histidine residues at its $NH_2$-terminus. The recombinant vector was then transformed into *Escherichia coli* Rosetta™ DE3pLysS competent cells (Novagen, EMD Chemicals Inc., Darmstadt, Germany) for recombinant RPH3AL protein expression. After isopropyl-β-D-1-thiogalactopyranoside induction for 3 h, bacteria were collected by centrifugation (12,000 rpm for 20 min) and disrupted in BugBuster® Protein Extraction Reagent (Novagen, EMD Chemicals Inc., Darmstadt, Germany) at 4 °C. The recombinant RPH3AL (rRPH3AL) protein was further purified using a HiTrap™ Chelating HP column (GE Healthcare, Munich, Germany) and then examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting assay using a rabbit anti-human RPH3AL polyclonal antibody (15297-1-AP, ProteinTech Group, Inc., Chicago, USA).

### 2.3. Identification the rRPH3AL protein by MALDI-TOF mass spectrometry analysis

The rRPH3AL protein was digested with sequencing grade modified porcine trypsin (20 mg/ml; Promega, Madison, WI) overnight at 37 °C. The digested peptides were extracted with acetonitrile containing 0.5% (v/v) trifluoroacetic acid and spotted onto an MTP AnchorChip®600/384 TF (Bruker Daltonics, Bremen, Germany). The samples were then analysed on an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The obtained peptide mass fingerprint spectral

data were then fed into the MASCOT search engine (ver 2.1, Matrix Science, London, UK) and searched against the National Center for Biotechnology nonredundant (NCBInr) protein sequence database.

### 2.4. Detection of RPH3AL autoantibodies in sera by Western blotting

The presence of circulating autoantibodies against RPH3AL was detected by Western blot assay. Briefly, the purified rRPH3AL was electrophoresed on an SDS-PAGE gel, transferred onto a polyvinylidene fluoride (PVDF) membrane and then blocked overnight at 4 °C in blocking buffer (25 mmol/l Tris, 190 mmol/l NaCl, 0.5% (v/v) Tween 20, pH 7.5 and 5% skim milk). The membrane was then cut into strips, which were incubated separately with individual sera samples (1:500 dilution) or anti-$His_6$ mouse monoclonal antibody (Calbiochem, Darmstadt, Germany) for 1 h at 37 °C with agitation. After washing the strips six times with washing buffer (PBS with 0.1% Tween-20), the strips were incubated with horseradish peroxidase-conjugated anti-human IgG (1:2500 dilution; Jackson ImmunoResearch Inc., West Grove, PA) or horseradish peroxidase-conjugated bovine anti-mouse IgG (1:5000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h at 37 °C. Immunoreactive bands were detected using the 3,5,5 tetramethylbenzidine (TMB) membrane peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) according to the manufacturer's instructions. All plasma samples were analysed in triplicate, and the mean values were calculated. Two samples with known expression levels (positive and negative controls) were included in each batch to verify the reproducibility of the assays. The reactive protein bands were visualised by incubating with enhanced chemiluminescence western reagents and photographed with Kodak Biomax light films. Immunoblot images were acquired with Imagemaster (Amersham Pharmacia Biotech, Piscataway, NJ) and analysed with the Multi Gauge ver 2.0 software (Fuji PhotoFilm, Tokyo, Japan). The intensities of the reactive bands are presented as arbitrary units (AU).

### 2.5. Statistical analysis

Statistical analysis was performed using the SPSS for Windows programme ver 12.0 (SPSS Inc., Chicago, IL). The differences in the serum levels of RPH3AL autoantibodies between groups were evaluated by the non-parametric Mann–Whitney test. A comparison of the frequency of antibodies between groups was performed using the chi-square test or, when the sample size was small, a Fisher's exact test. The discriminative power was assessed using the area under the receiver operating characteristic (ROC) curve (AUC), which was constructed by plotting the sensitivity *versus* (1-specificity). For each statistical analysis, a $P<0.05$ was considered significant.

## 3. Results

### 3.1. Expression and purification of the recombinant RPH3AL protein

The constructed RPH3AL-pET 30b vector, containing the 855 bp full-length *RPH3AL* gene, was used to transform competent *E. coli* Rosetta cells for recombinant RPH3AL protein expression (Fig. 1A). The 40.6-kDa recombinant protein was purified and confirmed by Western blot analysis with an anti-RPH3AL antibody (Fig. 1B) and by Matrix Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF) Mass Spectrometry analysis (Supplementary Table 1).

### 3.2. The prevalence of sera autoantibodies against RPH3AL

In total, 147 study subjects, including 84 CRC patients and 63 non-tumour controls, were tested for the presence of RPH3AL autoantibodies by Western blot analysis. The sera autoantibody level, as shown in Fig. 2, was significantly higher in the CRC group (median: 233; 25% and 75% percentiles: 149 and 321.5) when compared with that in the control group (median: 67; 25% and 75% percentiles: 36 and 137) ($P<0.001$). Furthermore, when the patients were divided into early stage and advanced stage CRC according to the biopsy data, the autoantibody levels of early stage (median: 236; 25% and 75% percentiles: 123 and 391) and advanced stage (median: 232; 25% and 75% percentiles: 178 and 303) patients were also significantly higher compared with that in the control group ($P<0.001$). However, the difference between early stage and advanced stage CRC was not significant ($P=0.964$).

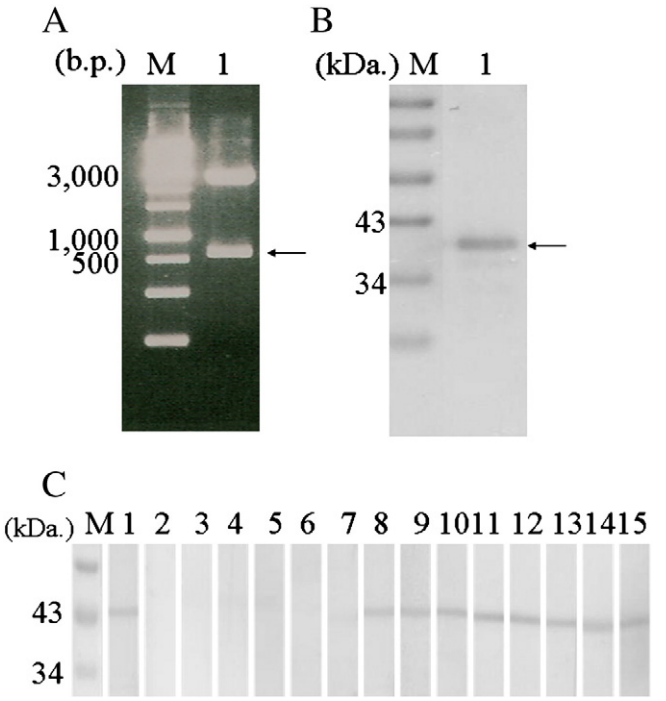


**Fig. 1.** (A) Cloning of the human *RPH3AL* gene. Lane M, nucleic acid markers. Lane 1, the full-length human *RPH3AL* gene (855 bp) is shown. (B) Identification of the recombinant RPH3AL protein by Western blotting assay. Lane M, protein molecular weight markers. Lane 1, the 40.6-kDa recombinant RPH3AL protein was confirmed by reaction with the specific anti-RPH3AL antibody. (C) Representative results of detection of sera autoantibodies to RPH3AL by Western blotting assay. Lane M, protein molecular weight markers. Lane 1, a positive control that shows the presence of autoantibodies to RPH3AL. Lane 2, a negative control that shows the absence of autoantibodies to RPH3AL. Lanes 3–7, five representative sera samples of non-tumour controls. Lanes 8–15, eight representative sera samples of patients with colorectal cancer.

### 3.3. The diagnostic value of anti-rRPH3AL autoantibodies and CEA level detection

With the optimum cut-off value of 166 AU, the frequencies of a positive reaction to the rRPH3AL in the CRC and control groups were 72.6% (61/84) and 15.9% (10/63), respectively (Table 2). The sensitivity, specificity, positive predictive value and negative predictive value for the RPH3AL autoantibody detection for CRC diagnosis were 72.6%, 84.1%, 85.9% and 69.7%, respectively. To evaluate the diagnostic value of measuring anti-rRPH3AL autoantibodies and CEA levels for CRC diagnosis, a ROC curve was generated (Fig. 3). The area under the ROC curve (AUC) were 0.84 and 0.67 for the RPH3AL autoantibody and CEA level analyses, respectively. Furthermore, as shown in Fig. 4, when 5 ng/ml is used as the cut-off for the CEA measurement, the frequencies of RPH3AL autoantibodies were 69.4% (34/49) in CEA-negative CRC patients and 77.1% (27/35) in CEA-positive CRC patients. The positive rates of RPH3AL autoantibody detection and CEA level measurement for CRC diagnosis are shown in

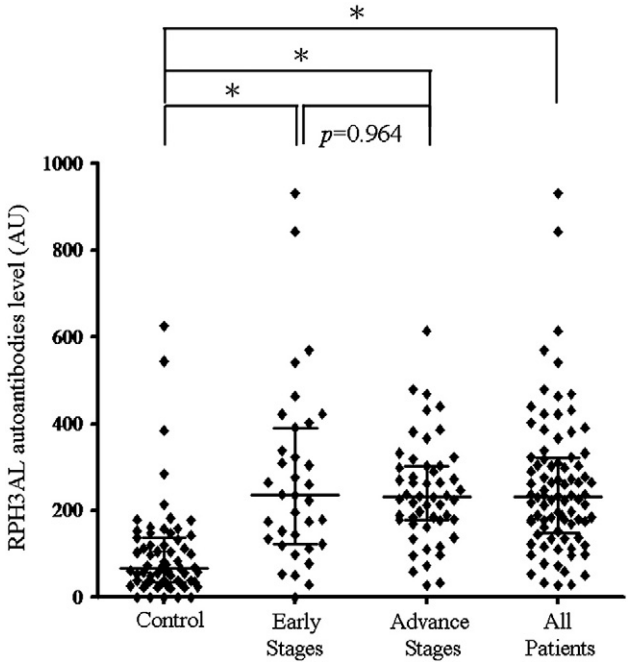


**Fig. 2.** Levels of RPH3AL autoantibodies in different study cohorts. The distribution of RPH3AL autoantibody levels is expressed as arbitrary unit (AU) obtained from Western blotting assays. The median and the 25% and 75% percentiles of the autoantibody levels are shown in each group (horizontal bar); Early stages: Dukes' stage A and B; Advanced stages: Dukes' stage C and D; *, $P<0.001$, using the Mann–Whitney test.

Table 2. The positive rates for detection of RPH3AL autoantibodies in early stages, advanced stages and all CRC patients were 64.7%, 78.0% and 72.6%, respectively. The positive rate for early stage CRC detection increased to 82.4% if the measurements of RPH3AL autoantibodies and CEA levels were combined.

### *3.4. Relationship between RPH3AL autoantibody seropositivity and clinicopathological parameters of patients with CRC*

To investigate the correlation between RPH3AL autoantibody detection and the clinicopathological characteristics of CRC, we analysed the clinical parameters of age, gender, tissue status, clinical status (Dukes' classification), tumour stage, lymph node metastasis and distal metastasis. The results revealed that no significant differences were observed between the incidence of RPH3AL autoantibody seropositivity and the clinicopathological parameters examined in the present study (Table 1).

**Table 2**
The positive rates of anti-RPH3AL autoantibodies and CEA level measurements for CRC diagnosis.

| Dukes' stage | Number tested | Anti-RPH3A | CEA[a] | Anti-RPH3A or CEA | *P* value |
|---|---|---|---|---|---|
| A | 5 | 3 (60.0%) | 0 (0.0%) | | |
| B | 29 | 19 (65.5%) | 11 (37.9%) | | |
| Early stages (A+B) | 34 | 22 (64.7%) | 11 (32.4%) | 28 (82.4%)[b] | <0.001 |
| C | 34 | 27 (79.4%) | 12 (35.3%) | | |
| D | 16 | 12 (75.0%) | 10 (62.5%) | | |
| Advanced stages (C+D) | 50 | 39 (78.0%) | 22 (44.0%) | 41 (82.0%)[b] | <0.001 |
| All stages (A+B+C+D) | 84 | 61 (72.6%) | 33 (39.3%) | 69 (82.1%)[b] | <0.001 |

Anti-RPH3A: autoantibodies against Rabphilin-3A-like protein.
CEA: carcinoembryonic antigen.
[a] CEA level ≧5 ng/ml.
[b] Compare with CEA measurement alone.

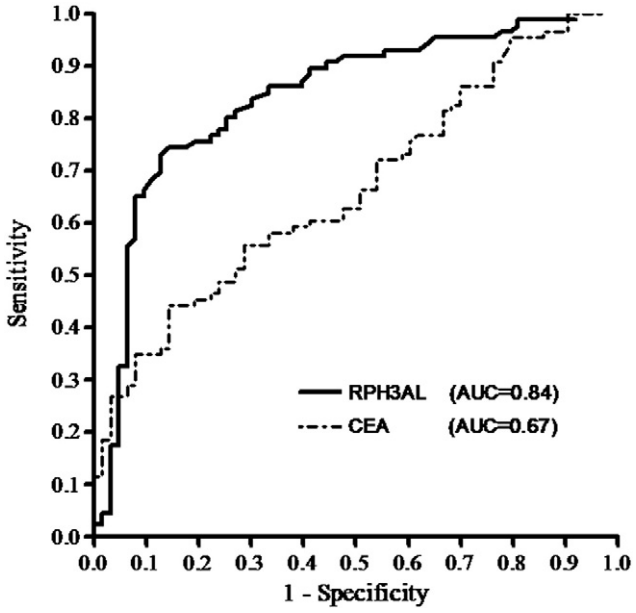


**Fig. 3.** Receiver operating characteristic curves for CEA and RPH3AL autoantibodies for colorectal cancer detection. Sera concentrations of CEA and RPH3AL autoantibody levels among 84 colorectal cancer patients and 63 non-tumour controls were determined by Western blotting assay. The diagnostic potentials of CEA and RPH3AL autoantibodies were assessed by ROC curves. The areas under the curve (AUC) values were 0.67 and 0.84, respectively.

## 4. Discussion

We demonstrated the presence of anti-RPH3AL autoantibodies in patients with CRC and showed that the RPH3AL protein elicits humoral immune responses in CRC patients, suggesting that the presence of anti-rRPH3AL autoantibodies could be a potential biomarker for early detection of CRC. Identification of differentially expressed proteins between normal and malignant tissues could provide clues to potential novel biomarkers for CRC diagnosis. Thus, systematic investigation of the protein expression profiles of tumour cells is critical and might provide insight into the mechanisms of carcinogenesis. In addition, because of the heterogenic nature of CRC

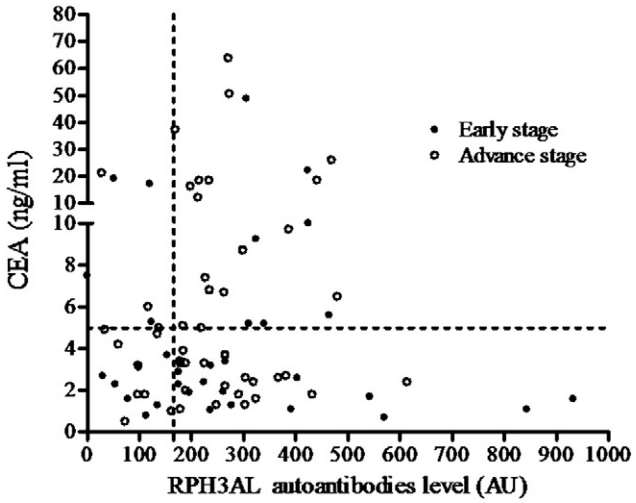


**Fig. 4.** Analysis of RPH3AL autoantibody levels and CEA concentrations in sera from patients with CRC ($n=86$). The vertical and horizontal dashed lines indicate cut-off values for RPH3AL seropositivity (arbitrary units = 166) and CEA concentrations (5 ng/ml), respectively. ●, Patients with early stage cancer (Dukes' stage A and B); ○, Patients with advanced stage cancer (Dukes' stage C and D).

and the great diversity of immunoreactivity of patients with the same type of cancer, it is crucial to discover new biomarkers to be included in a panel for CRC detection by different approaches. To explore novel biomarkers for CRC diagnosis, our group has quantitatively analysed the expression profiles of membrane proteins in paired CRC tissue samples by different experimental technologies [28,29]. We have identified several proteins that show discrepancies in expression between normal and malignant tissues, including the RPH3AL protein, which was found to be overexpressed in 82.8% (24/29) of the tissue samples tested (data not shown).

Until now, few reports have explored the biological function of RPH3AL. Some studies suggested that this protein plays roles in intracellular vesicle transport and calcium-dependent neurotransmitter exocytosis [30,31]. One previous study analysed the coding region of the RPH3AL gene for genetic aberrations in 50 primary colorectal cancer patients; this study identified several somatic *RPH3AL* mutations and suggested that it may play a role as a tumour suppressor gene in a subset of CRC patients [27]. In another study, the authors analysed the incidence of a single nucleotide polymorphism at the −25 position in the 5′ untranslated region in a retrospectively collected CRC sample cohort. They reported that CRC patients who exhibited homozygosity for the A or C alleles had a significantly increased risk of CRC recurrence and mortality [26].

The immune system has the capability to sense aberrant structures, locations, quantities, and functions of cellular proteins involved in carcinogenesis, and it produce autoantibodies in response to the presence of these aberrant proteins [14,32]. Although the mechanism by which modifications of proteins trigger the immune response is still not entirely clear, autoantibodies have been described in various cancers, including anti-survivin and anti-livin in lung and breast cancers, anti-thyroglobulin in thyroid cancers, anti-CENP-B and anti-SS-B in breast cancers, and anti-p53 in multiple types of cancer [23,33–39]. Mutations in the tumour suppressor *p53* have been associated with many types of human cancer, and autoantibodies to p53 in the sera of cancer patients have also been reported [34]. These autoantibodies not only serve as reporters of malignant transformation but could also be used as biomarkers for early detection [40,41]. Although the association between *RPH3AL* gene mutations and the production of its corresponding autoantibodies is still unclear, our results provide evidence that autoantibodies against RPH3AL exist in the sera of CRC patients and possess the potential to be a diagnostic marker for CRC detection.

The measurement of CEA levels is commonly clinically used to assess the prognosis of CRC patients rather than for the early detection of colorectal cancer. However, from a research point of view, it is worthwhile to explore the potential diagnostic usefulness of the combination of the CEA and anti-RPH3AL autoantibody assays, which has been described in many previous papers [42–44]. Although several CRC-associated autoantibodies, such as anti-HER-2/neu, anti-Calnuc, anti-p16, anti-p53, anti-mucin 5AC, anti-GA733-2, anti-sFasL and anti-annexin A, have been identified, clinical application of the detection of these autoantibodies has been restrained by low sensitivity [9,18,20,22,23,45,46]. For example, the seropositive rate for the p53 autoantibody in sera of CRC patients varied between 13 and 32% [12,34]. Intriguingly, the discovery of the anti-RPH3AL autoantibody as a serological CRC marker is extremely valuable because the overall seropositive rate of 72.6% for CRC detection was highly significant by comparison. For screening purposes, it is also important to discuss the performance of the combination of anti-RPH3AL antibody and CEA level analyses as a CRC biomarker. Intriguingly, in comparison to CEA, the incidence of RPH3AL autoantibodies was remarkably higher in patients in the early stages of CRC, which might imply that it is diagnostically valuable for the early detection of CRC. Furthermore, our results showed that combining these two assays could elevate the detection rate of early stage CRC to 82.4% (Table 2). Similarly, it has been reported that combining serum CEA and metallopeptidase inhibitor 1 tests increases the detection frequency to 66% among patients with early stage CRC [42]. Our findings suggest that the RPH3AL autoantibody may serve as a candidate marker when combined with other serological markers to improve the detection of early stage CRC, particularly in individuals with low CEA levels.

The potential clinical application of RPH3AL autoantibody detection has also been examined thoroughly in the present study. Our results showed that detection of autoantibodies against RPH3AL did not significantly correlate with the clinical parameters assessed in the present study (Table 1). We found a trend of elevated RPH3AL autoantibody levels in patients with advanced stage cancer, metastasis status and higher CEA concentrations, but the differences were not significant. We also found a trend towards a lower survival rate in patients who were positive for circulating RPH3AL autoantibodies, but this trend was also not significant (Kaplan–Meier test; $p = 0.23$). The mean survival periods during the 24-month follow-up period were 19.3 and 21.8 months in patients with or without RPH3AL autoantibodies, respectively (data not shown). These results are similar to a previous report that showed no association between serum anti-p53 antibodies and other clinical parameters [47]. More studies are needed to explore the exact roles of RPH3AL autoantibodies in the tumorigenesis of CRC.

The relationship between circulating autoantibodies and clinicopathological parameters is controversial [47]. In this study, no significant difference was found when comparing the RPH3AL autoantibodies positive rate of Dukes A stage patients with that in patients with advanced stage (Dukes B, C and D) ($P = 0.61$, Fisher's exact test). The phenomenon that autoantibodies against RPH3AL are present in patients with early stage CRC but are not correlated with tumour progression may be explained in part by the principles of tumour immunology. Autoantibodies against aberrant tumor-associated antigens could be produced several months to years before the manifestation of clinical signs of malignance and be persistently present in all stages of tumorigenesis. On the contrary, the production of autoantibodies also may decrease due to immunosuppression during the course of tumour progression [14]. Hence, a prospective study with a large cohort of samples, including patients with different types of cancer or inflammatory and autoimmune diseases and healthy subjects, is needed to validate the use of RPH3AL autoantibodies for clinical applications in the future.

In conclusion, we report that autoantibodies against the RPH3AL protein are present in sera from patients with CRC at significantly higher rates compared with sera from healthy controls and from malignant patients who are negative for the CEA marker. Our results suggest that RPH3AL autoantibodies have potential clinical applications as a novel biomarker when used as a complementary test for CRC detection.

Supplementary materials related to this article can be found online at doi: 10.1016/j.cca.2011.04.020.

*List of abbreviations*

| | |
|---|---|
| RPH3AL | Rabphilin-3A-like |
| CRC | colorectal cancer |
| FOBT | faecal occult blood test |
| TAA | tumor-associated antigen |
| PVDF | polyvinylidene fluoride |
| TMB | 3,5,5 tetramethylbenzidine |
| AU | arbitrary units |

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