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Identification of Rho GTPase Activating Protein 6 Isoform 1 Variant as a New Molecular Marker in Human Colorectal Tumors

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Abstract The early diagnosis of colorectal cancer (CRC) is important because it is one of the most readily curable of all cancers, if detected early. However, the sensitivity of current markers is low. Immunostaining intensity for the monoclonal antibody Hb3 in CRC cell lines and tissues was stronger than in controls. Interestingly, this was associated with a low level of tumor differentiation. We used Hb3-coupled affinity chromatography to search for a corresponding Hb3 antigen as a candidate biomarker for early detection, and identified a Rho GTPase activating protein 6 (RhoGAP6) isoform 1 variant as an Hb3 antigen by mass spectrometry. Using reverse transcription polymerase chain reaction and western blot analysis, we confirmed that the expression levels of this variant were elevated in aberrant cells and tissues. Thus, the RhoGAP6 isoform 1 variant might serve as a biomarker for the development and progression of CRC.

Keywords RhoGAP6 isform 1 variant · Colorectal cancer · Mass spectrometry · Biomarker · Monoclonal antibody

Abbreviations

CRC	Colorectal cancer
RhoGAP6	Rho GTPase activating protein 6
mAb	monoclonal antibody
MALDI-TOF MS	Matrix assisted laser desorption ionization time-of-flight mass spectrometry

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in the world. It is estimated that 783,000 new cases are diagnosed each year; a number that has increased rapidly since 1975 [1]. CRC is the third most common form of cancer and the second leading cause of cancer-related mortality in the Western world, and the incidence of CRC in China has also increased rapidly during the past few decades [2].

Despite curative surgery, nearly 40% patients with CRC experience disease relapse, 20% develop liver metastases and 8.3% develop pulmonary metastases [3]. The early diagnosis of CRC is of paramount importance as it is one of the most readily curable of all cancers if detected early. Understanding the genes and pathways that cause CRC will contribute to better surveillance and early diagnosis, and thus help reduce cancer morbidity and mortality [4]. Early diagnosis is fundamental in reducing morbidity and mortality, as patients diagnosed at early stages demonstrate increased long-term survival. Therefore, candidate biomarkers are needed for early detection and prediction of this cancer.

Some genetic markers might play a role in the prognosis for patients with CRC. For example, epidermal growth factor receptor (EGFR) expression has been studied in relation to prognosis, but results are inconsistent and an independence from tumor stage has not been proven. Thus, a clear relationship between EGFR expression and

CRC prognosis has not been established [5–7]. Serum markers such as carcinoembryonic antigen and CA19-9 have been investigated in patients with CRC, but their sensitivity is so low that their use is limited to surveillance after surgery [8]. Furthermore, current treatment plans might fail to discriminate the biological behavior of the tumor, and selection of the most beneficial treatment regimen is still hindered by a lack of validated predictive and prognostic markers [9]. Clearly, the identification of molecular predictive and prognostic markers for CRC is critical for decision making regarding when to treat, which drugs to use, how much to apply and what combination to use. The development and validation of predictive and prognostic markers will enable oncologists to select patients more effectively for the most successful and least toxic treatment strategies, based on the molecular profile of both the patient and their tumor.

In previous studies, monoclonal antibody (mAb) Hb3, a murine IgM-type antibody constructed in our laboratory, could mediate complement dependent cytotoxicity (CDC) against human CRC cells and was able to kill CRC cells when coupled with the ricin A chain (RTA). However, normal cells did not suffer damage [10–12]. The positive rate of radioimmunoimaging with ^{131}I labeled Hb3 in a nude mouse model of colorectal cancer was 92% (13/14) [13]. Both intact Hb3 and its F (ab′) fragment have some practical value in radioimmunoimaging of CRC [14]. Moreover, using immunocytochemistry and immunohistochemistry for Hb3 in tumor tissues and cells could help in identifying Hb3 antigen as a prospective marker.

Materials and Methods

Cell Lines and Culture Conditions

Human umbilical vein endothelial cells (HUVEC) and the CRC cell lines HCT-116, HT-29, SW480 and LS1747 were cultured in our laboratory. All the cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Proliferating confluent human cancer cells were harvested and the culture supernatants were cleared by centrifugation.

Human CRC Specimens

A total of 150 formalin-fixed paraffin-embedded tumor and normal adjacent tissue sections were obtained from the Department of Pathology in the First Hospital of Changsha, P. R. China. Specimens were diagnosed histopathologically and staged according to the International Union Against Cancer TNM classification system. Informed consent was obtained from all of the patients.

Immunocytochemistry

CRC and HUVEC cell lines grown on coverslips overnight were fixed for 10 min in a mixture of cold methanol and acetone (1:1 *v*/*v*) at 4°C and the subsequent immunohistochemistry steps were performed as below.

Immunocytochemistry

Paraffin wax sections were deparaffinized with xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide at room temperature (RT) for 10 min. Nonspecific binding was blocked with phosphate buffered saline with Tween 20 (PBST) containing 10% goat serum for 2 h at RT. Hb3 mAb was added to each slide and incubated at 4°C overnight. Following three washes, slides were incubated with EnVision (Dako, Glostrup, Denmark) for 40 min at RT. Diaminobenzidine was used as the chromogen. Sections were counterstained with hematoxylin, dehydrated and mounted. Evaluation of immunohistochemical slides was done using a Nikon Eclipse E800 microscope at 100 × magnification. Samples were evaluated under light microscopy independently by two pathologists without prior knowledge of the patients' clinical data. The intensity of the staining was scored on a scale of 0 to 3+ where 0, 1, 2 and 3 represented no staining and weak, moderate or strong staining, respectively.

mAb Hb3 and Crude CRC Antigen Preparation

The mAb Hb3 was prepared using an Hb3 hybridoma cell line to inoculate BALB/c mice. The extracted ascites were first purified using ammonium sulfate and were then applied to Sephacryl S-300 columns to collect the peak of antibody activity, which was identified using an enzyme linked immunosorbent assay (ELISA; see below). The crude antigen was extracted from human CRC HCT-116 cells using ice-cold mono-detergent lysis buffer (50 mM Tris pH 8.0, 1% Triton-X-100, 1 mM EDTA, 150 mM NaCl, 1 μg/mL aprotinin, 1 μg/μl PMSF).

Affinity Chromatography

After mAb Hb3 had been coupled to CNBr-activated Sepharose 4B (GE Healthcare) in a column according to the manufacturer's protocol, an equal volume of crude antigen was added to the column. The mixture was incubated at RT for 1 h and then at 4°C overnight. The unbound antigen was washed out using phosphate-buffered saline (PBS) and then the bound antigen was eluted with 0.1 M glycine (pH 2). The collected elution peak of antigen was neutralized by adding 0.2 M PBS (pH 8.6).

Sandwich ELISA

High-binding polystyrene plates were coated overnight at 4°C with 4 μg/ml mAb Hb3. After blocking with 50 mg/ml fat-free milk in PBS for 2 h at RT and three washes with PBST, the purified colon antigen at different concentrations was added at 50 μl/well, incubated for 2 h, and 50 μl of horseradish peroxidase (HRP)-labeled Hb3 was applied to each well. The plates were incubated for 3 h at RT with shaking. All samples were analyzed in duplicate and the absorbance was measured at 405 nm on a microplate reader. The results were used to construct a standard curve of antigen concentration and the isolated 'A405-nm antibody'.

Dot-blotting

Spots of 3 μl and 5 μl of purified CRC antigen were placed onto nitrocellulose (NC) membranes. The control samples were bovine serum albumin (BSA), also at 3 μl and 5 μl. After the membrane had been dried, nonspecific sites were blocked by soaking in 5% BSA in phosphate buffered saline (PBS) for 1 h at RT. Membranes were incubated with Hb3 dissolved in PBS overnight at 4°C and washed three times with PBST. They were then incubated with HRP-conjugated goat anti-mouse IgG/IgM (Sigma-Aldrich, St Louis, MO, USA) for 1 h at RT. Membranes were washed three times with PBST then once with PBS (5 min each) then incubated with enhanced chemiluminescence (ECL) reagent (KKPL, Inc., Gaithersburg, MD, USA) and exposed to X-ray film in the dark. The membrane was developed in 0.01 M PBS containing 0.05% 3',3-diaminobenzidine (DAB) and 0.01% hydrogen peroxide (H_2O_2).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blotting

Purified CRC antigen in the cold lysate buffer (50 mmol/L Tris-Cl pH 8.0, 150 mmol/L NaCl, 10 mmol/L Triton X-100, 10 mmol/L PMSF) was electrophoresed on 10% gradient SDS-polyacrylamide gels in the presence of β-mercaptoethanol. The proteins were transblotted onto a NC membrane, blocked with 50 mg/mL fat-free milk and

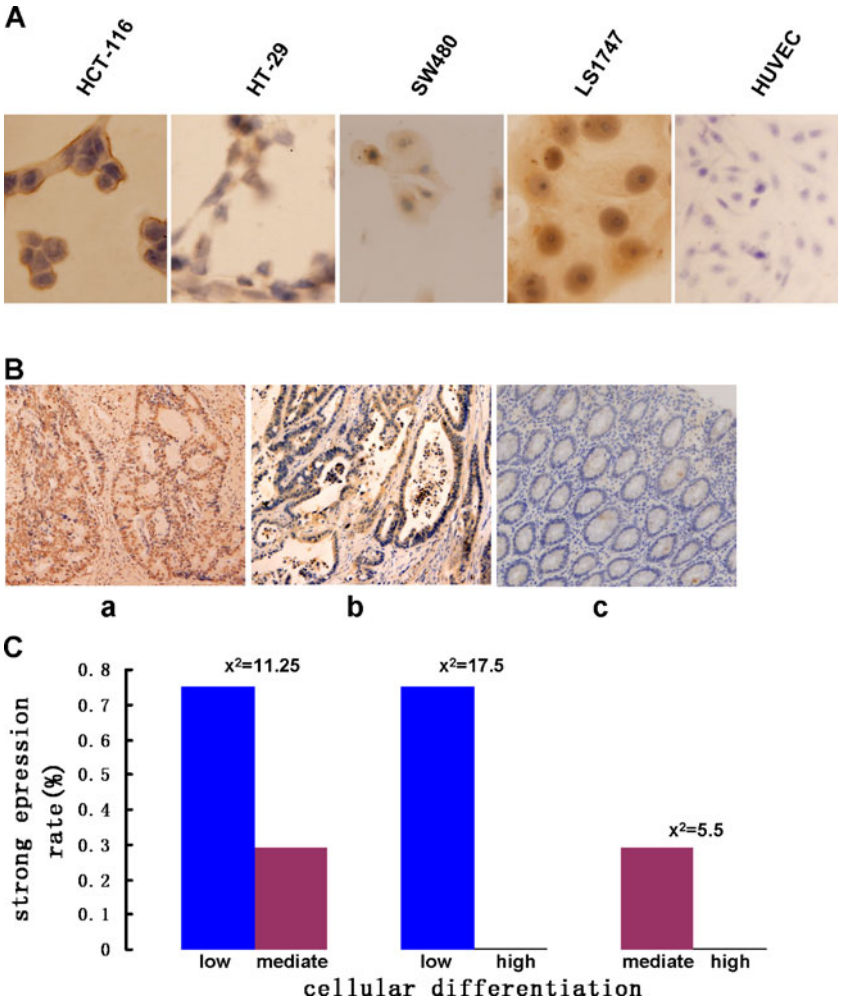

Fig. 1 Immunocytochemical and immunohistochemical staining for Hb3. **a** The intensity of immunostaining Hb3 in colorectal cancer (CRC) cell lines (HCT-116, HT-29, SW480, LS1747) was strong whereas it was weak in human umbilical vein endothelial cells (HUVECs). Forty percent of CRC cells expressed moderate grade 2 staining (**b**-*b*) and 32% of the tumors expressed the highest grade of staining (grade 3) (**b**-*a*), whereas the normal tissues were unstained (**b**-*c*). **c** The expression of Hb3 antigen was strong (grade 3) in 14 of 20 low differentiation samples, but grade 3 staining was seen in only 10 of 40 tissues with moderate differentiation and no such staining was seen in highly differentiated samples. (magnification in **a**, 200×; in **b**, 100×)

Table 1 Characteristics in 75 patients with colorectal cancer and Rho GAP6 isform 1 variant expression

	Number of patients	Rho GAP6 isoform 1 variant expression	χ^2
		0–1–2 versus 3	
Rho GAP6 isoform 1 variant expression	75	51(68%) 24(32%)	
Gender			
Male	40	26(70%) 14(30%)	0.35
Female	35	25(77%) 10(23%)	
Age			
<60	20	14(75%) 6(25%)	0.05
>60	55	37(67%) 18(33%)	
Dukes' stage			
A+B	25	18(72%) 7(28%)	0.28
C+D	50	33(66%) 17(34%)	
Differentiation			
Low	20	6(30%) 14(70%)	11.25
Median	40	30(75%) 10(25%)	
High	15	15(100%) 0(0%)	
Number of analysed lymph nodes			
<12	41	29(71%) 12(29%)	0.31
≥12	34	22(65%) 12(35%)	

100 ml PBS for 2 h and then incubated with the primary antibody (Hb3) overnight at 4°C. The membrane was washed and then incubated with HRP-conjugated goat anti-mouse IgG/IgM (Sigma-Aldrich) at RT for 1 h. The membrane was washed again and the antigen–antibody reaction was visualized using an ECL detection system.

Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Purified proteins prepared in the previous step were stained with Coomassie blue. Bands of interest were excised from gels and digested overnight with sequence grade trypsin

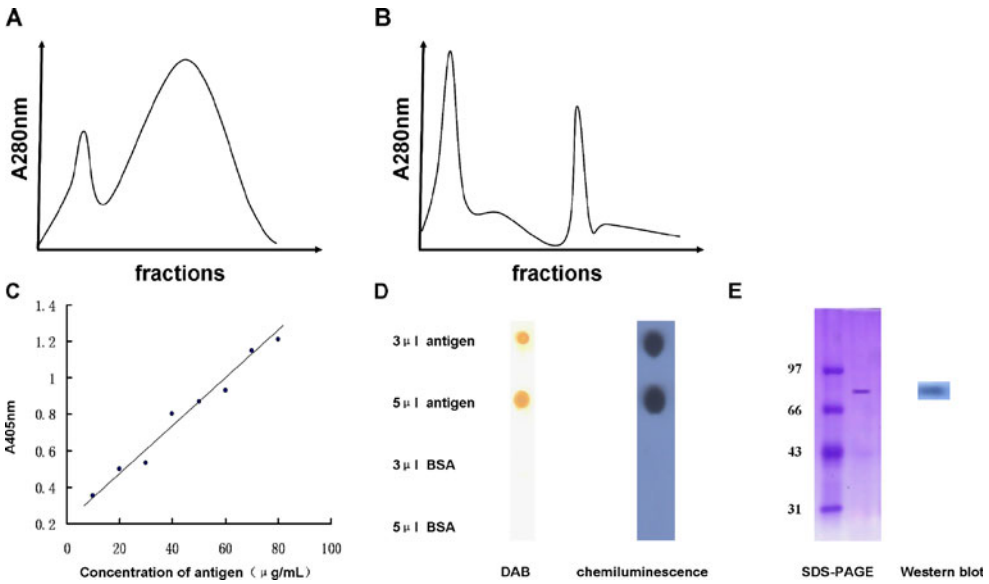

Fig. 2 Purification and biological characteristics of colon cancer antigen. **a** After the extracted Hb3 ascites had been purified using ammonium sulfate, there were two protein peaks eluted by Sephacryl S-300 chromatography. **b** There were two elution peaks after affinity chromatography. **c** The purified colon antigen was detected using a sandwich enzyme-linked immunosorbent assay (ELISA). **d** Dot-blot testing showed that the purified colorectal carcinoma antigen reacted specifically with Hb3. **e** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of the purified colorectal carcinoma antigen and western blot analysis for Hb3

(Promega, Madison, WI, USA). The eluate was analyzed with a Micromass Q-TOF Micro mass spectrometer (Waters, Milford, MA, USA). Protein identification was performed by searching the nonredundant NCBInr protein database of Mascot (http://www.matrixscience.com/).

Reverse Transcription Polymerase Chain Reaction (RT–PCR)

RNA isolated from tissues and cells was reverse-transcribed and amplified using the One-Step RT–PCR System (Fermentas, Vilnius, Lithuania). Primer sequences used were: sense 5′–GGATTTGGGTTGCATATTTGC–3′ and antisense 5′–GGCTTTTTCACCATCTCATTT–3′ for the Rho GTPase activating protein 6 (RhoGAP6) isoform 1 variant. A 587-bp GAPDH fragment was amplified as an internal control. For GAPDH, the forward primer was 5′–AATCC CATCACCATCTTCCA–3′ and the reverse primer was 5′–CCTGCTTCACCACCTTCTTG –3′. After heating at 95°C for 1 min, samples were exposed to 30 cycles (GAPDH, 25 cycles) of 95°C for 30 s, 60°C for 30 s and 68°C for 1 min 30 s with a final extension at 68°C for 10 min.

Statistical Analysis

All statistical analyses were done using the SPSS 10.0 software package (SPSS Inc., Chicago, IL, USA). Differences between groups were compared using χ^2 tests. All tests were two-tailed and were considered significant at $P<0.05$.

Results

Specificity of Hb3 in CRC Tissues and Cell Lines

The immunostaining intensity of Hb3 in the CRC cell lines tested (HCT-116, HT-29, SW480, LS1747) was strong whereas the intensity in HUVECs was weak (Fig. 1a). To assess whether Hb3 could be utilized in clinical applications, we performed immunohistochemistry on in human CRC and normal tissue samples. Of 75 samples from patients with CRC, 68 (91%) displayed clear cytoplasmic and plasmalemmal staining of Hb3 in malignant cells at different expression levels but no immunoreactions were observed in normal epithelia adjacent to tumor tissues (Fig. 1b). Immunohistochemical detection of the Hb3 antigen was often heterogeneous in the samples. Nine percent of the tumors did not express the antigen (grade 0), whereas 19% of the tumors expressed grade 1, 40% expressed grade 2 (Fig. 1b-b) and 32% expressed the highest grade of staining (grade 3; Fig. 1b-a). The expression of Hb3 antigen was independent of patient gender, age and tumor stage (Table 1). However, the protein expression varied greatly among different differentiation stages. Thus, Hb3 antigen was expressed strongly (grade 3) in 14 of 20 low differentiation samples, but this level was only seen in 10 of 40 moderate differentiation samples and not at all in high differentiation samples (Fig. 1c).

Purification of the CRC Antigen

Affinity chromatography was used to isolate the Hb3 antigen. After the extracted Hb3 ascites had been purified, using ammonium sulfate, there were two protein peaks found by Sephacryl S-300 chromatography. The first peak was identified to have antibody activity by ELISA (Fig. 2a). The protein peak was coupled to CNBr-activated Sepharose 4B and the crude antigen was added to the column. This procedure produced two elution peaks,

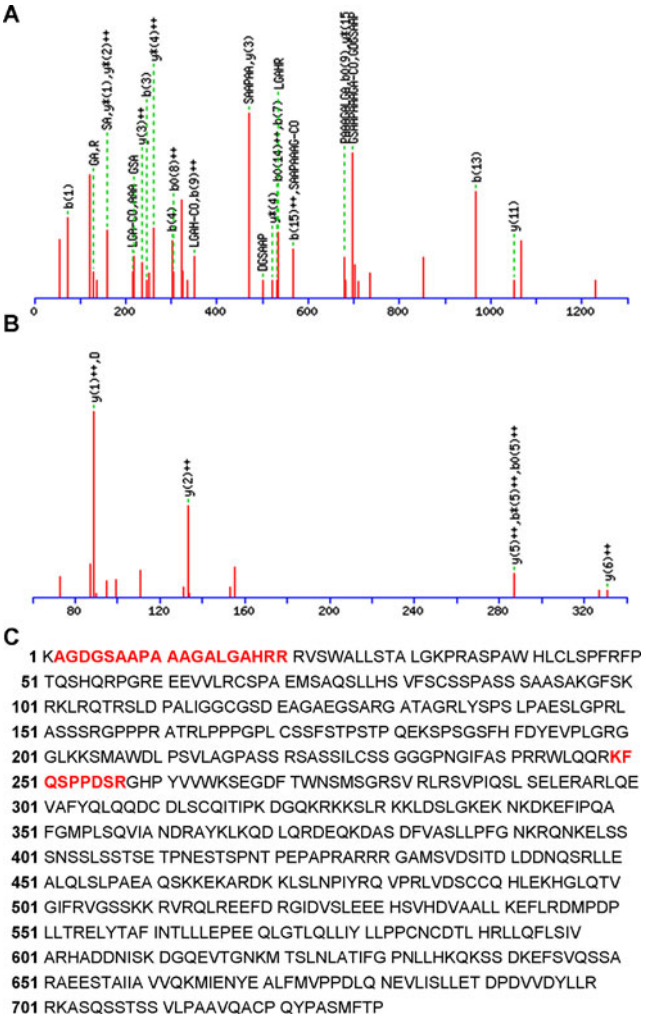

Fig. 3 Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) identified Rho GTPase activating protein 6 isoform 1 variant as an antigen recognized by Hb3. **a**, **b** MALDI-TOF MS analysis of RhoGAP6 isoform 1 variant. **c** Protein sequence of RhoGAP6 isoform 1 variant. Matched peptides are shown in *bold red*

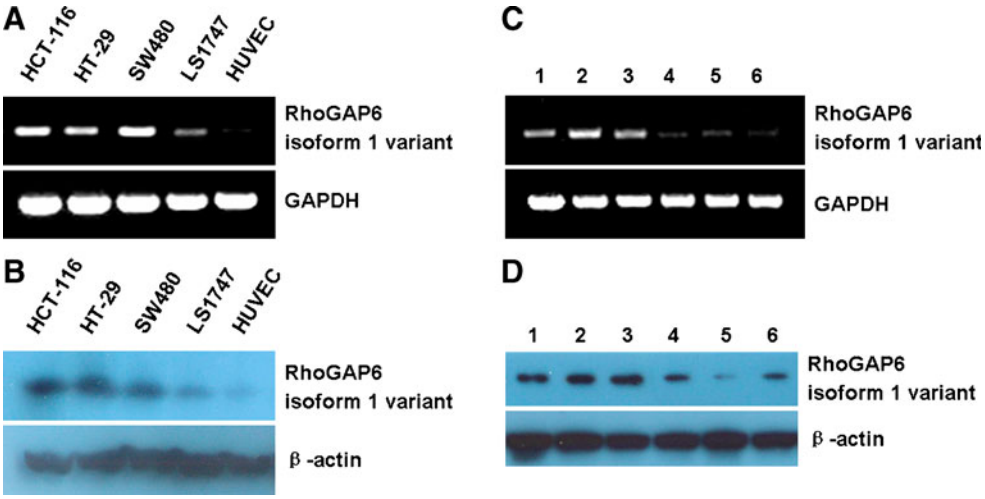

Fig. 4 Reverse transcription polymerase chain reaction (RT–PCR) and western blot analyses of the expression levels of RhoGAP6 isoform 1 variant. **a**, **b** The expressions of RhoGAP6 isoform 1 variant in colorectal cancer cell lines (HCT-116, HT-29, SW480, LS1747) were higher than in HUVECs. **c**, **d** The expression levels of RhoGAP6 isoform 1 variant in tumor tissues were higher than in normal tissues. (1–3, tumor tissues; 4–6, normal tissues)

of which the second was an Hb3-specific antigen eluted by glycine–HCl (Fig. 2b). The concentration of the concentrated antigen was 83 μg/ml.

Biological Characteristics of the Colon Cancer Antigen Detected by Hb3

After the purified colon antigen had been detected using sandwich ELISA, we constructed a standard curve based on the concentrations of antigen and the A405-nm antibody (Fig. 2c). The concentrations of antigen and A405-nm were correlated linearly in the range of 5.5–83 μg/ml.

Spots of 3 μl and 5 μl of purified CRC antigen and BSA on the nitrocellulose membranes were visualized using an ECL and DAB detection system. The spots of CRC antigen showed a color reaction whereas the control BSA spots did not (Fig. 2d). This confirmed that the purified CRC antigen was specifically recognized by mAb Hb3. The purified CRC antigen was electrophoresed on SDS–PAGE and the proteins were stained with Coomassie blue. One specific electrophoretic band of about 80 kDa was found. Western blot analysis also illustrated that the protein band combined with Hb3 (Fig. 2e).

Mass Spectroscopy Identification of Antigen Recognized by Hb3

The identity of the antigen recognized by Hb3 was determined using MALDI-TOF MS after trypsin digestion. After database searching on Mascot for MS/MS ions, the antigen protein was identified as the RhoGAP6 isoform 1 variant. This was consistent with the biological characteristics of the antigen recognized by Hb3 (Fig. 3). Figure 3a and b show MALDI-TOF MS analysis of this variant. Matched peptides are shown in bold red (Fig. 3c).

Expression Levels of RhoGAP6 Isoform 1 Variant in CRC Cell Lines, HUVECs, Human CRC and Normal Tissues

Using RT–PCR analysis, mRNA levels of RhoGAP6 isoform 1 variant in colorectal cancer cell lines (HCT-116, HT-29, SW480, LS1747) were higher than in HUVECs (Fig. 4a). Western blot analysis showed protein signals corresponding to the RhoGAP6 isoform 1 variant in CRC cell lines and HUVECs. The protein was expressed in CRC cell lines HCT-116, HT-29, SW480 at a high level, at a moderate level in LS1747, but was at lower levels in HUVECs. This was consistent with the RT–PCR results (Fig. 4b). Then we did RT–PCR and Western blot to examine RhoGAP6 isoform 1 variant levels in human colorectal cancer and normal tissue samples. Both RT–PCR and western blot analysis showed that the mRNA and protein levels of this protein were higher in CRC tissues than in normal tissues (Fig. 4c, d).

Discussion

The identification of useful markers for the detection and diagnosis of human colon cancer is a major goal in cancer research and provides valuable information on tumorigenesis and development. We used mAb Hb3 to find the responding antigen, RhoGAP6 isoform 1 variant, and then tested its expression level in different cells and tissues. This antigen was

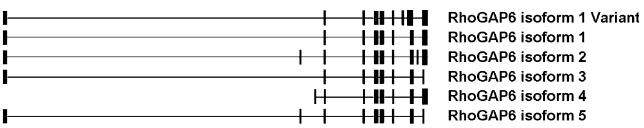

Fig. 5 The nucleotide sequence similarity between RhoGAP6 isoform 1 variant and five isoforms of RhoGAP6 is shown, based on BLAST searching

expressed at higher levels in CRC cells and tissues than in normal colon cells and tissues. Thus RhoGAP6 isoform 1 variant is a potential marker for CRC. This is particularly important as it provides new insights into the biology of human colorectal carcinogenesis and may prove to have clinically useful applications.

RhoGAP6 isoform 1 variant belongs to the RhoGAP6 superfamily, which operate as molecular switches that couple changes in the external environment to intracellular signal transduction pathways. Thus, they provide critical links between membrane receptors, the cytoskeleton and the nucleus [15–17]. RhoGAP6 has a 'rhoGAPlike' domain and three proline-rich motifs with consensus SH_3-binding sites [18]. It is expressed in multiple human tissues, including the heart [19]. While RhoGAP6 was initially characterized for its RhoGAP function, nonenzymatic functions for RhoGAP6 have been identified, including acting as a cytoskeletal protein to promote actin remodeling. More recently, RhoGAP6 has been shown to enhance PLC activity in a mammalian overexpression system and to co-immunoprecipitate with PLC-δ1 in extracts of transfected Cos-7 cells [20].

RhoGAP6 has five isoforms produced by alternative splicing. The isoform 1 variant is similar to RhoGAP6. The nucleotide sequence similarity between Rho GAP isoform 1 variant and the five other isoforms of RhoGAP6 identified by BLAST searching is shown in Fig. 5. Most nucleotides of the RhoGAP6 isoform 1 variant are in common with RhoGAP6. It also has the RhoGAP domain of RhoGAP6-like proteins.

Full-length *Homo sapiens*, *Pan troglodytes*, *Mus domesticus*, *Canis familiaris* and *Equus caballus* sequences show extensive homology with conserved amino acids and few conservative changes. Partial sequences derived from *Ornithorhynchus* and *Xenopus* were also found to have extensive homology. The extensive homology in different species and the higher expression of this variant in tumor tissues and cells than normal ones suggest that it might be a new biomarker for CRCs. At present, it appears that RhoGAPs have multiple roles. For example, the deleted in liver cancer 1 (*DLC-1*) gene encodes a GTPase activating protein that acts as a negative regulator of the Rho family of small GTPases [21]. Rho proteins transduce signals that influence cell morphology and physiology and their aberrant up-regulation is a key factor in the neoplastic process, including metastasis. Given the ubiquitous nature of most Rho proteins, it has been challenging to develop anti-cancer drugs based on their action. Tissue-specific GAPs may offer an interesting avenue to controlling Rho GTPase activity in cancer.

Immunohistochemistry showed that the RhoGAP6 isoform 1 variant expression was markedly increased in CRC cells compared with normal tissues. Thus, RhoGAP6 isoform 1 variant may play a significant role in CRC progression and could be a potential therapeutic target for treating patients with CRC. Interestingly, increased RhoGAP6 isoform 1 variant expression was associated with a low level of tumor differentiation. A variety of pathological changes are all closely associated with the cellular differentiation in CRC, including gross typing, growth pattern, depth of infiltration and metastasis into lymph nodes [22–24]. A low level of differentiation is positively correlated with a poor patient survival rate. Thus, this RhoGAP6 isoform 1 variant might be a new diagnostic molecular marker for human colorectal tumors.

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