

# RhoA activity increases due to hypermethylation of *ARHGAP28* in a highly liver-metastatic colon cancer cell line

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**Abstract.** Certain cell lines exhibit metastatic ability (highly metastatic cell lines) while their parent cell lines have no metastatic ability. Differences in methylation, which are not derived from differences in the gene sequence between cell lines, were extensively analyzed. Using an established highly metastatic cell line, KM12SM, and its parent cell line, KM12C, differences in the frequency of methylation were analyzed in the promoter regions of ~480,000 gene sites using Infinium HumanMethylation450. The promoter region of the Rho GTPase-activating protein 28 (*ARHGAP28*) gene was the most markedly methylated region in KM12SM compared with KM12C. *ARHGAP28* is a GTPase-activating protein (GAP), and it converts activated RhoA to inactivated RhoA via GTPase. RhoA activity was compared between these two cell lines. The activated RhoA level was compared using western blot analysis and G-LISA. The activated RhoA level was higher in KM12SM compared to KM12C for western blot analysis and G-LISA analysis. RhoA is a protein involved in cytoskeleton formation and cell motility. RhoA, for which *ARHGAP28* acts as a GAP, is possibly a factor involved in the metastatic ability of cancer.

## Introduction

Clinical cancer lesions are masses of heterologous cancer cells. The mainstream hypothesis of cancer metastasis is that 'a population of highly malignant cells acquires metastatic ability due to the accumulation of gene mutations forming metastatic lesions' (1), i.e., it is hypothesized that highly malignant cancer cells with metastatic ability and low-malignant cancer cells with no metastatic ability are present in the same cancer lesion, and metastatic lesions are mainly formed by

the former. Certain established cancer cell lines have a high metastatic ability (highly metastatic cell lines) that is similar to clinical cancers. To establish a highly metastatic cell line, an animal model of metastasis is prepared and cells collected from metastatic lesions are transplanted into another animal, and this procedure is repeated. A highly metastatic cell line is established by passaging only a cell population that has metastasized in the metastasis model, however, why such a highly malignant cell line is established from a genetically cloned cell line through this passaging method is questionable. It is unlikely that a new change in a gene sequence, such as gene mutation, occurs through only several passages even in a metastasis model. Cancer cell lines are manufactured on the assumption that cells will be passaged a specific number of times. When a gene mutation occurs within a small number of passages, this contradicts the stability of the cancer cell line. Therefore, the difference between a highly metastatic cell line and its parent cell line with no metastatic ability is of interest and we hypothesize that the difference is not a genetic mutation.

Recent studies identified the presence of the alternation of cell phenotypes, termed epigenetic abnormality, in cancers, in addition to genetic mutation. The representative epigenetic change is DNA methylation, particularly the methylation of gene promoter regions. The state of methylation is inherited even after cell division, and its abnormality (hypermethylation or genome-wide hypomethylation) in various types of cancer has been reported (2). Highly malignant human cancer cell lines established by the passage method include a highly metastatic human colon cancer cell line, KM12SM, and its parent cell line, KM12C, with no metastatic ability (3). We hypothesized that abnormal methylation is not present in the parent cell line KM12C, and it has occurred in highly metastatic KM12SM and altered certain gene and protein expression levels (increase or decrease), resulting in the difference in biological behavior between the two cell lines. Therefore, the differences in the frequency of methylation of the gene promoter regions were extensively analyzed between the highly metastatic and parent cell lines.

## Materials and methods

**Cell lines and assay.** Two human colon cancer cell lines, KM12C and KM12SM, derived from human colon cancer, which were kindly provided from Dr Nakajima at SBI

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Pharmaceuticals Co., Ltd. (Tokyo, Japan) were used. Cells were cultured at 37°C in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO, USA) and 1% penicillin and streptomycin (Life Technologies) under a humidified atmosphere containing 5% CO<sub>2</sub>. DNA sample preparation was as follows: Genomic DNA was isolated from the cell lines using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany). To confirm the quality of the samples, the concentration of DNA ( $\mu\text{g}/\mu\text{l}$ ) and absorbance ratio (260/280 nm) were measured by a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). DNA of 81.8  $\mu\text{g}/\mu\text{l}$  with a ratio of 1.98 for KM12C, and 81.1  $\mu\text{g}/\mu\text{l}$  with a ratio of 1.93 for KM12SM was prepared. These samples were confirmed under electrophoresis on a 1% agarose gel to check the size of the fragments at 200 V of constant power for 25 min. The extent of abnormal methylation of the promoter region was assessed by bead array analysis. Bead array analysis was as follows: analysis was performed following the protocol of the Illumina Infinium HD Methylation assay. As the software, GenomeStudio version V2011.1 and Methylation Module version 1.9.0 were used. Regarding KM12SM and KM12C as comparative and control groups, respectively, the level of methylation was determined at ~480,000 sites throughout the genome, grading no methylation and methylation of only one and both alleles as 0, 0.5 and 1, respectively, and the average (AVG) was calculated for each gene. In addition, the difference between KM12SM-AVG and KM12C-AVG was calculated using the following formula:  $\text{Log-ratio} = (\log_2 \text{KM12SM-AVG}) - (\log_2 \text{KM12C-AVG})$ . When the difference was <0.6, the gene was regarded as equally methylated (EM), and when it was  $\geq 0.6$ , the gene was regarded as differently methylated (DM).

**Western blot analysis of activated-RhoA.** For analysis, the RhoA Activation Assay Biochem kit™ (Cytoskeleton, Inc., Denver, CO, USA) was used following the manufacturer's protocol. The cell lysates for KM12SM and KM12C were seeded in culture flasks at  $5 \times 10^4$  cells/ml and cultured in 5% CO<sub>2</sub> at 37°C. After 3 days, cells were lysed with 500  $\mu\text{l}$  of cell lysis buffer [50 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.5 M NaCl and 2% IPEGAL] and centrifuged at 10,000  $\times$  g, and the supernatant was collected as a lysate. The protein level was measured using the BCA™ Protein Assay kit-Reducing Agent Compatible (Thermo Fisher Scientific).

For the sample preparation, the KM12C and KM12SM lysates were aliquoted into 3 portions, adjusting the protein content to 700  $\mu\text{g}$ . To 2 of the 3 aliquots, 1/100 volumes of GTP $\gamma$ S (200 mM solution) and GDP (100 mM solution) were added as positive- and negative-control samples, respectively. The remaining sample was used as an untreated sample. For the pulldown assay, to each sample 50  $\mu\text{g}$  (60  $\mu\text{l}$ ) of Rhotekin-Rho-binding domain beads was added, the sample was incubated at 4°C for 1 h, and the supernatant was removed. Following washing, the precipitate was resuspended with 20  $\mu\text{l}$  of 2X Laemmli sample buffer [125 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 0.005% bromophenol blue and 5%  $\beta$ -mercaptoethanol] and boiled for 2 min. For the western blot assay, following the pulldown assay, 15  $\mu\text{l}$  of each sample was applied on 4-20% Mini-PROTEAN® TGX™ gel (Bio-Rad Laboratories, Hercules, CA, USA) and

electrophoresed at 200 V for 32 min, followed by transfer to a PVDF membrane at 75 V for 45 min. Following blocking, the membrane was reacted with an anti-RhoA monoclonal antibody as a primary antibody (ARH03; Cytoskeleton, Inc.), and chemiluminescence emitted by the target band using electrochemiluminescence was exposed to an X-ray film. The area of bands detected on the X-ray film was calculated using Manual ROI Selection (Draw Polygons) of Tissue Studio 4.0 (Definiens, München, Germany).

**Analysis of activated small G-protein.** Activated-RhoA was quantified using the G-LISA® RhoA Activation Assay Biochem kit™ (Cytoskeleton, Inc.). Preparation of the cell lysates and protein assay was performed as described previously. For the G-LISA assay, the assay was performed following the manufacturer's protocol. Briefly, 120  $\mu\text{l}$  of cell lysis buffer combined with 1.2  $\mu\text{l}$  of the protease inhibitor cocktail and 120  $\mu\text{l}$  of the binding buffer were mixed and used as a buffer blank, and a mixture of 24  $\mu\text{l}$  of the RhoA control protein, 96  $\mu\text{l}$  of the cell lysis buffer, and 120  $\mu\text{l}$  of the binding buffer was used as a RhoA-positive control. KM12SM and KM12C lysates were adjusted to 0.5, 1.0 and 1.5 mg/ml, and 90  $\mu\text{l}$  of each lysate sample was mixed with 90  $\mu\text{l}$  of the binding buffer. These samples, the buffer blank and the RhoA-positive control (50  $\mu\text{l}$ ) were distributed to 3 wells each. The anti-RhoA monoclonal antibody was added as a primary antibody and detected using horseradish peroxidase, and the absorbance was measured at 490 nm.

## Results

**Methylation in KM12SM and KM12C cells.** In the overall investigated regions, EM and DM in KM12SM compared to methylation in KM12C were 78.44 and 21.56%, respectively. Limiting to promoter regions, EM and DM were 84.15 and 15.85%, respectively. DM exhibited hypermethylation and hypomethylation in KM12SM compared with those in KM12C in 37.70 and 62.30% of the overall regions, respectively, and 25.98 and 74.00% of the promoter regions, respectively. The promoter regions with the top 15 methylation levels, including the two overlap in KM12SM and in its parent cell line KM12C, are shown in Fig. 1. Of these, the highest methylation level in KM12SM was detected in the Rho GTPase-activating protein 28 (*ARHGAP28*) gene.

ARHGAP28 was identified as a GTPase-activating protein (GAP) of a low molecular weight G-protein, RhoA protein (4). Activated RhoA that has bound to guanosine triphosphate (GTP) is converted to inactivated RhoA bound to guanosine diphosphate (GDP) through the action of a GAP, ARHGAP28. The hypermethylated state of the *ARHGAP28* gene indicates that activated-RhoA cannot return to inactivated-RhoA, as the GAP of RhoA, ARHGAP28, is not functioning, which results in the persistence of activated-RhoA (hyperactivated-RhoA).

The cDNA sequence of RhoA was analyzed to investigate whether or not mutation of the RhoA gene occurred prior to activated-RhoA expression in the two cell lines. Briefly, the method and findings were as follows: RNA was extracted from KM12SM and KM12C, converted to cDNA, and exons 1-5 were exhaustively sequenced. The base sequence of RhoA

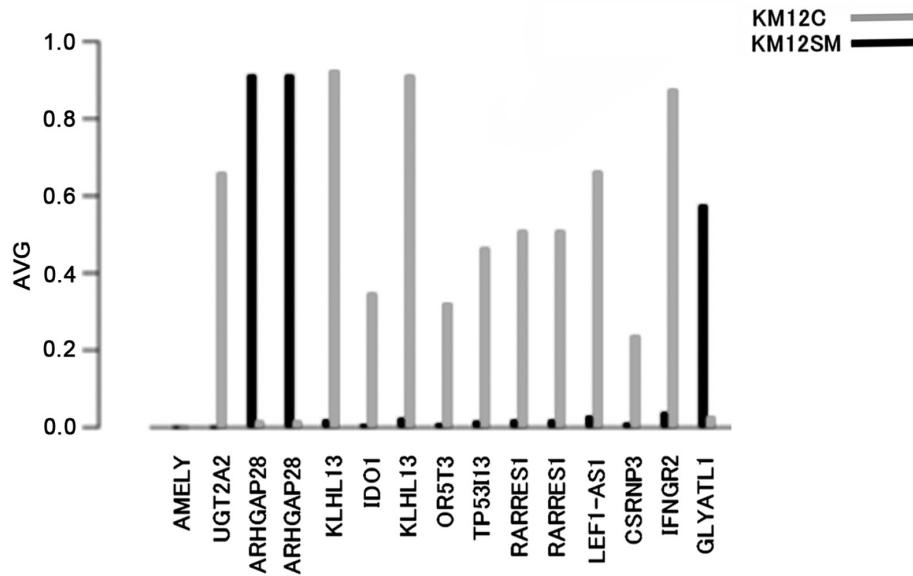


Figure 1. Methylation of the promoter region. The y-axis shows the average (AVG) methylation in each gene. The x-axis shows the top 15 methylation levels of the promoter regions, including the two overlap. *AMELY*, amelogenin, Y-linked; *UGT2A2*, glucuronosyl transferase 2 family, polypeptide A2; *ARHGAP28*, Rho GTPase-activating protein 28; *KLHL13*, kelch-like 13 (*Drosophila*); *IDO1*, indoleamine 2,3-dioxygenase 1; *OR5T3*, olfactory receptor, family 5, sub-family T, member 3; *TP53I13*, tumor protein p53-inducible protein 13; *RARRES1*, retinoic acid receptor responder (tazarotene induced) 1; *LEF1-AS1*, LEF1 antisense RNA 1; *CSRP3*, cysteine-serine-rich nuclear protein 3; *IFNGR2*, interferon- $\gamma$  receptor 2 (interferon- $\gamma$  transducer 1); *GLYT1L*, glycine-N-acyl-transferase-like 1.

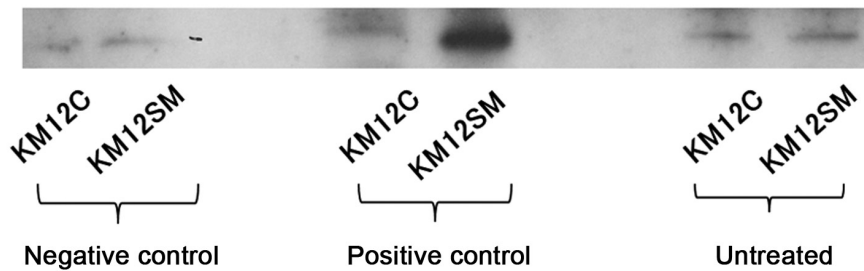


Figure 2. Western blot analysis of activated-RhoA. Bands were detected at 21 kDa. The band on the untreated blot represents RhoA-guanine triphosphate (activated-RhoA).

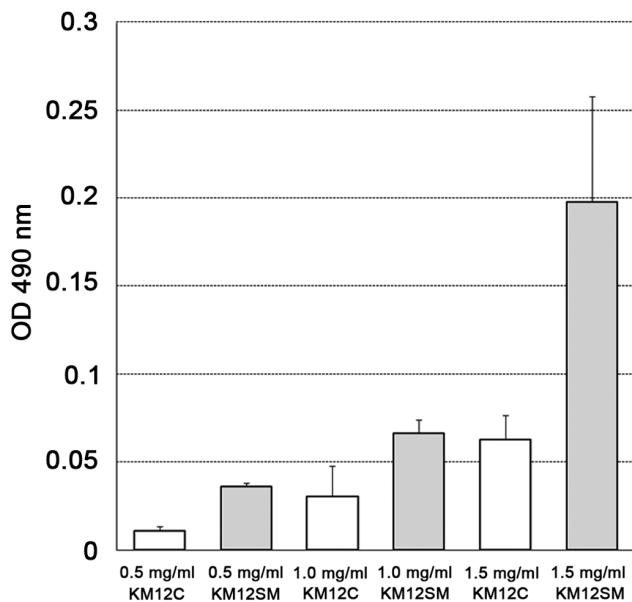


Figure 3. G-LISA assay for activated-RhoA. The y-axis shows the optical density at 490 nm, and the x-axis shows the concentration of the cell lysate.

cDNA was identical between KM12SM and KM12C, but the sequences with G and T at position 464 were mixed in the two cell lines, and due to this mutation, the amino acid sequences with Gly (GGG) and Val (GTG) at position 155 were simultaneously present.

**Western blot analysis.** The western blot analysis of RhoA-GTP in the parent cell line, KM12C, and KM12SM is shown in Fig. 2. Bands were detected at the target molecular weight, 21 kDa. The band area measured using the Tissue Studio was 13,713.47  $\mu\text{m}^2$  in KM12SM and 11,781.86  $\mu\text{m}^2$  in KM12C, showing that it was ~1.2-fold wider in KM12SM compared to KM12C.

**G-LISA analysis.** The optical density at 490 nm on the G-LISA assay was compared between KM12C and KM12SM by the concentration of the cell lysate. The absorbances were 0.011±0.005 vs. 0.036±0.004 at 0.5 mg/ml, 0.030±0.035 vs. 0.066±0.016 at 1.0 mg/ml, and 0.063±0.028 vs. 0.198±0.120 at 1.5 mg/ml, respectively, showing that RhoA-GTP, i.e., the activated-RhoA level, was higher in KM12SM compared to KM12C at all 3 concentrations (Fig. 3).

## Discussion

Epigenetic changes are originally mechanisms that efficiently use genes, and DNA methylation and histone modifications are representative mechanisms. In DNA methylation, a methyl group (-CH<sub>3</sub>) is added to a cytosine of a CpG sequence, which strongly inhibits the transcription of downstream genes. In histone modification, a specific site of the DNA scaffold protein, termed histone, is methylated, which inhibits DNA transcription. It has long been known that exposure to various environmental factors with aging is a cause of abnormality of these mechanisms; however, bacterial infection, cigarette smoking and hormones are also environmental factors inducing epigenetic abnormalities. Abnormal DNA methylation is roughly determined by what tissue is exposed to what environmental factor. For example, DNA methylation is different between esophageal cancer exposed to cigarette smoking and gastric cancer induced by *H. pylori* infection. In colorectal cancers, the cancer phenotype with augmentation of enhanced DNA methylation in CpG islands in numerous genes is termed the CpG island methylator phenotype (CIMP), and gene instability due to inactivated *BRAF* and *MLH1* genes in CIMP-high colon cancers, *KRAS* gene abnormality in CIMP-low colon cancers, and the *TP53* gene abnormality in CIMP-negative colon cancers have been reported (5).

A merit of investigating DNA methylation is the superior stability of methylated states. mRNA and protein expression levels markedly vary depending on the environment of cancers and cell cycle. For example, slight changes in temperature and the composition and condition of culture medium *in vivo* alter mRNA and protein expression levels. By contrast, DNA methylation is mostly stable anytime it is measured, and the cell condition can be determined based on it. In addition, the number of methylations is 0 (no methylation), 0.5 (only one allele is methylated) or 1 (both alleles are methylated). Thus, the value is unlikely to be biased in cancer cell populations, which are heterogeneous. This is useful for the pathological diagnosis of cancer. Extensive analysis of DNA methylation has recently been performed for early discovery and identifying a risk group of cancer. Regarding the benefit of extensive analysis, previous studies clarified that DNA was non-methylated in normal tissue and hypermethylated in cancers (silencing by methylation), i.e., non-expressed genes were identified. The genes silenced by methylation are considered as tumor suppressor genes. However, the identification of genes expressed at a low level in normal cells even though they are not methylated has become possible with technological advancements. Analysis of various types of cancer using Infinium HumanMethylation is the most rapidly progressing. The present study used Infinium HumanMethylation450 of Illumina for extensive analysis, which simultaneously measured the methylation level at ~480,000 sites throughout the genome. The methylation state was most markedly different in the *ARHGAP28* gene in the highly metastatic cell line, KM12SM, compared with the parent cell line, KM12C. The methylation frequency of the *ARHGAP28* gene was nearly 1.0 in KM12SM, showing that both alleles were methylated in the majority of KM12SM cells. By contrast, the methylation level was mostly 0 in this region in KM12C, clarifying that the promoter region of the *ARHGAP28* gene is hypermethylated in the highly metastatic human colon cancer cell line KM12SM

compared with that in the parent cell line KM12C. *ARHGAP28* is not a direct cause of carcinogenesis or malignancy, i.e., the abnormality is not a driver gene mutation. Thus, it is likely that methylation of the *ARHGAP28* gene is a passenger methylation due to the phenomenon of metastasis.

Low molecular weight G-proteins are classified into 5 groups: the Ras, Rho, Rab, Ran and Sar/Arf families. The Rho family includes 3 typical molecules: RhoA, Rac1 and Cdc42, and several molecules exit downstream of each. *ARHGAP28* is involved in a Rho family, RhoA, as discussed in the following. RhoA is a 21-kDa protein consisting of 193 amino acids, and it is involved in cytoskeleton formation and cell migration (6). The core effector domains of RhoA, Y42 and G17, are highly conserved among the Rho family proteins. G17 is a pocket that GNP enters. Y42 and R5 are present on the external surface of RhoA, and Y42 is important as the binding site of RhoA-activity regulatory protein. RhoA is normally bound to GDP: RhoA-GDP = inactivated-RhoA; and GDP is replaced with GTP by the GDP/GTP exchange factor in response to certain stimulation, converting the molecule to RhoA-GTP = activated-RhoA. When the active form becomes unnecessary, GTPase activity of RhoA itself is enhanced by *ARHGAP28* and the bound GTP is hydrolyzed to GDP, converting the molecule to inactivated-RhoA. A highly activated-RhoA level in KM12SM with a highly metastatic ability observed in the western blot analysis and G-LISA analysis, and a hypermethylated state of the gene of *ARHGAP28*, which is GAP of RhoA, in the methylation analysis do not contradict each other. Thus, we considered that the RhoA activity level increased in a cell population with a hypermethylated *ARHGAP28* gene (possibly a small number) in KM12C, and the population acquired metastatic ability. Therefore, the RhoA gene may be involved in metastasis, and there are studies supporting this. It has been reported that a constantly active mutant of the RhoA gene may be a driver gene, and cases with RhoA gene mutation showed typical features of diffuse gastric cancer with an alveolar structure of lesions comprised of one to several cells in the infiltrated region (7). Cells were scattered in diffuse gastric cancer, and a budding cell group was present in the deep infiltrated region of colon cancer, being involved in metastasis (8). If these findings were involved in the cell scattering process, these do not contradict our consideration. RhoA kinase is present downstream of RhoA, and the inhibition of colon cancer cell migration by RhoA kinase inhibitor has been reported (9), but the absence of an association between RhoA activity and infiltration of colon cancer has also been reported (10). In either case, a question arises as to whether there is merit in analyzing only one driver gene. In 2004, Müller *et al* (11) reported a study using methylation abnormality observed in CpG islands of the promoter region of the secreted frizzled-related protein 2 (*SFRP2*) gene as a biomarker, in which the sensitivity was high (77%, 10/13), although the number of cases was only 13. Takeda *et al* (12) performed an additional study on these findings, in which when a ≥1% methylation rate was judged as methylated, it was observed in 30 and 85% of normal mucosa and colon cancer, respectively. *ARHGAP28* gene methylation observed in the highly metastatic cell line KM12SM may lead to findings similar to those observed in *SFRP2*. To investigate this, future studies should screen clinical samples for the activity level of RhoA, on which *ARHGAP28* acts as a GAP, as a liver metastasis-predicting factor.



In conclusion, a high level of RhoA, which is involved in cell migration, is due to hypermethylation of the gene of *ARHGAP28*, which is a GAP of RhoA, and it may be associated with a highly metastatic ability.

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### References

1. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr and Kinzler KW: Cancer genome landscapes. *Science* 339: 1546-1558, 2013.
2. Yamashita S, Hosoya K, Gyobu K, Takeshima H and Ushijima T: Development of a novel output value for quantitative assessment in methylated DNA immunoprecipitation-CpG island microarray analysis. *DNA Res* 16: 275-286, 2009.
3. Morikawa K, Walker SM, Nakajima M, Pathak S, Jessup JM and Fidler IJ: Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. *Cancer Res* 48: 6863-6871, 1988.
4. Yeung CY, Taylor SH, Garva R, Holmes DF, Zeef LA, Soininen R, Boot-Handford RP and Kadler KE: *Arhgap28* is a RhoGAP that inactivates RhoA and downregulates stress fibers. *PLoS One* 9: e107036, 2014.
5. Yamamoto E, Suzuki H, Yamano HO, Maruyama R, Nojima M, Kamimae S, Sawada T, Ashida M, Yoshikawa K, Kimura T, *et al*: Molecular dissection of premalignant colorectal lesions reveals early onset of the CpG island methylator phenotype. *Am J Pathol* 181: 1847-1861, 2012.
6. Ishizaki T, Morishima Y, Okamoto M, Furuyashiki T, Kato T, Narumiya S: Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1. *Nat Cell Biol* 3: 8-14, 2001.
7. Kakiuchi M, Nishizawa T, Ueda H, Gotoh K, Tanaka A, Hayashi A, Yamamoto S, Tatsuno K, Katoh H, Watanabe Y, *et al*: Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. *Nat Genet* 46: 583-587, 2014.
8. Ueno H, Murphy J, Jass JR, Mochizuki H and Talbot IC: Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Gastroenterology* 127: 385-394, 2004.
9. Al-haidari AA, Syk I, Jirstrom K and Thorlacius H: CCR4 mediates CCL17 (TARC)-induced migration of human colon cancer cells via RhoA/Rho-kinase signaling. *Int J Colorectal Dis* 28: 1479-1487, 2013.
10. de Toledo M, Anguille C, Roger L, Roux P and Gadea G: Cooperative anti-invasive effect of Cdc42/Rac1 activation and ROCK inhibition in SW620 colorectal cancer cells with elevated blebbing activity. *PLoS One* 7: e48344, 2012.
11. Müller HM, Oberwalder M, Fiegl H, Morandell M, Goebel G, Zitt M, Mühlthaler M, Ofner D, Margreiter R and Widschwendter M: Methylation changes in faecal DNA: A marker for colorectal cancer screening? *Lancet* 363: 1283-1285, 2004.
12. Takeda M, Nagasaka T, Dong-Sheng S, Nishie H, Oka T, Yamada E, Mori Y, Shigeyasu K, Morikawa T, Mizobuchi S, *et al*: Expansion of CpG methylation in the SFRP2 promoter region during colorectal tumorigenesis. *Acta Med Okayama* 65: 169-177, 2011.