SPANDIDOS PUBLICATIONS

Analysis of RIN1 gene expression in colorectal cancer

KATSUNORI SENDA, TAKANORI GOI, YASUO HIRONO, KANJI KATAYAMA and AKIO YAMAGUCHI

First Department of Surgery, University of Fukui, 23-3 Eiheiji-cho, Yoshida-gun, Fukui, Japan

Received November 4, 2006; Accepted December 7, 2006

Abstract. The RIN1 gene, located on chromosome 11q13.2, is a molecule consisting of a coding region of 2352 bp, has a domain on the 3' side that binds to H-Ras protein, and is presumed to be an important molecule in an intracellular signaling pathway. Since the RIN1 molecule belonging to the effector molecules of H-Ras has not been reported in colorectal or other digestive tract cancers to date, we investigated how the RIN1 gene was involved in colorectal cancer. Fifty-two (51.5%) of 101 colorectal cancer specimens strongly expressed the RIN1 gene compared to the adjacent normal colorectal tissue. The 5-year survival rate of patients positive for the expression of the RIN1 gene was significantly poorer at 55% than that (83%) of patients negative for the expression of the RIN1 gene. Also, we confirmed that RIN1 protein was localized chiefly in the cytoplasm of colorectal cancer cell lines, and bound to 14-3-3 protein, but not to Ras protein. These results indicate that the RIN1 gene serves as an important signal transduction system for evaluating the malignancy of colorectal cancer.

Introduction

The first step for extracellular stimulators such as cytokines, growth factors, and ultraviolet light to transmit information into cells is their binding to receptors on cell membranes. Various types of receptors such as tyrosine kinase, cytokine, T cell, and G protein-coupled receptors have been identified (1-3). Receptor-mediated stimulation converts H-Ras protein to active H-Ras protein. Specifically, in response to stimulation, an increase in the activity of receptors themselves or receptor-coupled tyrosine kinases causes phosphorylation of the specific tyrosine residues of intranuclear substrates including receptors themselves, inducing the association of adaptor molecules such as SOS, Shc, and Grb2 with receptors through specific interaction between the SH2 (Src homology 2) or PTD (phosphotyrosine-binding) domain and phosphorylated tyrosine residue (4-8). Adaptor molecules are permanently associated with H-Ras GDP/GTP exchange factors such as mSOS1 through the interaction between the SH3 domain and proline-rich specific sequences. Receptor stimulation induces the migration of these factors to cell membranes, and causes the activation of H-ras molecules (9). Downstream of H-Ras, RAF1, Ral GDS, AF6, Nore1, and PI3-kinase pathways are recognised (10-18). In addition, the RIN1 gene which binds to activated H-Ras has been discovered (19,20). This gene is located on chromosome 11q13.2, and represents a molecule consisting of a coding region of 2352 bp (783 amino acids) (21), which structurally contains sequences corresponding to a tyrosine-phosphorylating SH2 domain toward the N-terminal end, and binds to ABL protein, a non-receptor tyrosine kinase. A proline-rich SH3 domain is present in the middle of the molecule, and a domain which binds to H-Ras is present at the C-terminal end (22,23). It is known that at the C-terminal end, RIN1 protein interacts directly with H-Ras protein and competes with RAF1 (19), that 14-3-3 protein acts as a negative regulator of membrane localization of RIN1 protein (24), that the critical serine of RIN1 is a substrate for protein kinase D (24), and that RIN1 protein enhances the transforming properties of ABL (23). These observations have led to the speculation that RIN1 protein is a biologically important factor. However, to date, no studies have investigated the relationship between the RIN1 gene and digestive tract cancer. In this study, we examined how the RIN1 gene was involved in colorectal cancer.

Materials and methods

Cell culture. The human colorectal cancer cell lines, SW480, LoVo, DLD1, and HT29, were cultured at 37˚C in 5% CO_2 in RPMI-1640 containing 10% heat-inactivated fetal calf serum.

Patients and tissue samples. The tissue specimens examined were taken from 101 colorectal cancer patients who underwent resection. Immediately after resection, necrotic and ulcerated parts of the tumors were removed and normal colorectal mucosae were dissociated from the muscle and connective tissues. All tissue specimens were then frozen in liquid nitrogen and stored at -80˚C.

All patients had been treated at the First Department of Surgery, University of Fukui, between 1990 and 2000. One hundred and twenty patients consisted of 10 with stage I; 45 with stage II; 25 with stage III; and 21 with stage IV cancers

Correspondence to: Dr Takanori Goi, First Department of Surgery, University of Fukui, 23-3 Eiheiji-cho, Yoshida-gun, Fukui 910-1193, Japan
E-mail: tgoi@fmsrsa.fukui-med.ac.jp

Key words: colorectal cancer, RIN1 gene

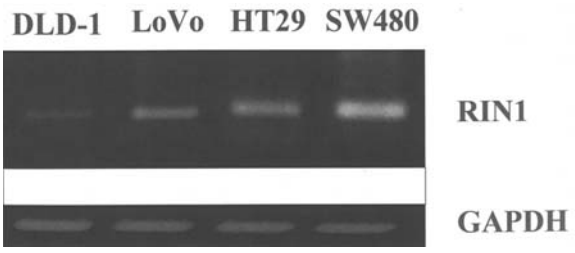

Figure 1. Expression of the RIN1 gene in colorectal cancer cell lines. The expression of RIN1 gene mRNA was detected by RT-PCR. The different levels of the expression were observed in the colorectal cancer cell lines: SW480, HT29, DLD1, and LoVo.

using the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) staging system (25). Cancers were reviewed and graded by a single pathologist using criteria recommended by the general rules of clinical and pathological studies on cancer of the colon, rectum and anus for histological type, lymphatic invasion and venous invasion (26).

Semi-quantitative RT-PCR analysis. Total RNA was extracted from colorectal tissues using guanidinium-thiocyanate (27). Single-strand cDNA prepared from 3 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Bethesda, MD) with an oligo (dT) primer-14 was used as the template for the polymerase chain reaction (PCR) (27). The primers for PCR to amplify the RIN1 gene-coding regions were as follows: The 5' primer, RIN1-AX, encompassed positions 337-354 of the published human RIN1 sequence (21), 5'-TGGCCCCTCCTTCGTCTC-3'. The 3' primer, RIN1-BX, encompassed positions 707-727 of sequence 5'-CTTGAATTTCTCCCGCTTGGT-3'. GAPDH amplification was used as the internal PCR control with 5'-GGGGAGCC AAAAGGGTCATCATCT-3' as the sense primer and 5'-GACGCCTGCTTCACCACCTTCTTG-3' as the antisense primer. Thirty cycles of denaturation (94˚C for 1 min), annealing (50˚C for 1.5 min), and extension (72˚C for 1 min) were carried out in a Thermal controller (PTC-100, NJ Research Inc, Bio-Rad, Japan). Tubes containing all ingredients except templates were included in all runs as negative reaction controls. Preparations of the RIN1-positive human colon cancer cell line, LoVo, were used as positive controls. Ten microliters of the PCR product was resolved by electrophoresis in polyacrylamide (12%) gels. Ethidium bromide staining of the gels identified a band of the RIN1 gene. To ensure reproducibility, all PCR amplifications were performed in duplicate. Densitometric analysis of the photographic negatives was used for band quantification (28).

The semi-quantitative detection of mRNA. In order to evaluate the amplified product quantitatively by PCR, preliminary experiments were carried out to determine a suitable number of cycles in the linear range of PCR amplification in representative cases (data not shown). A suitable number of PCR cycles for RIN1 was 30.

Statistical considerations. Survival time was calculated using the Kaplan-Meier method, and the log rank test was used to compare the curves of the survival times. Other characteristics of the two treatment arms were compared using the chi-square test. Values of $P<0.05$ were considered as statistically significant.

Ethics. The procedures of our study received ethical approval from the institutional committee responsible for human experimentation at the university of Fukui and all those who participated in the study did so voluntarily, having given their informed consent. The research was performed in accordance with the humane and ethical rules for human experimentation that are stated in the Declaration of Helsinki.

Membrane fraction. LoVo cells were washed three times with phosphate-buffered saline and disrupted with 10 mmol/l Tris-HCl (pH 7.8) containing 1% Nonidet P 40 (Sigma Chemical Co., MO), 0.15 mol/l NaCl, 1 mmol/l EDTA, and 2 mmol/l phenylmethylsulfonyl fluoride for 30 min at 40˚C. Cells were pelleted by centrifugation at 100000 rpm for 15 min and boiled for 3 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Western blot analysis. LoVo cells were washed three times with phosphate-buffered saline and disrupted with 10 mmol/l Tris-HCl (pH 7.8) containing 1% Nonidet P 40 (Sigma), 0.15 mol/l NaCl, 1 mmol/l EDTA, and 2 mmol/l phenyl-methylsulfonyl fluoride for 30 min at 4˚C. Cells were pelleted by centrifugation at 10000 rpm for 15 min and boiled for 3 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. PAGE in the presence of SDS was performed previously (27). The protein was run on 8% SDS-polyacrylamide gel and transferred to the PVDF membrane. After the electrophoretic transfer, the membrane was blocked overnight at 4˚C with 5% BSA, then washed once with Tris-buffered saline containing 0.05% Tween-20 (T-TBS) and incubated with the primary antibody. After incubation, the PVDF membrane was washed three times with T-TBS and incubated with the horseradish second antibody. Signals were visualized by enhanced chemiluminescence (27).

Antibody. The anti-RIN1 or anti-14-3-3 antibody were obtained from UBI (Upstate Biotechnology Inc, NY).

Immunoprecipitation. The cell lysates were incubated with the agarose-conjugated RIN1 antibody and immune complexes were collected by centrifugation. Agarose beads precoupled with the RIN1 antibody were washed 4 times with lysis buffer.

Results

Expression of the RIN1 gene in colorectal cancer cell lines. The expression of RIN1 gene mRNA was detected by RT-PCR using specific primers. As shown in Fig. 1, the expression was confirmed at 391 bp. The different levels of expression were confirmed in all 4 other cell lines: DLD1, LoVo, HT29, and SW480 (Fig. 1).

RIN1 gene expression rates in human colorectal cancers by clinicopathological parameters. The RIN1 gene expression

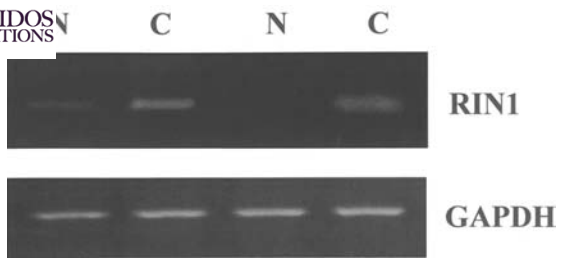

Figure 2. RIN1 gene expression rates in human colorectal cancers. No expression was observed in the colorectal mucosae (N), whereas the expression was confirmed in human colorectal cancers (C).

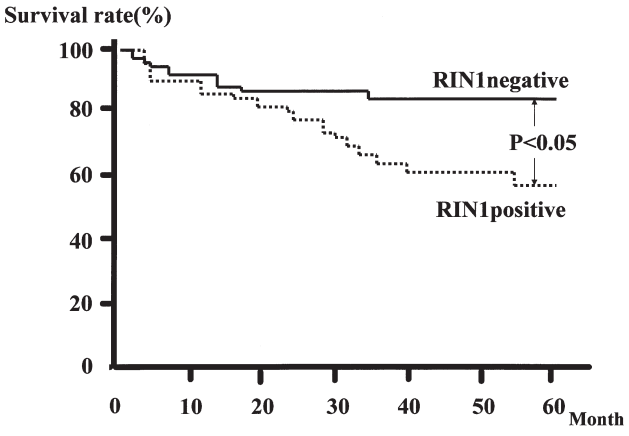

Figure 3. Relationship between RIN1 gene expression rates and survival rates in colorectal cancers. The 5-year survival rate was 83% in RIN1-negative cancers, but significantly poorer at 55% in RIN1-positive cancers.

level in 52 (51.5%) of 101 colorectal cancer specimens was 5 times higher than that in the adjacent normal colorectal tissue (the RIN1 gene in the adjacent normal colorectal tissue was not or weakly expressed) (Fig. 2). Clinicopathological analysis confirmed that the RIN1 gene expression positivity rate of cancers with venous invasion was significantly higher at 62% than that (37%) of cancers with no venous invasion. Abnormal RIN1 gene expression rates did not significantly correlate with other clinico-pathological parameters such as differentiated type, tumor depth, lymphatic invasion, lymph node metastasis, liver metastasis, or TNM classification (Table I).

Relationship between RIN1 gene expression and survival rates in colorectal cancer. The 5-year survival rate in 52 patients positive for expression of the RIN1 gene was significantly poorer at 55% in comparison to 83% in 49 patients negative for the expression when expression levels higher in primary colorectal cancer lesions in comparison to those in the adjacent normal colorectal tissue were regarded as positive (Fig. 3).

Localization of RIN1 protein expression in colorectal cancer cell lines. The RIN1 gene-expressing colorectal cancer cell lines, LoVo, were examined for the location of RIN1 protein by Western blotting with antibody. Membrane fraction staining of RIN1 gene-expressing colorectal cancer cell lines for cellular

Table I. The correlation between clinicopathological findings and expression of the RINl gene.

		No. of all cases	Positive cases (%)	P-value
Differentiated type	Well	45	27 (55.6)	N.S.
	Moderate	45	21 (46.7)	
	Other	11	5 (45.5)	
Tumor depth (serosal invasion)	Negative	59	29 (49.2)	N.S
	Positive	42	23 (54.8)	
Lymphatic invasion	Negative	17	6 (35.3)	N.S.
	Positive	84	46 (54.8)	
Venous invasion	Negative	43	16 (37.2)	P=0.01
	Positive	58	36 (62.1)	
Lymph node metastasis	Negative	62	30 (48.4)	N.S.
	Positive	39	22 (56.4)	
Liver metastasis	Negative	85	43 (50.6)	N.S.
	Positive	16	9 (56.3)	
TNM classification	I	10	3 (30.0)	N.S.
	II	45	23 (51.1)	
	III	25	14 (56.0)	
	IV	21	12 (57.1)	

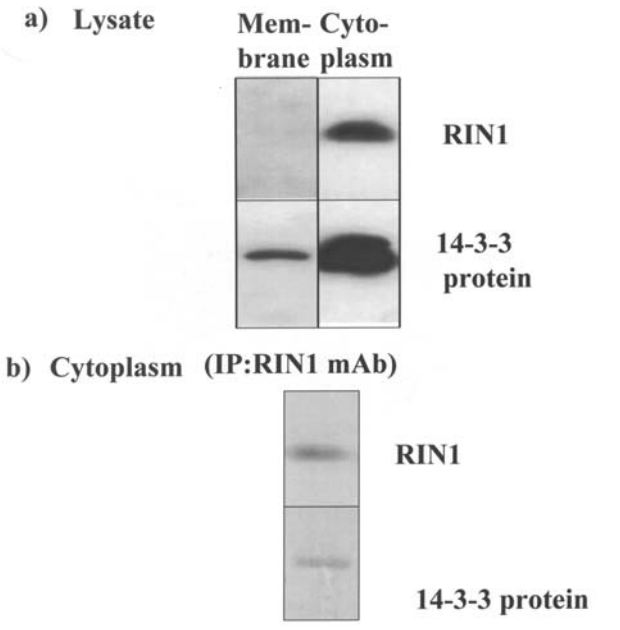

Figure 4. (a) Localization of RIN1 protein expression in colon cancer cell line. Membrane fraction staining of RIN1 gene-expressing colorectal cancer cell lines showed that RIN1 protein was expressed in the cytoplasm whereas no expression of RIN1 protein was observed in the membrane. (b) Binding of RIN1 protein to 14-3-3 protein in colon cancer cell line. RIN1 protein was bound to 14-3-3 protein in colon cancer cell lines by immunoprecipitation with anti-RIN1 antibody followed by Western blotting with anti-14-3-3 antibody.

localization showed that RIN1 protein was expressed in the cytoplasm (Fig. 4a).

Binding of RIN1 protein to 14-3-3 protein in colorectal cancer cell line. The RIN1 gene-expressing colorectal cancer cell line was examined for binding of RIN1 protein to 14-3-3 protein by immunoprecipitation with anti-RIN1 antibody followed by Western blotting with anti-14-3-3 antibody. The results showed that RIN1 protein was bound to 14-3-3 protein (Fig. 4b).

Discussion

Many studies have reported that the malignant transformation of colorectal tissue involves various gene changes including the adenoma-carcinoma sequence proposed by Vogelstein *et al* (29). It has been confirmed that several molecules such as growth factors, adhesion molecules and matrix metalloproteases (MMPs) are involved in the proliferation, invasion, and metastasis of colorectal cancer (30-38). However, no therapy to control these molecules has been established. Various factors determine cell functions such as proliferation, differentiation, and invasion, most of which are thought to involve the activation of intracellular signal transduction molecules to exert their function. In the 1990s, the currently well-known main pathways of proliferation signal transduction from the cell membrane to cytoplasm, i.e. growth factor- tyrosine kinase receptor-low molecular weight G protein (Ras)-(RAF1, Ral GDS, AF6, Nore1, and PI3-kinase pathway) were identified, and their importance was widely recognized (11-18). These pathways were well conserved throughout evolution from yeasts to mammals, and are said to be

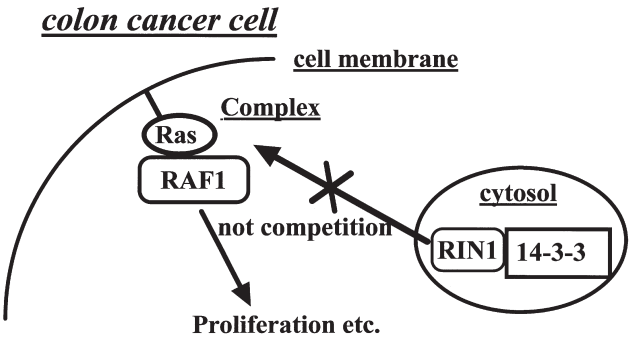

Figure 5. Model of the correlation between RIN1 protein and associated signaling molecule in colon cancer. RIN1 protein was bound to 14-3-3 protein, presumably resulting in its detention in the cytoplasm (no expression in the cell membrane), which suggests that the failure of RIN1 protein to compete, as has been reported, with H-Ras protein and RAF1 protein precludes it from fulfilling its original function.

involved in not only the proliferation but also the fate and function control of various cells.

Also, the RIN1 gene related to the H-Ras gene has been found (19-21). This gene encodes RIN1 protein, which structurally contains an SH2 domain toward the N-terminal end, and binds to ABL protein, a non-receptor tyrosine kinase. An SH3 domain is present in the middle of the molecule, and a domain which binds to H-Ras and 14-3-3 protein is present at the C-terminal end. It is known that RIN1 protein interacts directly with H-Ras and competes with RAF1, that 14-3-3 protein acts as a negative regulator of membrane localization of RIN1 protein, that the critical serine of RIN1 is a substrate for protein kinase D, and that RIN1 protein enhances the transforming properties of ABL (19,23,24). These observations have led to the speculation that RIN1 protein is a biologically important factor. First, we analyzed the state of RIN1 gene expression in colorectal cancer cell lines, and found that the RIN1 gene was expressed at different levels in all 4 cell lines used.

Next, analysis of RIN1 expression in specimens of colorectal cancers resected in our department showed that the expression was increased in 51.5% of primary colorectal cancer lesions compared with the adjacent normal colorectal mucosa. Increased RIN1 gene expression was associated with a significantly lower survival rate, suggesting that RIN1 gene overexpression contributes to the malignant potential of colorectal cancer.

The state of RIN1 protein expression in the cytoplasm or cell membrane has been reported to be important for its function (to inhibit the cell growth) (24). We examined its localization in colorectal cancer, and found that it was expressed primarily in the cytoplasm with no expression in the cell membrane, which suggests that the failure of RIN1 protein to compete, as has been reported, with H-Ras protein and RAF1 protein precludes it from fulfilling its original function (Fig. 5). Next, to investigate the causes of the failure of RIN1 protein to migrate to the cell membrane, we examined its relationship with 14-3-3 protein, a known negative regulator, and found that RIN1 protein was bound to 14-3-3 protein, presumably resulting in its detention in the cytoplasm (Figs. 4 and 5).

ugh detailed studies of the RIN1 gene are needed ture, we first found in this study that the RIN1 gene was abnormally expressed in many colorectal cancer specimens and that its abnormal expression was associated with a decreased survival rate. These findings will serve as a step forward to defining the degree of malignancy of colorectal cancer.

References

1. Ullrich A and Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. Cell 61: 203-212, 1990.
2. Yamashita M, Kimura M, Kubo M, *et al*: T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. Proc Natl Acad Sci USA 96: 1024-1029, 1999.
3. Satoh T, Minami Y, Kono T, *et al*: Interleukin 2-induced activation of Ras requires two domains of interleukin 2 receptor beta subunit, the essential region for growth stimulation and Lck-binding domain. J Biol Chem 267: 25423-25427, 1992.
4. Hubbard SR, Mohammadi M and Schlessinger J: Autoregulatory mechanisms in protein-tyrosine kinases. J Biol Chem 273: 11987-11990, 1998.
5. Heldin CH: Dimerization of cell surface receptors in signal transduction. Cell 80: 213-223, 1995.
6. Pawson T: Protein modules and signalling networks. Nature 373: 573-580, 1995.
7. Buday L and Downward J: Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 73: 611-620, 1993.
8. Hunter T: Oncoprotein networks. Cell 88: 333-346, 1997.
9. Boriack-Sjodin PA, Margarit SM, Bar-Sagi D and Kuriyan J: The structural basis of the activation of Ras by Sos. Nature 394: 337-343, 1998.
10. Buday L, Warne PH and Downward J: Downregulation of the Ras activation pathway by MAP kinase phosphorylation of Sos. Oncogene 11: 1327-1331, 1995.
11. Vojtek AB and Der CJ: Increasing complexity of the Ras signaling pathway. J Biol Chem 273: 19925-19928, 1998.
12. Herrmann C, Horn G, Spaargaren M and Wittinghofer A: Differential interaction of the ras family GTP-binding proteins H-Ras, Rap1A, and R-Ras with the putative effector molecules Raf kinase and Ral-guanine nucleotide exchange factor. J Biol Chem 271: 6794-6800, 1996.
13. Pacold ME, Suire S, Perisic O, *et al*: Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. Cell 103: 931-943, 2000.
14. Deng T and Karin M: c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. Nature 371: 171-175, 1994.
15. Rodriguez-Viciana P, Warne PH, Dhand R, *et al*: Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370: 527-532, 1994.
16. Feig LA, Urano T and Cantor S: Evidence for a Ras/Ral signaling cascade. Trends Biochem Sci 21: 438-441, 1996.
17. Kuriyama M, Harada N, Kuroda S, *et al*: Identification of AF-6 and canoe as putative targets for Ras. J Biol Chem 271: 607-610, 1996.
18. Vavvas D, Li X, Avruch J and Zhang XF: Identification of Nore1 as a potential Ras effector. J Biol Chem 273: 5439-5442, 1998.
19. Han L and Colicelli J: A human protein selected for interference with Ras function interacts directly with Ras and competes with Raf1. Mol Cell Biol 15: 1318-1323, 1995.
20. Wang Y and Colicelli J: RAS interaction with effector target RIN1. Methods Enzymol 332: 139-151, 2001.
21. Colicelli J, Nicolette C, Birchmeier C, Rodgers L, Riggs M and Wigler M: Expression of three mammalian cDNAs that interfere with RAS function in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 88: 2913-2917, 1991.
22. Han L, Wong D, Dhaka A, *et al*: Protein binding and signaling properties of RIN1 suggest a unique effector function. Proc Natl Acad Sci USA 94: 4954-4959, 1997.
23. Afar DE, Han L, McLaughlin J, *et al*: Regulation of the oncogenic activity of BCR-ABL by a tightly bound substrate protein RIN1. Immunity 6: 773-782, 1997.
24. Wang Y, Waldron RT, Dhaka A, *et al*: The RAS effector RIN1 directly competes with RAF and is regulated by 14-3-3 proteins. Mol Cell Biol 22: 916-926, 2002.
25. Fleming ID, Cooper JS, Henson DE, *et al*: AJCC Cancer Staging Manual. 5th edition. Lippincott-Raven, Philadelphia, 1997.
26. Japanese Society for Cancer of the Colon and Rectum: Japanese Classification of Colorectal Carcinoma. 1st English edition. Kanehara, Tokyo, 1997.
27. Goi T, Yamaguchi A, Nakagawara G, Urano T, Shiku H and Furukawa K: Reduced expression of deleted colorectal carcinoma (DCC) protein in established colon cancers. Br J Cancer 77: 466-471, 1998.
28. Sato T, Yamaguchi A, Goi T, *et al*: Heparanase expression in human colorectal cancer and its relationship to tumor angiogenesis, hematogenous metastasis, and prognosis. J Surg Oncol 87: 174-181, 2004.
29. Vogelstein B, Fearon ER, Hamilton SR, *et al*: Genetic alterations during colorectal-tumor development. N Engl J Med 319: 525-532, 1988.
30. Takeuchi T, Hisanaga M, Nagao M, *et al*: The membrane-anchored matrix metalloproteinase (MMP) regulator RECK in combination with MMP-9 serves as an informative prognostic indicator for colorectal cancer. Clin Cancer Res 10: 5572-5579, 2004.
31. Onogawa S, Kitadai Y, Tanaka S, Kuwai T, Kimura S and Chayama K: Expression of VEGF-C and VEGF-D at the invasive edge correlates with lymph node metastasis and prognosis of patients with colorectal carcinoma. Cancer Sci 95: 32-39, 2004.
32. Goi T, Koneri K, Katayama K, Hirose K and Yamaguchi A: Evaluation of clinicopathological factors and the correlation between the adhesion molecule CD44 variant 9 expression and pulmonary metastases from colorectal cancers. Int Surg 87: 130-136, 2002.
33. Yamaguchi A, Goi T, Taguchi S, *et al*: Clinical significance of serum levels of CD44 variant exons 8-10 protein in colorectal cancer. J Gastroenterol 33: 349-353, 1998.
34. Waas ET, Wobbes T, Lomme RM, DeGroot J, Ruers T and Hendriks T: Matrix metalloproteinase 2 and 9 activity in patients with colorectal cancer liver metastasis. Br J Surg 90: 1556-1564, 2003.
35. Holten-Andersen MN, Christensen IJ, Nielsen HJ, *et al*: Total levels of tissue inhibitor of metalloproteinases 1 in plasma yield high diagnostic sensitivity and specificity in patients with colon cancer. Clin Cancer Res 8: 156-164, 2002.
36. Takamura M, Ichida T, Matsuda Y, *et al*: Reduced expression of liver-intestine cadherin is associated with progression and lymph node metastasis of human colorectal carcinoma. Cancer Lett 212: 253-259, 2004.
37. Ueda T, Shimada E and Urakawa T: Serum levels of cytokines in patients with colorectal cancer: possible involvement of interleukin-6 and interleukin-8 in hematogenous metastasis. J Gastroenterol 29: 423-429, 1994.
38. Kawamoto K, Onodera H, Kan S, Kondo S and Imamura M: Possible paracrine mechanism of insulin-like growth factor-2 in the development of liver metastases from colorectal carcinoma. Cancer 85: 18-25, 1999.