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KPNA2 Over-Expression is a Potential Marker of Prognosis and Therapeutic Sensitivity in Colorectal Cancer Patients

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Background: Karyopherin α 2 (KPNA2) is a member of the Karyopherin α family and has recently been reported to play an important role in tumor progression. The aim of the current study was to elucidate the clinicopathological significance of KPNA2 over-expression in colorectal cancer (CRC).

Patients and Methods: KPNA2 expression was evaluated by immunohistochemistry in 122 surgically resected CRC and 13 biopsy specimens obtained at colonoscopy during screening for preoperative hyperthermochemoradiation therapy (HCRT). The association between KPNA2 expression and clinicopathological features and preoperative HCRT efficacy were examined.

Results: The high and low KNPA2 expression groups were comprised of 91 (74.6%) and 31 CRC patients, respectively. A significant association was observed between high expression and lymphatic invasion ($P = 0.0245$). KPNA2 high expression group had decreased overall survival ($P = 0.00374$). Multivariate analysis demonstrated high KPNA2 expression was independently associated with poor prognosis. Histological examinations revealed 11 (84.6%) and 2 (15.4%) of cases were KPNA2 positive and negative, respectively. Pathological complete response (pCR) was observed in 9.1% of KPNA2-positive cases and 100% of KPNA2-negative cases.

Conclusion: High KPNA2 expression was found to be associated with poor prognosis and resistance to HCRT.
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KEY WORDS: hyperthermo-chemo-radiation therapy; HCRT; tumor progression

INTRODUCTION

The incidence of colorectal cancer (CRC) is increasing worldwide [1,2] and currently represents one of the most common cases of cancer death [1]. The development of multimodal therapy has improved CRC prognosis [3]; however, there is hope of further improvements and treatment breakthroughs in the near future.

Preoperative radiotherapy (RT) is often necessary in cases of rectal cancer. Preoperative RT is effective in controlling primary lesions [4,5], and chemotherapy and hyperthermia have been shown to improve the effectiveness of RT [6,7]. Pathological complete response (pCR) is reportedly observed in 10–20% of patients who undergo preoperative RT [6,8]. These findings indicate the prediction of therapeutic effect prior to RT may allow curative treatments rectal cancer without the need for resection. However, predictors of the therapeutic effect of RT have yet to be demonstrated.

Karyopherin αs are members of the importin/karyopherin superfamily and function in regulation the transportation of proteins from the cytoplasm into the nucleus. Karyopherin αs bind the nuclear localization signals of proteins and karyopherin β and mediate the transport of proteins weighing greater than 50 kDa via nuclear pore complexes [9,10]. Karyopherin α 2 (KPNA2) is as isoform of Karyopherin α and has been reported as a marker of poor prognosis in a number of solid tumor types [11–13]. In vitro studies have shown KPNA2 to be essential in repairing DNA double strand breaks (DSBs) caused by ionizing radiation[14]. However, only small scale analyses of KPNA2 expression have been performed in CRC [15].

The aim of the present study was to elucidate the significance of KPNA2 as a marker of prognosis and therapeutic efficacy in CRC patients. We examined the expression levels of KPNA2 in CRC tissue samples using immunohistochemistry to determine the utility of KPNA2 expression in cancer tissues as a prognostic biomarker for CRC. Further, we examined the relationship between RT therapeutic grade and KPNA2 expression.

MATERIALS AND METHODS

Clinical Samples

Surgical specimens from 122 patients (74 males and 48 females) who had underwent resection of a primary CRC lesion at the Department of General Surgical Science, Graduate School of Medicine, Gunma University between 1999 and 2009 were included in the present study. No patients had received irradiation or chemotherapy prior to surgery. Biopsy specimens from 13 patients

Abbreviations: CRC, colorectal cancer; KPNA2, karyopherin α 2; RT, radiotherapy; HCRT, hyperthermochemoradiation therapy; pCR, pathological complete response; DSB, double strand break.

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with rectal cancer were obtained and all of these patients underwent preoperative hyperthermochemoradiation therapy (HCRT). All biopsy specimens were collected during pre-therapeutic colonoscopy for HCRT. All clinical data in the present study were collected, stored, and used in accordance with institutional guidelines and the Helsinki Declaration after obtaining written informed consent from all participants.

Preoperative HCRT Protocol

Radiation treatment was delivered by 10-MV x-rays using a three-field box technique. Clinical target volumes encompassed the primary tumor and all mesorectal tissues. The total radiation dose was 50 Gy given in daily fractions of 2.0 Gy on five consecutive days per week. Chemotherapy consisted of capecitabine (1,700 mg/m^2 per day) administered for five days a week with radiation. Two to five hyperthermia sessions were performed once a week with 8 MHz radiofrequency capacitive heating equipment (Thermotron-RF 8, Yamamoto Vinita Co., Ltd., Osaka, Japan).

Immunohistochemical Staining

Immunohistochemistry was performed as previously described [13]. Slides were incubated with primary antibodies against KPNA2 (1: 400; from Abcam) for 24 hr at 4°C. Negative controls were incubated without primary antibody, with and no detectable staining observed.

Immunohistochemical slides were scanned and evaluated by two experienced researchers. The intensity of nuclear KPNA2 staining was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The proportion of cells with nuclear KPNA2 staining was evaluated by examining at least 2,000 cancer cells in 5 representative areas. The proportion of cells with nuclear KPNA2 staining was scored as follows: 0, no staining; 1, 1–10% positive; 2, 11–50% positive; and 3, 51–100% positive. The KPNA2 evaluation score was defined as the proportion of nuclear-stained cells multiplied by the intensity score (0, 1, 2, 3, 4, 6, or 9). The optimal cut-off point, as defined by ROC curve analysis, was used to classify cases into high (6 and 9) and low (0, 1, 2, 3, and 4) expression groups. For biopsy specimens, the proportion of cells with nuclear KPNA2 staining was evaluated using a single representative area as specimens were too small to apply the full scoring method. Nuclear KPNA2 expression was used to classify cases into KPNA2-positive (nuclear staining score, 2–3) and negative (nuclear staining score, 0–1) groups.

Statistical Analyses

For continuous variables, data were expressed as means ± standard deviation (SD). Comparisons between the high and low KPNA2 expression groups were performed using Student's *t*-test, the χ^2 test, and ANOVA. Survival times were plotted according to the Kaplan–Meier method, with the log-rank test used for comparisons. The differences were considered statistically significant at the level of $P < 0.05$. Relative multivariate significance of potential prognostic variables was examined. Cox proportional hazards regression was used to test the independent prognostic contribution of clinicopathological factors. All statistical analysis was performed with R script generated by EZR [16].

RESULTS

Immunohistochemical Analysis of KPNA2 Expression in CRC Tissues

Immunohistochemical staining were used to evaluate KPNA2 expression in CRC specimens. KPNA2 expression was predominantly observed in the nuclei of tumor cells, with little expression observed in the cytoplasm of either normal or tumor cells (Fig. 1a). The high

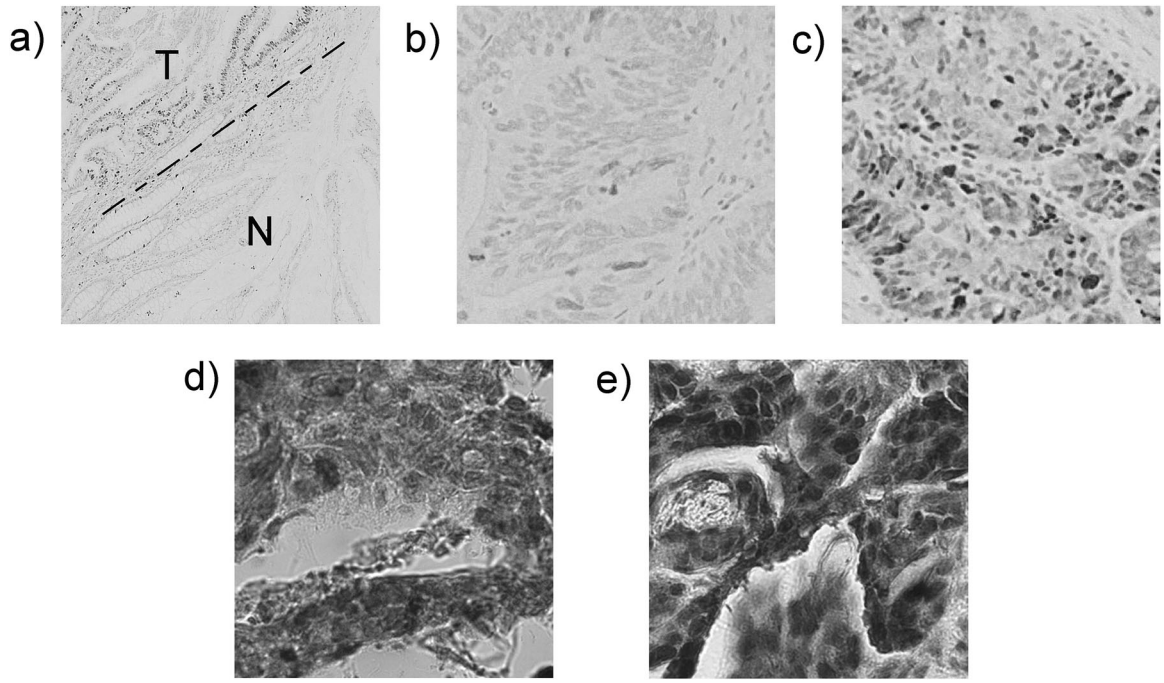

Fig. 1. Immunohistochemical analysis of KPNA2 expression in CRC tissue samples. Deparaffinized sections of CRC tumors were stained with anti-KPNA2 antibodies and counterstained with hematoxylin. a) Normal mucosa and tumor (T, tumor; N, normal mucosa). KPNA2 was highly expressed in tumoral tissues and rarely in normal mucosa. b) CRC patient from the low KPNA2 expression group. c) CRC patient from the high KPNA2 expression group. d) KPNA2-negative biopsy specimen. e) KPNA2-positive biopsy specimen.

KPNA2 expression group was comprised as 91 specimens (91/122; 74.6%) and the low KPNA2 expression group was comprised of 31 (31/122; 25.4%) specimens according to the KPNA2 evaluation score (Fig. 1b,c, and Supplementary Fig. S1). KPNA2 expression was also evaluated in biopsy specimens, with 11 specimens assigned to the KPNA2-positive group (11/13; 84.6%) and 2 specimens assigned to the KPNA1-negative group (2/11; 15.4%; Fig. 1d,e).

Association Between KPNA2 Expression and Clinicopathological Features of CRC

Table I shows the correlation between KPNA2 expression and patient clinicopathological characteristics (age, gender, tumor histology, T factor, lymph node metastasis, lymphatic and venous invasion, liver metastasis, peritoneal dissemination, and pathological stage).

Significantly greater lymphatic invasion was observed in the high KPNA2 expression group ($P = 0.0245$). No significant difference were observed in other factors; however, there was a trend toward more undifferentiated cellular histology ($P = 0.121$) and higher pathological stage ($P = 0.0817$) in the high KPNA2 expression group.

Prognostic Significance of KPNA2 Expression in CRC Patients

Overall survival rates in the high and low KPNA2 expression groups are shown in Figure 2a. The 5-year survival rate was significantly lower in the high KPNA2 expression group (69.6%) compared to the low KPNA2 expression group (89.5%; $P = 0.0374$). The disease-free survival rates of stage I–III patients who underwent curative resection are shown in Figure 2b. No significant difference in disease-free survival rate was observed between cases with high KPNA2 expression and cases with low KPNA2 expression ($P = 0.754$). Survival rates after recurrence are shown in Figure 2c. No significant difference in survival rate after recurrence was observed between cases with high KPNA2 expression and cases with low KPNA2 expression; however, there was a trend toward increased survival in cases with high KPNA2 expression compared to those with low KPNA2 expression (median survival time, 3.98 yeas vs. 6.74 years; $P = 0.184$).

Univariate and multivariate analyses of overall survival times are shown in Table II. Lymph node metastasis, liver metastasis, peritoneal dissemination, and high nuclear KPNA2 expression were found to be independent prognostic factors ($P < 0.05$).

TABLE I. Backgrounds and Clinicopathological Characteristics of the Patients

Factors	KPNA2		*P*-value
	Low expression n = 31	High expression n = 91	
Age	63.5 ± 1.6	64.5 ± 12.0	0.681
Gender			
Male	18	56	0.832
Female	13	35	
Histology			
wel	14	22	0.121
mod	17	62	
por	0	4	
muc	0	3	
T factor			
Tis, T1, T2	9	19	0.458
T3, T4	22	72	
Lymph node metastasis			
Absent	15	46	1
Present	16	45	
Lymphatic invasion			
Absent	10	11	0.0245*
Present	21	80	
Venous invasion			
Absent	15	33	0.288
Present	16	58	
Liver metastasis			
Absent	24	69	1
Present	7	22	
Peritoneal dissemination			
Absent	30	87	1
Present	1	4	
Pathological stage			
0	3	0	0.0817
I	4	10	
II	7	27	
III	11	30	
IV	6	24	

The Immunohistochemical Analysis of Biopsy Specimens was Associated With the Effect of HCRT

We evaluated KPNA2 expression in biopsy specimens obtained during colonoscopy before HCRT. The pCR rate was 100% in KPNA2-negative cases and 9.1% in KPNA2-positive cases (Table III; $P = 0.0385$).

DISCUSSION

The results of the present study demonstrated KPNA2 expression was significantly higher in the nucleus of cells in cancer tissues compared with normal mucosa. High KPNA2 expression was correlated greater lymphatic invasion and shorter survival time, and was found to be an independent prognostic factor. KPNA2-positive cases were also more likely to be resistant to HCRT. To the best of our knowledge, this is the first report of a correlation between KPNA2 expression and the efficacy of radiation therapy in CRC.

Our results corroborate those of previous studies which have reported KPNA2 as a marker of poor prognosis in other solid tumor types [11–13]. In the present study, there was a trend toward lower degree of differentiation and higher pathological states in the high KPNA2 expression group. KPNA2 is known to transport cell cycle regulators, such as cMyc [17] and RAC1 [18]. Myc is a target of the Wnt and RAS signaling pathways and has been shown to be highly expressed in CRC [19,20]. MYC deletion has been shown to suppress colon tumorigenesis in a murine model [21]. RAC1 [22] is expressed in CRC [23] and is reportedly a marker of poor prognosis in several solid tumor types [24–26]. RAC1 activates a number of oncogenes, including RAS and Tiam1, and promotes cancer development and progression [27,28]. As KPNA2 functions in recruiting oncoproteins into the nucleus, we believe high KPNA2 expression plays a key role in cancer development and progression. The results of the present study are consistent with this hypothesis and KPNA2 may have utility as a prognostic marker in CRC.

In the present study, the high KPNA2 expression group had poorer prognosis; however, no difference in disease free survival was observed between the high and low KPNA2 expression groups. Survival times after recurrence tended to be shorter in the high KPNA2 expression group. Furthermore, KPNA2-positive cases were more likely to be resistant to HCRT. These findings indicate KPNA2 expression is associated with the therapeutic efficacy of RT in cases of CRC. NBS1 is a cargo protein of KPNA2 [14] and forms a complex with MRE11 and RAD50 (the MRN complex). The MRN complex activates ATM and is an important trigger for DSB repair cascades [14]. The suppression of the MRN complex enhances the effect of radiation in head and neck cancers [29]. Further, the blockade of RAD50, a component of the MRN complex, enhances the efficacy of platinum agents in squamous cell carcinoma [30]. Chk2 is also a cargo protein of KPNA2 and function in the activation of ATM and promotion of DNA DSB repair [31]. Clinical

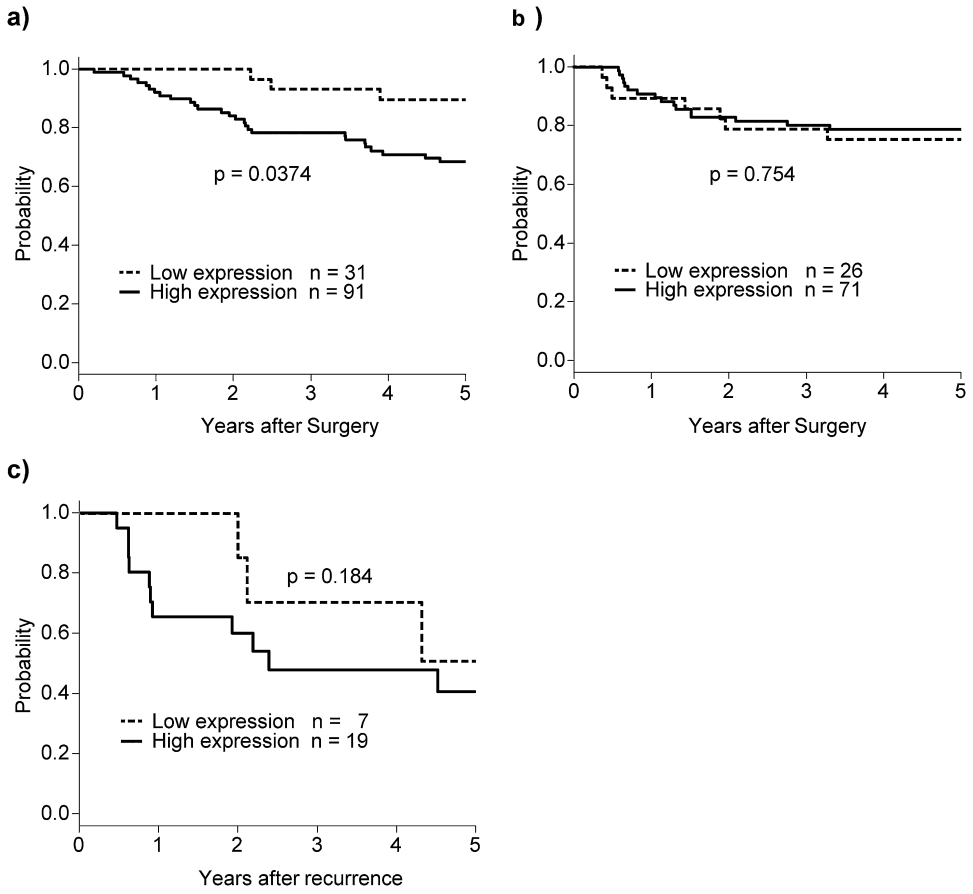

Fig. 2. a) Overall survival after surgery. b) Disease-free survival after surgery. Patients with stage I–III disease are included. c) Overall survival after recurrence. Patients with stage I–III disease who had recurrence are included.

TABLE II. Univariate and Multivariate Analyses of Overall Survival

	Univariate analysis			Multivariate analysis		
Clinicopathological variables	HR	95%CI	*P*-value	HR	95%CI	*P*-value
Age	0.982	0.956–1.01	0.186	—	—	—
Gender	0.684	0.331–1.41	0.304	—	—	—
T factor (Tis, T1, T2, T3/T4)	3.02	1.50–6.09	<0.01*	1.14	0.526–2.47	0.741
Lymph node metastasis (negative/positive)	3.37	1.57–7.26	<0.01*	2.50	1.13–5.51	0.023*
Lymphatic invasion (negative/positive)	3.94	0.942–16.5	0.060	—	—	—
Venous invasion (negative/positive)	2.97	1.29–6.86	0.011*	1.36	0.568–3.28	0.487
Liver metastasis (negative/positive)	7.97	3.89–16.3	<0.01*	6.02	2.71–13.4	<0.01*
Peritoneal dissemination (negative/positive)	9.88	3.35–29.1	<0.01*	6.43	1.90–21.8	<0.01*
KPNA2 expression (low/high)	2.89	1.02–8.22	0.047*	4.25	1.44–12.6	<0.01*

HR, hazard ratio; CI, confidence interval.
*$P < 0.05$.

TABLE III. KPNA2 Expression and Effect of HCRT

	KPNA2 expression		
	Negative	Positive	
Non-pCR	0	10	
pCR	2	1	$P = 0.0385$

pCR, pathological complete response.

studies evaluating Chk2 as a therapeutic target are currently in progress [32,33]. KPNA2 functions in recruiting these proteins, which promote DNA DSB repair, and high expression of KPNA2 may increase the activity of DSB repair and enhance the chemo- and radio-resistance of cancer cells. We propose KPNA2 as a potential marker of therapeutic effect and believe KPNA2 suppression may enhance the efficacy of HCRT through reducing the activity of DNA DSB repair factors in the nucleus. KPNA2 may have utility as a therapeutic target in conjunction with HCRT and may allow curative treatment of CRC without the need for resection.

CONCLUSIONS

KPNA2 over-expression may have utility as a marker of poor prognosis and therapeutic resistance in CRC patients represents a potential therapeutic target for enhancing RT efficacy.

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REFERENCES

1. Torre LA, Bray F, Siegel RL, et al.: Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87–108.
2. Jemal A, Bray F, Center MM, et al.: Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
3. Bosman FT, Hamilton SR, Lambert R: In: Stewart W B, Wild C P, editors. Colorectal Cancer in World Cance Report 2014. Geneve: World Health Organization; 2014.pp. 392–402.
4. Cammà C, Giunta M, Fiorica F, et al.: Preoperative radiotherapy for resectable rectal cancer A meta-analysis. JAMA 2000;284: 1008–10015.
5. Colorectal Cancer Collaborative Group: Adjuvant radiotherapy for rectal cancer: A systematic overview of 8,507 patients from 22 randomised trials. Lancet 2001;358:1291–304.
6. Tsutsumi S, Tabe Y, Fujii T, et al.: Tumor response and negative distal resection margins of rectal cancer after hyperthermochemoradiation therapy. Anticancer Res 2011;31:3697–3963.
7. Shoji H, Motegi M, Osawa K, et al.: A novel strategy of radiofrequency hyperthermia (neothermia) in combination with preoperative chemoradiotherapy for the treatment of advanced rectal cancer: A pilot study. Cancer Med 2015.
8. Wadlow RC, Ryan DP: The role of targeted agents in preoperative chemoradiation for rectal cancer. Cancer 2010;116:3537–3548.
9. Davis LI: The nuclear pore complex. Annu Rev Biochem 1995;64:865–896.
10. Lange A, Mills RE, Lange CJ, et al.: Classical nuclear localization signals: Definition, function, and interaction with importin alpha. J Biol Chem 2007;282:5101–5105.
11. Gluz O, Wild P, Meiler R, et al.: Nuclear karyopherin alpha2 expression predicts poor survival in patients with advanced breast cancer irrespective of treatment intensity. Int J Cancer 2008;123: 1433–1438.
12. Sakai M, Sohda M, Miyazaki T, et al.: Significance of karyopherin-{alpha} 2 (KPNA2) expression in esophageal squamous cell carcinoma. Anticancer Res 2010;30:851–856.
13. Altan B, Yokobori T, Mochiki E, et al.: Nuclear karyopherin-α2 expression in primary lesions and metastatic lymph nodes was associated with poor prognosis and progression in gastric cancer. Carcinogenesis 2013;34:2314–2321.
14. Tseng SF, Chang CY, Wu KKJ, et al.: Importin KPNA2 is required for proper nuclear localization and multiple functions of NBS1. J Biol Chem 2005;280:39594–39600.
15. Rachidi SM, Qin T, Sun S, et al.: Molecular profiling of multiple human cancers defines an inflammatory cancer-associated molecular pattern and uncovers KPNA2 as a uniform poor prognostic cancer marker. PLoS ONE 2013;8:e57911.
16. Kanda Y: Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant 2013;48:452–458.
17. Nadler SG, Tritschler D, Haffar OK, et al.: Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences. J Biol Chem 1997;272:4310–4315.
18. Sandrock K, Bielek H, Schradi K, et al.: The nuclear import of the small GTPase Rac1 is mediated by the direct interaction with karyopherin alpha2. Traffic 2010;11:198–209.
19. Cancer Genome Atlas Network: Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012; 487:330–337.
20. Wetering van de M, Sancho E, Verweij C, et al.: The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241–250.
21. Sansom OJ, Meniel VS, Muncan V, et al.: Myc deletion rescues Apc deficiency in the small intestine. Nature 2007;446:676–679.
22. Pankov R, Endo Y, Even-Ram S, et al.: A Rac switch regulates random versus directionally persistent cell migration. J Cell Biol 2005;170:793–802.
23. Jordan P, Brazåo R, Boavida MG, et al.: Cloning of a novel human Rac1b splice variant with increased expression in colorectal tumors. Oncogene 1999;18:6835–6839.
24. Du X, Wang S, Lu J, et al.: Wang,Clinical value of Tiam1-Rac1 signaling in primary gallbladder carcinoma. Med Oncol 2012;29: 1873–1878.
25. Wang SM, Ooi LLPJ, Hui KM: Upregulation of Rac GTPase-activating protein 1 is significantly associated with the early recurrence of human hepatocellular carcinoma. Clin Cancer Res 2011;17:6040–6051.
26. Yuan K, Qian C, Zheng R: Prognostic significance of immunohistochemical Rac1 expression in survival in early operable non-small cell lung cancer. Med Sci Monit 2009;15:313–319.
27. Qiu RG, Chen J, Kirn D, et al.: An essential role for Rac in Ras transformation. Nature 1995;374:457–459.
28. Malliri A, van der Kammen RA, Clark K, et al.: Collard, Mice deficient in the Rac activator Tiam1 are resistant to Ras-induced skin tumors. Nature 2002;417:867–871.
29. Malley BWO', Li D, Carney J, et al.: Molecular disruption of the MRN(95) complex induces radiation sensitivity in head and neck cancer. Laryngoscope 2003;113:1588–1594.
30. Abuzeid WM, Jiang X, Shi G, et al.: Molecular disruption of RAD50 sensitizes human tumor cells to cisplatin-based chemotherapy. J Clin Invest 2009;119:1974–1985.
31. Jiang H, Reinhardt HC, Bartkova J, et al.: The combined status of ATM and p53 link tumor development with therapeutic response. Genes Dev 2009;23:1895–1909.
32. Bucher N, Britten CD: G2 checkpoint abrogation and checkpoint kinase-1 targeting in the treatment of cancer. Br J Cancer 2008;98:523–528.
33. Matthews TP, Jones AM, Collins I: Structure-based design, discovery and development of checkpoint kinase inhibitors as potential anticancer therapies. Expert Opin Drug Discov 2013;6: 621–640.