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BRIEF ARTICLE

Interleukin-24 is correlated with differentiation and lymph node numbers in rectal cancer

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Abstract

AIM: To assess the significance of interleukin (IL)-24 and vascular endothelial growth factor (VEGF) expression in lymph-node-positive rectal cancer.

METHODS: Between 1998 and 2005, 90 rectal adenocarcinoma patients with lymph node involvement were enrolled. All patients received radical surgery and postoperative pelvic chemoradiotherapy of 50.4-54.0 Gy. Chemotherapy of 5-fluorouracil and leucovorin or levamisole was given intravenously during the first and last week of radiotherapy, and then monthly for about 6 mo. Expression of IL-24 and VEGF was evaluated by immunohistochemical staining of surgical specimens, and their relations with patient characteristics and survival were analyzed. The median follow-up of surviving patients was 73 mo (range: 52-122 mo).

RESULTS: IL-24 expression was found in 81 out of 90 patients; 31 showed weak intensity and 50 showed strong intensity. VEGF expression was found in 64 out of 90 patients. Negative and weak intensities of IL-24 expression were classified as negative expression for analysis. IL-24 expression was significantly reduced in poorly differentiated tumors in comparison with well or moderately differentiated tumors (P = 0.004), N2b to earlier N stages (P = 0.016), and stage Ⅲc to stage Ⅲa or Ⅲb (P = 0.028). The number of involved lymph nodes was also significantly reduced in IL-24-positive patients in comparison with IL-24-negative ones. There was no correlation between VEGF expression and patient characteristics. Expression of IL-24 and VEGF was not correlated with survival, but N stage and stages were significantly correlated with survival.

CONCLUSION: IL-24 expression was significantly correlated with histological differentiation, and inversely correlated with the degree of lymph node involvement in stage Ⅲ rectal cancer.

Key words: Interleukin-24; Rectal cancer; Lymph node; Histological differentiation; Vascular endothelial growth factor

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INTRODUCTION

Treatment outcomes of rectal cancer have been improved with the use of adequate combination therapies

and the identification of effective targets, but relapses are still frequent in advanced-stage patients. The survival rate of patients whose tumors are confined to the rectal wall at diagnosis (stage Ⅰ and Ⅱ) is > 75%, but those rates are reduced to 30%-60% in higher-stage patients, according to the degree of penetration into the wall, and lymph node involvement[1,2]. In order to yield more credibility and power to a study, we analyzed stage Ⅲ patients for the identification of new therapeutic targets.

The melanoma differentiation associated gene-7, later renamed as interleukin (IL)-24, was identified by subtraction hybridization from human melanoma cells stimulated with interferon-β and mezerein[3]. The expression of IL-24 is inversely related to human melanoma progression. That is, it is highest in melanocytes and lowest in metastatic melanomas[3,4]. Transfection of IL-24 into melanoma cells reduces growth without a similar effect on normal cells[3], and this antiproliferative activity of IL-24 has also been detected in a variety of cancer cells, such as breast, prostate, cervix, colorectal, lung, and nasopharynx carcinomas, as well as glioblastoma[5-11]. In addition to the antiproliferative effect in cancer cell lines, favorable survival has been marginally identified in high-IL-24-expressing non small cell lung cancer (NSCLC) patients[12]. From a subset analysis, high IL-24 expression has been revealed as a significant prognostic factor in adenocarcinoma patients. Whether high IL-24 expression also has a significant impact on the survival of patients with types of cancers other than NSCLC is unknown.

Multiple anticancer mechanisms of IL-24 have been reported, including cancer-specific apoptosis induction, cell cycle regulation, an ability to inhibit angiogenesis, potent bystander antitumor activity, and a capacity to enhance the sensitivity of tumor cells to radiation and chemotherapy[13]. Among them, new vessel formation is required for tumor growth and metastasis[14]. Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors[15]. Expression of VEGF is proportional to the degree of carcinogenesis of the colorectum, ranging from 0% in dysplastic adenomas, to 62% in mucosal carcinomas, and 100% in submucosal carcinomas[16]. However, the prognostic significance of VEGF expression in rectal cancers has been inconclusive so far[17-21]. Therefore, we also analyzed VEGF expression in rectal cancer patients.

We carried out the first analysis of the correlation between IL-24 expression and prognostic features in rectal cancer patients. To resolve the unanswered question of the effect of VEGF expression on the survival of rectal cancer patients, while limiting biases related to treatment methods and patient heterogeneity, we restricted the analysis to rectal cancer patients with lymph node metastasis who were treated at a single institution.

MATERIALS AND METHODS

Patients

In this retrospective study, we reviewed 96 rectal adenocarcinoma patients with pathologic lymph node involvement, who had consecutively undergone radical surgery and postoperative chemoradiotherapy at Dong-a University Hospital, Busan, South Korea between 1998 and 2005. The analysis of these patients was approved by the Institutional Review Board. Ninety patients were included for analysis, while six patients were excluded; two for lack of surgical specimens, and four due to liver metastasis at diagnosis, familial adenomatosis polyposis, adenosquamous cell carcinoma, and mucinous adenocarcinoma, respectively (Table 1).

Table 1 Patients characteristics

	n (%)
Age (yr)	
Median	59
Range	34 - 77
Sex	
Male	47 (52.2)
Female	43 (47.8)
Histologic differentiation	
Well	46 (51.1)
Moderately	34 (37.8)
Poorly	10 (11.1)
T stage	
T1	1 (1.1)
T2	8 (8.9)
T3	79 (87.8)
T4	2 (2.2)
N stage	
N1a	24 (26.7)
N1b	26 (28.9)
N2a	16 (17.8)
N2b	24 (26.7)
Stage	
Ⅲa	8 (8.9)
Ⅲb	57 (63.3)
Ⅲc	25 (27.8)
VEGF expression	
Negative	26 (28.9)
Positive	64 (71.1)
Intensity of IL-24 expression	
Negative	9 (10.0)
Weak	31 (34.4)
Strong	50 (55.6)

IL: Interleukin; VEGF: Vascular endothelial growth factor.

Thirty-three patients underwent abdominoperineal resection, and 57 underwent low anterior resection. Patients received postoperative chemoradiotherapy from the fourth to sixth week after radical surgery. Patients were positioned in a prone position on a belly board for radiotherapy. Tumor beds were boosted up to 50.4-54 Gy (1.8 Gy, once daily) after 45 Gy pelvic irradiation with 15 MV X-ray using a three-field technique. Two cycles of 5-fluorouracil with levamisole or leucovorin were concurrently given for radiotherapy, and maintenance chemotherapy was done thereafter for about 6 mo. Patients were followed up at 3-mo intervals for 2 years, 4-mo intervals for the next 2 years, and then every 6 mo. The median follow-up period of the surviving patients was 73 mo (range: 52-122 mo). The seventh edition of the American Joint Committee on Cancer TNM staging system (2010) was used for patient analysis.

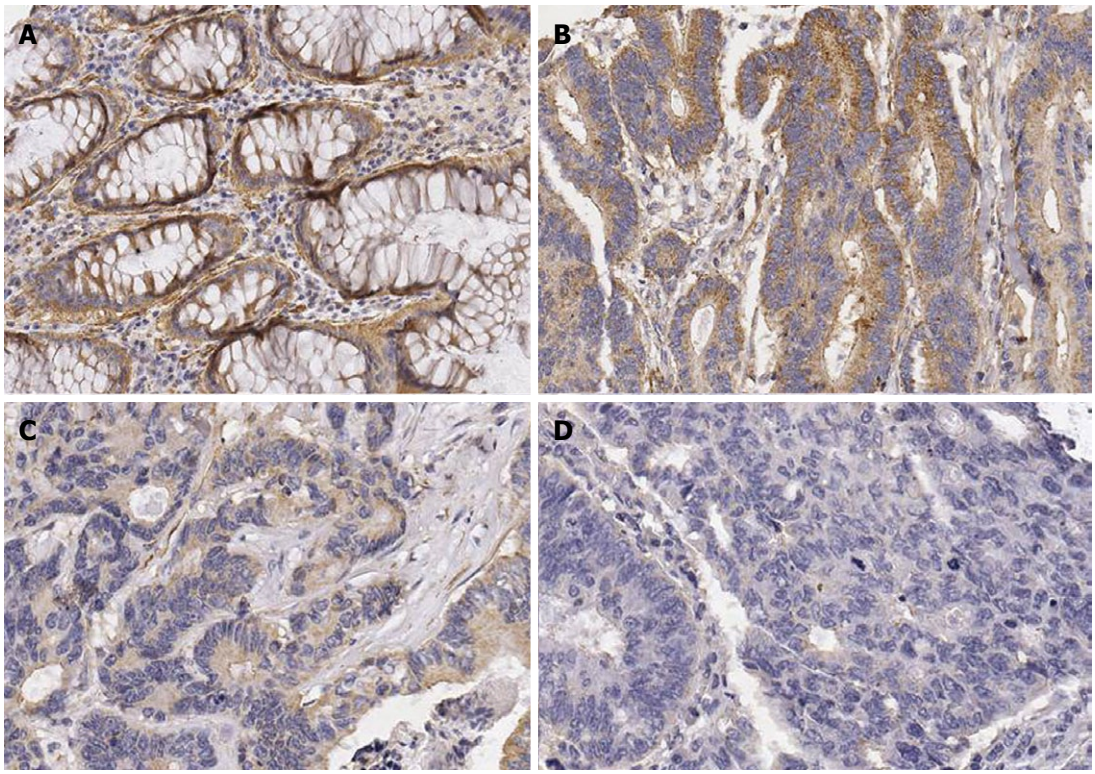

Figure 1 Immunohistochemical staining of interleukin-24 in rectal tissue (200 ×). A: In the normal rectal mucosa tissue, the non-neoplastic glandular epithelial cells were strongly positive for IL-24; B: In rectal cancer, a well-differentiated adenocarcinoma showed strong positive expression of IL-24; C: A moderately differentiated adenocarcinoma showed weak positive expression of IL-24; D: A poorly differentiated adenocarcinoma showed negative expression of IL-24. IL-24: Interleukin-24.

Immunohistochemical staining

The immunohistochemical studies for IL-24 and VEGF were performed on formalin-fixed, paraffin-embedded, 4-μm-thick tissue sections, using the avidin-biotin-peroxidase complex method. The primary antibodies were a goat polyclonal antibody against IL-24 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at 1:200 dilution and a rabbit polyclonal antibody against VEGF, which recognized the 121, 165 and 189 isoform (Santa Cruz Biotechnology) at a 1:100 dilution. Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was performed with a series of graded alcohol solutions. To enhance the immunoreactivity, microwave antigen retrieval was performed at 750 W for 30 min in Tris EDTA (pH 9.0). After the endogenous peroxidase activity was blocked with 5% hydrogen peroxidase for 10 min, incubation with the primary antibody was performed for 1 h at room temperature. An EnvisionTMChemTM Detection Kit (DakoCytomation, Carpinteria, CA, USA) was used for the secondary antibody at room temperature for 30 min. After the tissue samples were washed in Tris-buffered saline for 10 min, 3, 3'-diaminobenzidine was used as a chromogen, and then Mayer's hematoxylin counterstain was applied.

Evaluation of IL-24 expression: IL-24-positive samples were defined as those showing a cytoplasmic staining pattern of the lesional tissue. The staining intensity of IL-24 was graded as follows: 0, negative; 1, weak; 2, strong staining comparable to that seen in a positive control (adjacent normal glands of colonic mucosa) (Figure 1). The negative intensity stood for no stained cells. Weak intensity was allotted when the staining intensity was weaker than that of adjacent normal mucosa or < 5% of cells were stained, and strong intensity when the staining intensity was stronger than that of normal mucosa.

Evaluation of VEGF expression: Immunostaining of VEGF was considered to be positive if unequivocal staining of the membrane or cytoplasm was seen in > 10% of the tumor cells on the slides of the largest section of the tumor.

Statistical analysis

Survival was calculated from the date of surgery for rectal adenocarcinoma. The Kaplan-Meier method was used for survival analysis, and a log rank test was used for survival difference analysis. Correlation between patient characteristics and IL-24 or VEGF expression was evaluated by Fisher's exact test. The number of involved lymph nodes according to IL-24 intensity or expression states was analyzed by one-way ANOVA and *t* test, respectively. Differences were considered significant at $P < 0.05$. Statistical analyses were carried out with SPSS version 18 (Chicago, IL, USA).

RESULTS

Expression of IL-24 and VEGF

IL-24 expression was found in 81 out of 90 patients: nine

Table 2 Correlation of interleukin-24 and vascular endothelial growth factor expressions with patients' characteristics in lymph node positive rectal cancer patients

	IL-24			VEGF		
	Negative[1] (%)	Positive[1] (%)	*P* value	Negative (%)	Positive (%)	*P* value
Differentiation			0.009			0.634
Well	19 (41.3)	27 (58.7)		12 (26.1)	34 (73.9)	
Moderate	12 (35.3)	22 (64.7)		10 (29.4)	24 (70.6)	
Poor	9 (90.0)	1 (10.0)	0.004 (poor *vs* others)	4 (40.0)	6 (60.0)	
N stage			0.055			0.463
N1a	9 (37.5)	15 (62.5)		4 (16.7)	20 (83.3)	
N1b	11 (42.3)	15 (57.7)		8 (30.8)	18 (69.2)	
N2a	4 (25.0)	12 (75.0)		6 (37.5)	10 (62.5)	
N2b	16 (66.7)	8 (33.3)	0.016 (N1~N2a *vs* N2b)	8 (33.3)	16 (66.7)	
Stage			0.055			0.164
ⅢA	3 (37.5)	5 (62.5)		0 (0.0)	8 (100.0)	
ⅢB	22 (37.3)	37 (62.7)		18 (30.5)	41 (69.5)	
ⅢC	15 (65.2)	8 (34.8)	0.028 (ⅢA, ⅢB *vs* ⅢC)	8 (34.8)	15 (65.2)	

[1]Negative (n = 9) and weak (n = 31) intensities were classified as negative expression, and strong intensity (n = 50) as positive one. IL: Interleukin; VEGF: Vascular endothelial growth factor.

negative, 31 weak intensity, and 50 strong intensity. Most cancer cells belonged to the strong intensity group were stained diffusely, so the proportion of immunoreactions was not analyzed. VEGF expression was observed in 64 out of 90 patients. The staining intensity of VEGF was strong in most stained cells, so the difference in terms of intensity of immunoreaction was not assessed.

Correlation between IL-24 or VEGF expression and clinicopathological factors

IL-24 expression was weaker in poorly differentiated tumors compared to well or moderately differentiated tumors. When the negative and weak intensities of IL-24 expression were categorized as negative expression for analysis (Table 2), IL-24 expression was significantly reduced in poorly differentiated tumors compared to well or moderately differentiated tumors, N2b to earlier N stages, and stage ⅢC to stage ⅢA or ⅢB. These significant findings were maintained when another cut-off value of IL-24 was used for analysis (data not shown). There was no significant association between VEGF expression and patients' characteristics. This non-significance was sustained when other cut-off values of VEGF positivity were applied (data not shown).

Correlation between IL-24 expression and number of involved lymph nodes

IL-24 expression was inversely proportional to the N stages, so we compared the IL-24 expression status with the number of lymph nodes. The mean numbers of involved lymph nodes in the patients with negative, weak, and strong intensities of IL-24 expression were 12.11 ± 13.878, 5.48 ± 5.253, and 3.70 ± 3.346, respectively (P < 0.05) (Table 3, Figure 2). The numbers of involved lymph nodes in patients with weak and strong intensities were not different after a multiple comparison test using the Tukey B method.

Table 3 Correlation of interleukin-24 expressions with the number of lymph nodes in the node positive rectal cancer patients

	n	No. of lymph nodes	*P* value
Intensity of IL-24 expression			0.001
Negative	9	12.11 ± 13.878	
Weak	31	5.48 ± 5.253	
Strong	50	3.70 ± 3.346	
IL-24 expression			0.012
Negative (negative & weak intensity)	40	6.98 ± 8.282	
Positive	50	3.70 ± 3.346	
IL-24 expression			0.000
Negative	9	12.11 ± 13.878	
Positive (weak & strong intensity)	81	4.38 ± 4.238	

IL: Interleukin.

Survival analysis

There were significant differences in disease-specific survival (DSS), disease-free survival (DFS), local-recurrence-free survival (LRFS), and distant-metastasis-free survival (DMFS) with regard to *N* stages (Table 4). DSS, DFS and DMFS were different according to stages. A significant difference in LRFS was found according to histological differentiation. The expressions of IL-24 and VEGF had no effect on survival.

DISCUSSION

The stage has been known as the most important prognostic factor in colorectal cancer patients until now. The seventh AJCC TNM classification (2010) subdivides N stages according to the number of involved lymph nodes[22]. N1a is metastasis in one regional node, N1b in two or three, N2a in four to six, and N2b in seven or more. In this study, patients were distributed throughout

Table 4 Survival analysis in the lymph node metastatic rectal cancer patients

	n	5-yr disease specific survival	*P* value	5-yr disease free survival	*P* value	5-yr local recurrence free survival	*P* value	5-yr distant metastasis free survival	*P* value
Age (yr)									
Less than 60	48	60.4	0.963	51.8	0.918	60.3	0.683	57	0.483
60 or older	42	60.7		53.1		59.5		66.3	
Sex									
Male	47	60.3	0.742	49.2	0.528	57.4	0.903	60.3	0.760
Female	43	60.8		55.8		62.8		62.6	
Differentiation									
Well	46	74.9	0.060	59.3	0.333	73.8	0.008	64.7	0.459
Moderately	34	48.5		45.7		50		59.8	
Poorly	10	33.3		44.4		30		55.6	
T stage									
T1	1	100	0.441	100	0.427	100	0.385	100	0.298
T2	8	87.5		87.5		75		87.5	
T3	79	57.6		48.2		58.2		57.3	
T4	2	50		50		50		100	
N stage									
N1a	24	82.6	0.004	73.7	0.003	74.8	0.001	77	0.015
N1b	26	59.4		55.7		69.2		68.5	
N2a	16	68.8		50		68.8		56.3	
N2b	24	34.2		29.3		29.2		40.8	
Stage									
ⅢA	8	100	0.002	100	0.002	100	0.230	100	0.009
ⅢB	59	64.6		54.1		80.1		63	
ⅢC	23	35.8		30.7		64.5		42.7	
VEGF expression									
Negative	26	53.1	0.801	50	0.851	73.3	0.629	64.1	0.791
Positive	64	61.9		53.5		81.1		60.5	
IL-24 expression									
Negative	40	59.2	0.759	50.7	0.890	60	0.496	60.7	0.849
Positive	50	34.7		53.9		59.9		61.6	

VEGF: Vascular endothelial growth factor; IL-24: Interleukin-24.

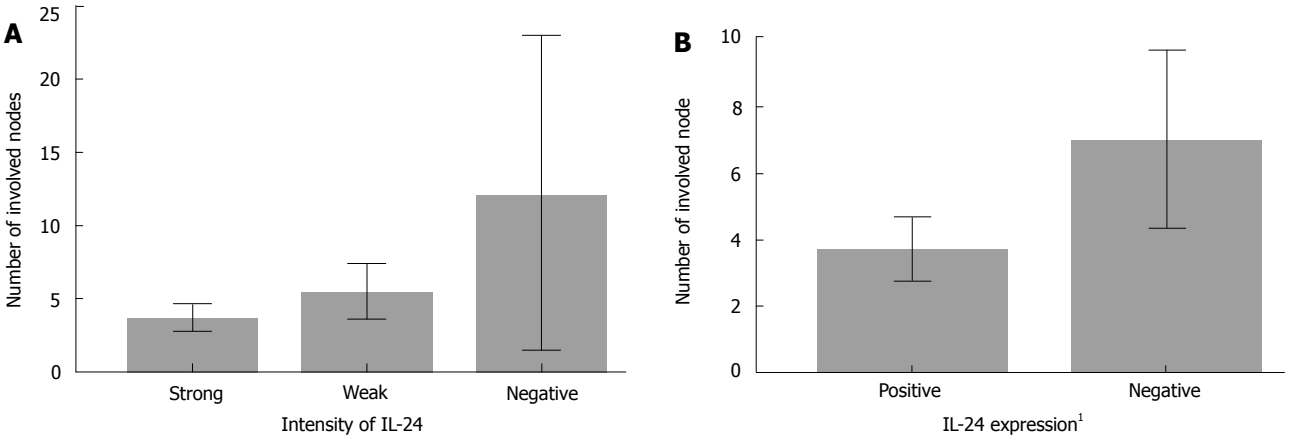

Figure 2 The number of involved lymph nodes according to interleukin-24 intensity (A) and expression status (B) in node-positive rectal cancer patients. [1]Negative (*n* = 9) and weak (*n* = 31) intensities were classified as negative expression, and strong intensity (*n* = 50) as positive. IL: Interleukin.

the N stages. That is, the proportions of the patient study group with cancer in the N1a, N1b, N2a and N2b stages were 26%, 29%, 18% and 26%, respectively. However, patients were not distributed evenly in terms of T stages: 79% were in stage T3. Therefore, to gain reasonable results from the T stage analysis was not possible, unlike the N stage analysis. The higher stages correlated significantly with poorer outcomes in terms of DSS, DFS, LRFS and DMFS ($P < 0.05$). In addition, higher N stages also had poorer survival rates compared to lower ones. The N stages and stages from the seventh AJCC staging system seem to be applicable, although the subgroup patient numbers were not large in our analysis.

IL-24 promotes growth suppression and induces apoptosis in a broad array of human cancers, after forced expression by means of a plasmid or a replication-competent

adenovirus, but it does not induce growth suppressive or toxic effects in normal cells[23]. To the best of our knowledge, the clinical significance of IL-24 expression in rectal cancer patients has not been assessed. This is believed to be the first study to analyze the association between IL-24 expression and patient prognostic features in lymph-node-involved rectal cancer patients.

IL-24 expression was weaker in the poorly differentiated tumors, N2b stage, and stage Ⅲc compared to well or moderately differentiated tumors, N1 or N2a stage, and stage Ⅲa or Ⅲb, respectively (Table 2). Moreover, the number of involved lymph nodes was significantly lower in patients with IL-24 expression compared to those without (Table 3). Even though IL-24 expression showed a significant correlation with these prognostic features, the status of IL-24 expression did not affect survival. This discrepancy may in part be explained by an inadequate patient number in the poor prognostic subgroups, and undetermined appropriate cut-off values for IL-24 positivity. Therefore, further studies with larger numbers of patients are necessary in order to develop an adequate grading system of IL-24 expression, and to verify whether IL-24 expression has a prognostic value.

The clinical significance of VEGF expression in rectal cancer is still open to debate. Several studies have insisted that increased VEGF expression is associated with poor prognosis, but some studies have shown that VEGF expression is not related to survival. Casinu *et al* [21]have claimed from their analysis of lymph-node-positive rectal cancer patients that patients with VEGF-positive tumors have lower event-free survival rates and more frequent distant metastases. However, Bertolini *et al*[18] have found from their study of locally advanced rectal cancer patients that VEGF expression obtained from pretreatment and post-chemoradiotherapy specimens does not show any significant correlation with DFS and overall survival (OS). In the study of Soumaoro *et al*[24], OS was worse in colorectal cancer patients with VEGF expression, but this prognostic independence disappeared after multivariate analysis. Our study used unhampered surgical specimens without exposure to any chemoradiotherapy for immunohistochemical staining. VEGF expression was found in 64 out of 90 lymph-node-involved rectal cancer patients. There was no significant survival difference in the rectal cancer patients with regard to VEGF expression in spite of applying several cut-off values for VEGF expression. To verify the influence of VEGF expression on survival in advanced rectal cancer patients, further studies with large numbers of patients are required.

VEGF expression has been reported to be significantly correlated with tumor size, lymph node metastasis, lymphatic invasion, and TNM stage in colorectal cancer patients[24,25]. It is also significantly associated with lymph node involvement in patients with locally advanced rectal cancer[26]. However, these associations with VEGF expression were not found in present study.

In addition to the VEGF analysis with tumor tissues, soluble VEGF in the serum or plasma of patients has also been investigated. Werther *et al*[27] have reported that preoperative soluble VEGF is of independent prognostic value in patients with colon cancer, but not in those with rectal cancer. Tsai *et al*[28] have found that patients with plasma VEGF elevation have worse DFS than those without plasma VEGF elevation in lymph-node negative colorectal cancer, but not in lymph-node-positive patients. Therefore, further studies are necessary to assess the role of soluble VEGF in rectal cancer patients.

In conclusion, we observed that IL-24 expression had a significant inverse relationship with N stage, overall stage, and the number of involved lymph nodes, but the status of IL-24 expression did not affect survival. Therefore, further studies with larger numbers of patients are needed in order to verify whether IL-24 expression has a prognostic value.

COMMENTS

Background

The survival rate of rectal cancer patients has been improved with the addition of chemoradiotherapy to surgery. However, that is still inadequate in stage Ⅲ patients. Therefore, much endeavor is needed to increase the outcome of those through identification of new therapeutic targets. Anticancer activity of interleukin (IL)-24 has been reported in various cancer cells, but it is not known whether IL-24 has clinical importance in rectal cancer patients. In addition, vascular endothelial growth factor (VEGF) is considered to be essential in tumorigenesis, but its prognostic significance is still inconclusive in rectal cancer patients.

Research frontiers

Favorable survival is marginally identified in high-IL-24-expressing non-small cell lung cancer (NSCLC) patients. From a subset analysis, high IL-24 expression has been revealed as a significant prognostic factor in adenocarcinoma patients. However, whether high IL-24 expression has a significant impact on the survival of patients with types of cancers other than NSCLC is unknown.

Innovations and breakthroughs

Selective anticancer effects of IL-24 have been reported *in vitro* and *in vivo* without significant toxic effects on normal cells. These interesting properties may make IL-24 a candidate therapeutic target.

Applications

From this study, correlation of IL-24 expression with histological differentiation and the degree of lymph node involvement in rectal cancer patients was found, but IL-24 expression was not significantly correlated with survival. Therefore, further studies with larger numbers of patients are required in order to assess the prognostic value of IL-24 expression in rectal cancer patients.

Terminology

The melanoma differentiation associated gene-7 was identified by subtraction hybridization from human melanoma cells stimulated with interferon-β and mezerein, and was renamed later as IL-24. Multiple anticancer properties have been identified in a variety of cancer cells without injury to normal cells. Therefore, IL-24 has been emerging as an interesting candidate treatment target in many cancers.

Peer review

Choi *et al* studied immunohistochemically the clinicopathological significance of IL-24 expression in rectal carcinoma. The experiments were conducted appropriately and the results were reasonable.

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