Expression of interleukin (IL)-11 and IL-11 receptor in human colorectal adenocarcinoma: IL-11 up-regulation of the invasive and proliferative activity of human colorectal carcinoma cells

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Abstract. Previous investigations have shown that interleukin (IL)-11/IL-11 receptor α-chain (IL-11Rα), a member of the PI3K, MAPK and JAK-STAT activating family of cytokines/receptors, correlates with the regulation of tumor progression. In this study, we established the IL-11/IL-11Rα expression profile in human colorectal adenocarcinoma (CRC) and clarified its signaling pathway and role in the invasion activity of CRC cell lines. To elucidate the role of IL-11/IL-11Rα, we examined 103 cases of CRC and 24 cases of colorectal adenoma by immunohistochemistry. In addition, we investigated the invasive activity of cell signaling pathway of CRC cell lines. The IL-11Rα expression was correlated with tumor invasion and lymphatic infiltration (p<0.01, respectively). Recombinant human IL-11 (rhIL-11) promoted the migration and proliferation of HT-29 cells and activated the PI3K and p44/p42 MAPK pathways. Wortmannin, a PI3K inhibitor, and PD98059, a p44/p42 MAPK inhibitor, significantly reduced the promotion of invasion and proliferation activity by rhIL-11, respectively. In summary, the IL-11Rα expression was correlated with clinicopathological features and IL-11 promoted the invasion via the PI3K and up-regulated the proliferation via the p44/p42 MAPK in CRC cells. These findings suggested that the IL-11/IL-11R pathway plays an important role in the progression of CRC.

Introduction

Colorectal adenocarcinoma (CRC) is one of the most common neoplasms and is a significant cause of morbidity and mortality in the world (1). The occurrence and progression of cancer is suggested to be related to a series of genetic events affecting the structure and/or expression of a number of oncogenes, anti-oncogenes and growth factors (2,3). The deep-invasive carcinomas, such as CRC, have higher rates of lymph duct and venous invasion as well as lymph node metastasis than less invasive types of cancer (4). However, the mechanisms of invasion and metastasis of CRC have not been fully elucidated.

IL-11 is expressed by a variety of tissues, such as gut, brain, spinal cord neurons, and testes, and hence may have a physiological role in these organs (5). Breast cancer cells have been shown to secrete IL-11, and its possible role in bone metastasis and poor prognosis of breast cancer has been studied (6,7). Recently, few reports have been published in the expression of IL-11 and IL-11R in colorectal cancer (8,9). However, the role of IL-11 and IL-11R in colorectal cancer is still unclear.

Interleukin (IL)-11 was originally cloned as a mediator of plasmacytoma cell proliferation and was later found to exhibit a wide variety of biological effects in neural cells as well as in the hematopoietic and immune systems (5,10). Although IL-11 shares gp130 as a signaling mechanism with several cytokines and growth factors, including IL-6, oncostatin M, leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF), it also has unique biological actions (5).

The IL-11 receptor α-chain (IL-11Rα) mediates the action of IL-11, a 19.1-KD pleiotropic cytokine that was initially cloned from a bone marrow stromal cell line (10). The hematopoietic effects of IL-11, which include stimulation of megakaryocyte maturation and platelet production, and growth stimulation of CD34+ hematopoietic progenitor cells, have been well studied. IL-11 has also been shown to mediate inhibition of adipogenesis, stimulation of osteoclasts, and cytoprotection of gut mucosa in gastric ulcers (11-14). The α subunit of the IL-11R is required for high affinity binding of the ligand; on ligand binding, gp130, the subunit responsible for signal transduction, is required for the formation of the receptor complex (15). It is known that IL-11Rα-associated gp130 undergoes homodimerization, as it does in the IL-6 receptor (16), but the nature of the signaling

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transduction systems used by IL-11Rα is currently under investigation.

Protein kinases that have been implicated in IL-11-mediated signaling include phosphatidylinositol-3 kinase (PI3K), MAP kinase (MAPK), Jak1 and Jak2 receptor-associated kinases, src-family tyrosine kinases including p60[src] and p62[yes] (17,18). In this study, to investigate the PI3K pathway, the phosphorylation of P70 S6 ribosomal protein (P70 S6 RP) was investigated. P70 S6 RP is a downstream target of PI3K-generated signals and becomes activated after phosphorylation of Ser 235/236 (19-21). To study the effect of IL-11 on the MAPK pathway, the phosphorylation of P90 RSK and P38 MAPK was investigated. P90 RSK is a downstream target of p44/p42 mitogen-activated protein kinases and becomes activated after phosphorylation of Ser 380/363 (22-24). P38 MAPKs is a downstream target of MKK3 and SEK generated signals and becomes activated after phosphorylation of Thr180 and Tyr182 (2,22,25). There is also evidence that the signal transduction and transcription factors (STAT) 1 and STAT3 are activated by IL-11Rα (16).

In this study, we investigated the expression of IL-11 and IL-11Rα in primary human CRC and attempted to determine, using cell line models, the biological actions of this cytokine in CRC.

Materials and methods

Clinical materials. Normal colorectal mucosa was obtained from a patient at autopsy. We studied 103 primary human CRC: 32 cases of submucosal invasion (T1), 9 cases of muscularis proprial invasion (T2), 43 cases of either subserosal or pericolic tissue invasion (T3), and 19 cases of invasion through the serosa or invasion of contiguous organs (T4). All tumor specimens were obtained from patients operated at Nagasaki University Hospital between 2001 and 2005. Each tumor was assigned a histological type and a grade of depth infiltration according to the TNM staging system (26,27). We also classified all tumors into four groups based on the modified Dukes' staging system: according to this system, there were 40 cases of Dukes' A, limited to the bowel wall; 31 cases of Dukes' B, involved extramural spread; 28 cases of Dukes' C, involved the lymph nodes; and 4 cases of Dukes' D tumors, involved distant metastasis (28). We used 24 adenomas as benign lesions with moderate dysplasia. Normal colonic mucosal tissues were evaluated as normal controls. Each parameter was defined as 'present' when invasion was identified with certainty, but defined as 'absent' when either not observed at all or not observed with certainty. Lymph node metastasis was defined as 'present' only when histologically proven.

Immunohistochemical analysis. Formalin-fixed and paraffin-embedded tissues were cut into 4 μm sections, deparaffinized in xylene and rehydrated in phosphate-buffered saline. Deparaffinized sections were preincubated with normal bovine serum and then incubated overnight at 4˚C with an optimal dilution (0.1 μg/ml) of a primary polyclonal rabbit antibody against human IL-11 (H-169) and IL-11Rα (N-20). Each antibody was bought from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The slides were sequentially incubated with a biotinylated and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody, and the reaction products were visualized using diaminobenzidine (DAB, Dako, Carpinteria, CA, USA) with methyl green as a counterstain and 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; Dako), respectively. Primary antibodies preabsorbed with excess recombinant peptides (Santa Cruz Biotechnology Inc.) were used as negative controls. Gastric ulcer tissue with mucosal proliferation served as the internal positive control. IL-11 and IL-11Rα expressions were classified into three categories depending on the percentage of cells stained: -, 0-10% positive cells; +, 10-50% positive tumor cells; and ++, >50% positive tumor cells.

Reagents and cell culture. Recombinant human IL-11 (rhIL-11) (Genetics Institute, Andover, MA, USA) was diluted in cell culture medium. DLD-1, HT-29, Caco-2, Lovo, and Colo-320DM cell lines derived from human colon cancer, were obtained from American Type Culture Collection (Manassas, VA, USA). DLD-1, HT-29, Caco-2, Lovo, and Colo-320DM cell lines were maintained in RPMI-1640, McCoy's 5a, minimum essential medium, Ham's F12, and Dulbecco's modified Eagle's medium, respectively (all media were purchased from Invitrogen Corp., Carlsbad, CA, USA). DLD-1 and Colo-320DM supplemented with heat inactivated 10% fetal calf serum (FCS) (Invitrogen Corp.), HT-29 supplemented with heat inactivated 10% fetal bovine serum (FBS, Invitrogen Corp.), Caco-2 supplemented with heat inactivated 20% FBS and Lovo supplemented with heat inactivated 20% FCS. All cell line incubations received 2 mM glutamine (Invitrogen Corp.) and incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Immunocytochemical analysis. Immunocytochemistry was performed on cells grown in 8-well glass chamber slides (Invitrogen Corp.). Cells were grown to confluence and incubated in serum-free medium alone for a further 24 h. Cells were fixed by absolute ethanol and blocked with 50% FCS for 1 h. The cell staining as described for the immuno-histochemistry and colorization proceeded with DAB. The gp130 (H-255), polyclonal rabbit antibody was bought from Santa Cruz Biotechnology Inc.

Stimulation and inhibition. The cells were first starved in medium with reduced serum for 24 h before stimulating with 25, 50 and 100 ng/ml rhIL-11 for 5 min. The inhibitors, Wortmannin (Sigma-Aldrich, St. Louis, MO, USA) and PD98059 (Cell Signaling Technology, Beverly, MA, USA), were added 30 min prior to IL-11 stimulation at concentrations of 100 nM and 50 μM, respectively. The inhibitors were dissolved in 0.001% dimethyl sulfoxide (DMSO, Sigma-Aldrich). This DMSO concentration was tested on each cell; no change in function was measured compared to untreated cells.

Western blot analysis. Western blot analysis was performed on human CRC tissues and CRC cell lines. The tissues and cells were then suspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and

0.05% SDS, pH 7.4). The supernatant was collected, and the protein concentration was quantified using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

After boiling, the proteins (20 μg) were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing, and transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, Arlington Height, IL, USA). The membranes blocked with 5% low-fat dried milk in TBS containing 0.1% Tween-20 (TBS-T), and then incubated for 1 h at room temperature with 1:500 dilutions of the anti-human IL-11 antibody (Santa Cruz Biotechnology Inc.), anti-human IL-11Rα antibody (Santa Cruz Biotechnology Inc.) and anti-human gp130 antibody (Cell Signaling Technology, Beverly, MA), and with a 1:1,000 dilution of the p-P70 S6 RP (Ser235/236), phospho-P90 RSK (p-P90 RSK) (Ser380) and phospho-P38 MAPK (p-P38 MAPK) (Thr180/Tyr182) antibodies (Cell Signaling Technology). We processed anti-human ß-actin antibody (Santa Cruz Biotechnology) as an indicator of the amounts of protein loaded. The membranes were incubated for 1 h with a 1:1,000 dilution of horseradish-peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Santa Cruz Biotechnology Inc.). The membranes were developed with a horseradish peroxidase chemiluminescence detection reagent (ECL Plus System, Amersham Biosciences), and then exposed to Hyperfilm ECL (Amersham Biosciences).

Cell growth. The cells were seeded in 6-well dishes (Coster, Cambridge, MA, USA) at 1x10⁴ cells per well and cultured in appropriate medium containing 0.1% FCS or FBS with/without the presence of 50 ng/ml rhIL-11. Normal rabbit IgG at the same concentration was used as a control for IL-11. To examine the growth stimulation by IL-11, the number of cells and viability were compared every 2 days using a conventional improved Neubauer hemocytometer (Bright-Line, American Optical Corp., Buffalo, NY, USA) (29).

Migration/chemotaxis assay. A 0.1 ml aliquot of HT-29 and Colo-320DM cell suspension at 1x10⁵ cells/ml was seeded to the upper chamber of transwell filters (8 μm pore) (Corning Inc., Corning, NY, USA), which was transferred to wells containing buffer with rhIL-11 or Wortmannin. 0.1% FBS-containing medium was added to the lower chamber and incubated for 18 h at 37°C. Non-migrating cells that remained on the upper surface of the filter were removed, and the remaining cells on the lower surface of the filter were fixed with 100% methanol, stained and counted under a light microscope in five different fields (Nikon E400, x200). Assays were performed in triplicate.

Matrigel invasion assay. The Matrigel invasion assay was carried out according to the manufacturer's instructions (BD Biosciences, Palo Alto, CA, USA). A 0.5 ml aliquot of HT-29 cell suspension at 1x10⁵ cells/ml was seeded in the upper chamber of Matrigel-coated transwell filters (8 μm pore), which were transferred to wells containing buffer with rhIL-11, FBS or Wortmannin. The conditioned medium without serum was added to the lower chamber and incubated for 72 h. Fixation, staining and counting as described for the migration and chemotaxis assay.

Statistical analysis. The StatView II program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analyses. Analyses comparing the degrees of IL-11 or IL-11Rα expressions were performed by the Mann-Whitney U and Spearman's tests.

Results

Expression of IL-11 and IL-11Rα in human CRC tissue/immunohistochemistry. We have summarized the immunohistochemical results in Table I in the expressions in human colorectal adenocarcinoma and adenoma. Normal colonic epithelium, fibroblasts and endothelial cells in stromal tissue of the tumor expressed IL-11 and IL-11Rα, although these expressions were very weak. Among 24 cases of adenoma, 15 cases (62.5%) expressed IL-11 and only 8 cases (33.3%) of adenoma expressed IL-11Rα. There were significant differences in IL-11 and IL-11Rα expressions between adenoma and adenocarcinoma, particularly in the expression of IL-11Rα (p=0.0025 and p<0.0001, respectively).

The expressed IL-11 was localized in the cytoplasm of the cancer cells (Fig. 1A). The expressed IL-11Rα was localized in the membrane of cancer cells (Fig. 1B). In IL-11 and IL-11Rα expression, the invasive parts of the primary tumor were intensely stained, as contrasted with the superficial area of the tumor in almost cases of invasive carcinomas. The invasion grade was assigned according to the TNM and Dukes' staging systems. In many cases, both IL-11 and IL-11Rα were expressed simultaneously in the carcinoma cell itself.

CRC cells revealed a statistically significant correlation between IL-11Rα expression and the depth of invasion (p=0.0059 in the TNM staging and p=0.0239 in the Dukes' staging). There was significant correlation between IL-11Rα expression and lymphatic invasion (p=0.0058). However, there were no correlation between the expression of IL-11Rα and the differentiation, lymph node metastasis and venous invasion of carcinoma. IL-11 expression in CRC did not correlate with these clinicopathological features (Table I).

Expression of IL-11, IL-11Rα, gp130, p-P70 S6 RP, p-P90 RSK and p-P38 MAPK in human CRC, adenoma and normal colorectal mucosal tissue/Western blot. The expressions of IL-11 by CRC were examined by Western blot analysis (Fig. 1C). Lanes 1 and 3 were human colorectal adenoma tissues, and lanes 2 and 4 were normal colorectal mucosa corresponding to the tumor tissues of lanes 1 and 3, respectively. Lanes 5 and 7 were CRC tissues, which were graded as T4 and/or Dukes' D. Lanes 6 and 8 were normal colorectal mucosa corresponding to the tumor tissues of lanes 5 and 7, respectively. The IL-11, IL-11Rα and gp130 were clearly detected in the two human CRC tissues. The expression of IL-11, IL-11Rα and gp130 were also detected in the other lanes, but their expressions were weaker than in CRCs.

The p-P70 S6 RP and p-P90 RSK were clearly detected in the two CRCs. However, adenomas and normal colorectal mucosa faintly expressed p-P70 S6 RP and p-P90 RSK. p-P38 MAPK was also clearly detected in the two human CRC tissues. p-P38 MAPK was also detected in adenomas and normal colorectal mucosa, but the expression was weaker than in CRCs. Moreover, the expressions of IL-11, IL-11Rα,

Table I. IL-11/IL-11R expression in colorectal adenocarcinoma (103 cases).

	n	IL-11 (%)			IL-11Rα (%)		
		-	+	++	-	+	++
Tobular adenoma		p=0.0025[a]			p<0.0001[b]		
	24	9 (37.5)	7 (29.2)	8 (33.3)	16 (66.7)	8 (33.3)	0 (0.0)
Total carcinoma	103	14 (13.6)	23 (22.3)	66 (64.1)	25 (24.3)	28 (27.2)	50 (48.5)
Differentiation		n.s			n.s		
Wel	51	7 (13.7)	13 (25.5)	31 (60.8)	15 (29.4)	14 (27.5)	22 (43.1)
Mod	41	5 (12.2)	7 (17.1)	29 (70.7)	6 (14.6)	13 (31.7)	22 (53.7)
Por	5	0 (0.0)	1 (20.0)	4 (80.0)	1 (20.0)	0.(0.0)	4 (80.0)
Muc	6	2 (33.3)	2 (33.3)	2 (33.3)	3 (50.0)	1 (16.7)	2 (33.3)
Deapth of invasion		n.s			p=0.0059		
T1	32	5 (15.6)	9 (28.1)	18 (56.3)	13 (40.6)	11 (34.4)	8 (25.0)
T2	9	1 (11.1)	2 (22.2)	6 (66.7)	2 (22.2)	2 (22.2)	5 (55.6)
T3	43	6 (14.0)	8 (18.6)	29 (67.4)	7 (16.3)	9 (20.9)	27 (62.8)
T4	19	2 (10.5)	4 (21.1)	13 (68.4)	3 (15.8)	6 (31.6)	10 (52.6)
Lymph node metastasis		n.s			n.s		
Present	32	5 (15.6)	6 (18.8)	21 (65.6)	7 (21.9)	7 (21.9)	18 (56.3)
Absent	71	9 (12.7)	17 (23.9)	45 (63.4)	18 (25.4)	21 (29.6)	32 (45.1)
Lymphatic invasion		n.s			p=0.0058		
Present	68	10 (14.7)	12 (17.6)	46 (67.6)	13 (19.1)	15 (22.1)	40 (58.8)
Absent	35	4 (11.4)	11 (31.4)	20 (57.1)	12 (34.3)	13 (37.1)	10 (28.6)
Venous invasion		n.s			n.s		
Present	40	5 (12.5)	9 (22.5)	26 (65.0)	7 (17.5)	10 (25.0)	23 (57.5)
Absent	63	9 (14.3)	14 (22.2)	40 (63.5)	18 (28.6)	18 (28.6)	27 (42.9)
Duke's stage		n.s			p=0.0239		
A	40	5 (12.5)	12 (30.0)	23 (57.5)	14 (35.0)	14 (35.0)	12 (30.0)
B	31	4 (12.9)	5 (16.1)	22 (71.0)	4 (12.9)	7 (22.6)	20 (64.5)
C	28	4 (14.3)	5 (17.9)	19 (67.9)	6 (21.4)	6 (21.4)	16 (57.1)
D	4	1 (25.0)	1 (25.0)	2 (50.0)	1 (25.0)	1 (25.0)	2 (50.0)

[a]A significant difference between adenoma and total carcinoma in the expression of IL-11; [b]A significant difference between adenoma and total carcinoma in the expression of IL-11R. n.s, not significant.

gp130, p-P70 S6 RP, p-P90 RSK and p-P38 MAPK in the two adenomas were of slightly higher intensity than in normal colorectal mucosa.

Expression of IL-11, IL-11Rα and gp130 in CRC cell lines. The expressions of IL-11, IL-11Rα and gp130 in five CRC cell lines, DLD-1, HT-29, Caco-2, Lovo and Colo-320DM, were examined by Western blot analysis (lane 1, DLD-1; lane 2, HT-29; lane 3, Caco-2; lane 4, Lovo; lane 5, Colo-320DM; Fig. 2A). These proteins were detected in all five human CRC cell lines. Caco-2 and Lovo cells highly expressed IL-11, while other cell lines expressed IL-11 at low level. HT-29 and Colo-320DM cells expressed IL-11Rα at high levels, while other cells expressed IL-11Rα at low level. Two CRC cell lines, HT-29 and Colo-320DM, were also examined by immunocytochemistry (Fig. 2B1-3, HT-29 and B4-6, Colo-320DM). The expressions of IL-11, IL-11Rα and gp130 were detected in both CRC cell lines. IL-11 was localized in the cytoplasm (Fig. 2B1 and B4), and IL-11Rα and gp130 were localized in the cell membrane and cytoplasm (Fig. 2B2, 3, 5 and 6).

Activation of signal transduction elements in response to rhIL-11 stimulation. As a first step in defining the IL-11 signaling pathways, the phosphorylation by IL-11 of the PI3 kinase, P70 S6 RP and the MAP kinases, P90RSK and P38 was determined. The cells were stimulated with 50 ng/ml of rhIL-11 for 5 min and extracted protein for different time periods (0, 5, 10, 15, 30 min, 1, 3, 6, 9 and 12 h). In two CRC cell lines, HT-29 and Colo-320DM, IL-11 had different effects
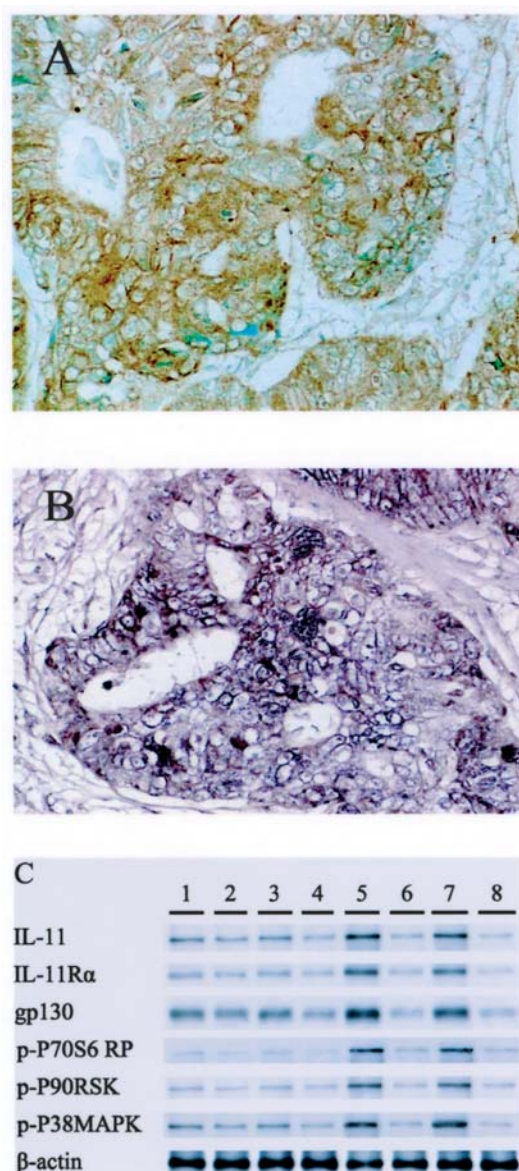
Figure 1. The investigation of IL-11/IL-11Rα, activated PI3K, and activated MAPK in human CRC tissues. Immunohistochemical staining in human colorectal carcinoma (A, IL-11 and B, IL-11Rα). (A, x400, DAB staining, B, x400, alkaline-phosphatase staining) Western blot analysis for IL-11, IL-11Rα, gp130, p-P70 S6 RP, p-P90 RSK and p-P38 MAPK in human CRC, adenoma and normal colorectal mucosa (C). Lanes 5 and 7 showed clearly expression of IL-11, IL-11Rα, gp130, p-P70 S6 RP, p-P90RSK and p-P38 MAPK. Lane 1, Case 1-adenoma; lane 2, Case 1-normal colorectal mucosa; lane 3, Case 2-adenoma; lane 4, Case 2-normal colorectal mucosa; lane 5, Case 3-CRC; lane 6, Case 3-normal colorectal mucosa; lane 7, Case 4-CRC and Lane 8, Case 4-normal colorectal mucosa. Case 3 and 4 were CRC of T4 and/or Dukes' D.

Figure 2. The investigation of IL-11/IL-11Rα, activated PI3K, and activated MAPK in human CRC cells. Western blot analysis for IL-11, IL-11Rα, gp130 and ß-actin in CRC cell lines (A). Caco-2 and Lovo cells highly expressed IL-11. HT-29 and Colo-320DM cells expressed IL-11Rα at high levels (lane 1, DLD-1; lane 2, HT-29; lane 3, Caco-2; lane 4, Lovo; lane 5, Colo-320DM). Immunocytochemistry for IL-11, IL-11Rα and gp130 in CRC cell lines (B1-3, HT-29 and B4-6, Colo-320DM). (B1-6, x400, DAB staining) Western blot for PI3K, MAPKs pathway in human colorectal carcinoma cell lines (C, HT-29 and D, Colo-320DM). In HT-29, the PI3K P70 S6 RP and p44/p42 MAPK P90 RSK was phosphorylated by stimulation of rhIL-11 (C). In Colo-320DM, the P70 S6 RP and P90 RSK was faintly phosphorylated in unstimulated cell, and IL-11 did not influence its phosphorylation (D).

(Fig. 2C, HT-29 and D, Colo-320DM). The MAPK P38 was not phosphorylated in unstimulated cells, and IL-11 did not influence the phosphorylation state in either cell line (Fig. 2C and D). In HT-29, PI3K P70 S6 RP became phosphorylated after 5-min stimulation with 50 ng/ml rhIL-11, and this activation continued for at least 1 h (Fig. 2C). The MAPK P90RSK became phosphorylated after 5-min stimulation with 50 ng/ml rhIL-11, and this activation continued for at least 3 h (Fig. 2C). On the other hand, in Colo-320DM, the 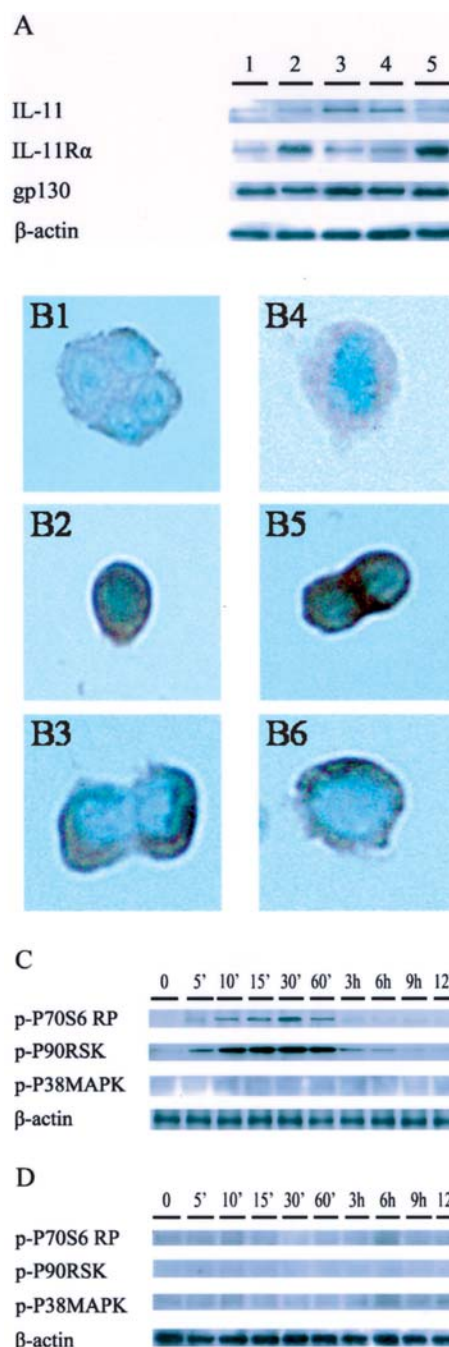MAPK P90RSK and PI3K P70 S6 RP was only slightly phosphorylated in unstimulated cells, and IL-11 did not influence their phosphorylation state (Fig. 2D).

Cell growth; effect of rhIL-11 on cell growth of CRC cell lines. We next examined the effect of exogenous IL-11 on the
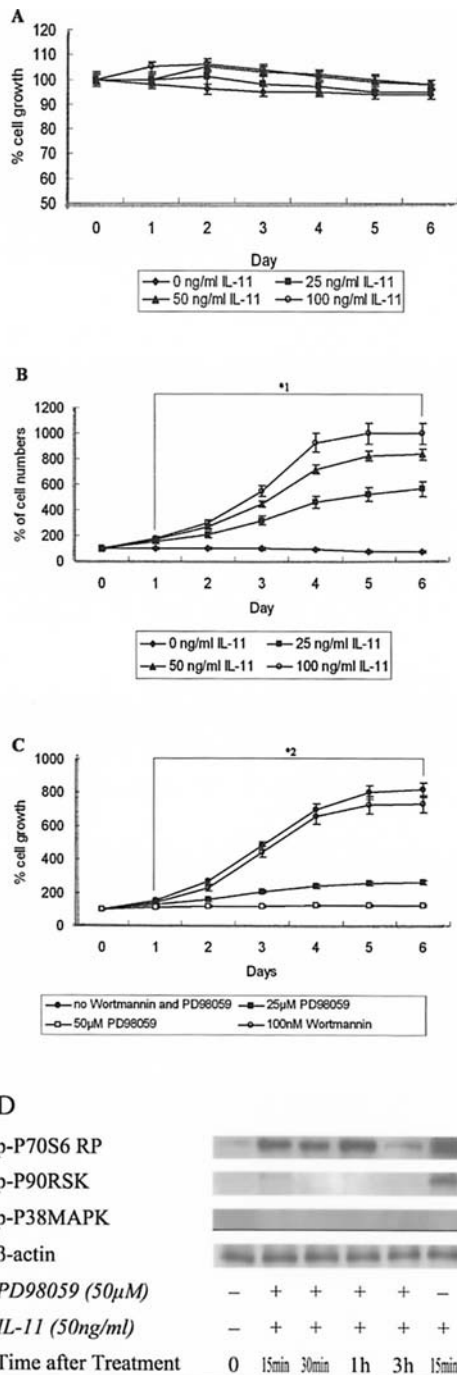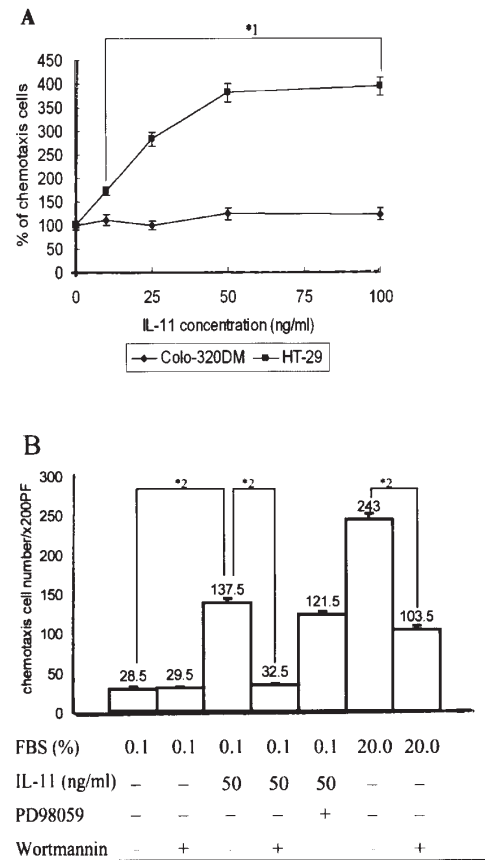
Figure 4. Chemotactic effect of IL-11 in human CRC cell lines. Chemotaxis assay in human colorectal carcinoma cells (A). Compared with Colo-320DM, HT-29 exhibited significant and dose-dependent chemotactic responses to rhIL-11 up to 50 ng/ml (p<0.0001). Chemotaxis assay in HT-29 treated with Wortmannin (100 nM) and PD98059 (50 μM) (B). The treatment of rhIL-11 up-regulated the number of cells undergoing chemotaxis (p<0.0001). Wortmannin suppressed the chemotactic response in HT-29 cells that were up-regulated by rhIL-11 and also 20% FBS (p<0.0001). *1, A significant correlation between Colo-320 and HT-29 cell numbers; *2, A significant suppression and/or upregulation.

Figure 3. Proliferative effect of IL-11 and phosphorylation of p44/p42 MAPK in human CRC cell lines. Proliferative effects of IL-11 in human CRC cells (A; Colo-320DM and B; HT-29). In Colo-320DM cells, rhIL-11 did not influence cells growth (A). In HT-29 cells, the cell numbers of each dose of rhIL-11 increased constantly until day 6 (B). In HT-29, there was a significant correlation between the dose of rhIL-11 and cell numbers (p<0.0001). Proliferative effect of IL-11 in HT-29 cells treated with PD98059 and Wortmannin (C). PD98059 significantly suppressed growth response in HT-29 cells dose dependently (p<0.0001). PD98059 reduced MAP-kinase activity in HT-29 (D). *1, A significant correlation between rhIL-11 dose and cell numbers.; *2, A significant correlation between PD98059 dose and cell numbers.

growth of CRC cells. We stimulated HT-29 and Colo-320DM cells by four different doses of rhIL-11. In Colo-320DM cells, the rhIL-11 did not influence growth (Fig. 3A). HT-29 cell exhibited a significant and dose-dependent growth response to rhIL-11 (p<0.0001) (Fig. 3B). To study the effect of IL-11 on the classical MAPK pathway which acts through p44/p42 MAPK and PI3K in HT-29, the growth response was investigated. The p44/p42 MAPK inhibitor, PD98059, significantly suppressed the growth response of 50 ng/ml rhIL-11 in HT-29 cells in a dose-dependent manner (p<0.0001) (Fig. 3C). Meanwhile, the PI3K inhibitor, Wortmannin, did not suppress growth response in HT-29 cells (Fig. 3C). In Western blot analysis, the phosphorylation of P90 RSK was induced by stimulation with rhIL-11 in the absence but not in the presence of PD98059 in HT-29 cells (Fig. 3D). PD98059 did not influence the state of P70 S6 RP and P38 MAPK.

Chemotaxis and invasion assay of CRC cells in response to rhIL-11. Transwell migration and matrigel invasion assays were performed to examine the mobilizing effect of rhIL-11 on Colo-320DM and HT-29 colorectal carcinoma cells. Fig. 4A shows the chemotactic response of CRC cells to various concentrations of rhIL-11. Compared with Colo-320DM cells, HT-29 cells exhibited significant and dose-dependent chemotactic responses to rhIL-11 (p<0.0001). To study the effect of IL-11 on the PI3K pathway in HT-29, the chemotactic response was investigated (Fig. 4B). The treatment of rhIL-11
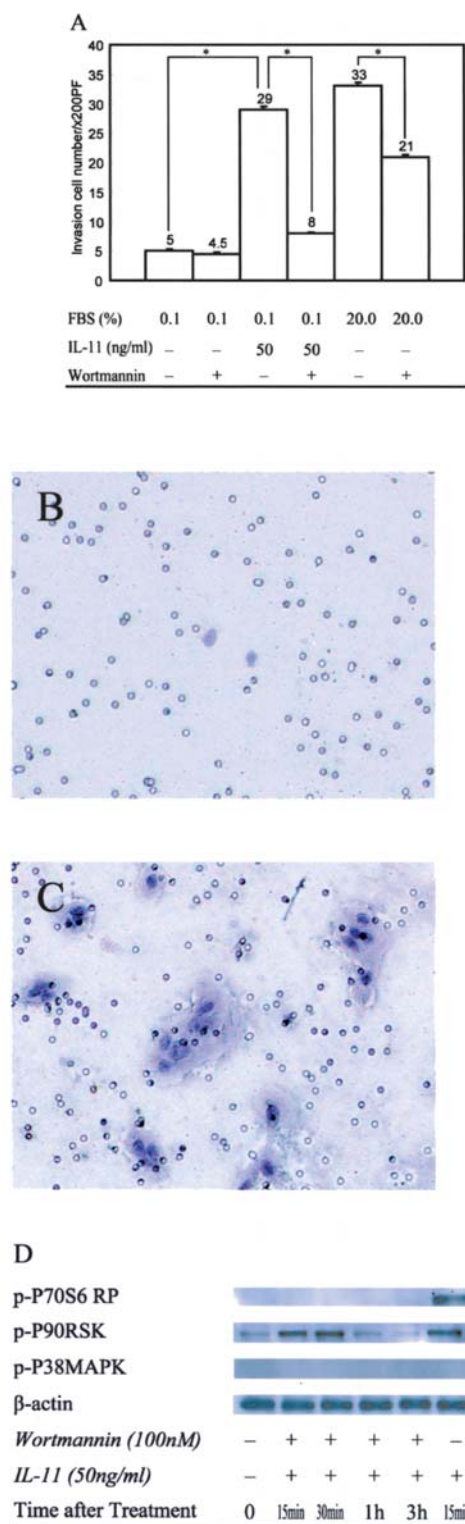
Figure 5. Invasion effect of IL-11 in human CRC cell lines. Invasion assay in HT-29 treated with Wortmannin (A-C). B, invaded cells with IL-11 0 ng/ ml with 0.1% FBS (x200, hematoxylin colorization). C, invaded cell with IL-11 50 ng/ml with 0.1% FBS (x200, hematoxylin). HT-29 cells exhibited high invasion responses, in a dose-dependent fashion, to rhIL-11 (p<0.0001) (A-C). Wortmannin (100 nM) suppressed invasion responses that were up-regulated by rhIL-11 and/or 20% FBS (p<0.0001, respectively) (A). Wortmannin reduced PI3 kinase activity which was induced by rhIL-11 in HT-29 (D). *A significant suppression and/or up-regulation.

up-regulated the number of cells undergoing chemotaxis (p<0.0001). The PI3K inhibitor, Wortmannin significantly suppressed the chemotactic response in HT-29 cells (p<0.0001). Wortmannin also suppressed a chemotactic response in 20% FBS (p<0.0001). However, the classical MAPK pathway inhibitor PD98059 did not suppress the chemotactic response in HT-29.

Next, we performed *in vitro* invasion assays to investigate the effects of these chemotactic responses on the invasiveness of CRC cells (Fig. 5A-C). Fig. 5A shows the number of cells that invaded through a reconstituted basement membrane in each of the five fields. We found that HT-29 cells exhibited highly invasive responses, in a dose-dependent fashion, to rhIL-11 through the reconstituted basement membrane (p<0.0001). To study the effect of IL-11 on the PI3K pathway in HT-29 cell, the invasive response was investigated. The PI3K inhibitor, Wortmannin, significantly suppressed the invasive response in HT-29 cells (p<0.0001). Wortmannin also inhibited the invasion activity in 20 %FBS (p<0.01). Next, to investigate the effect of Wortmannin on the PI3K pathway in CRC cells, the HT-29 cell was preincubated with Wortmannin in HT-29. Phosphorylation of P70 S6 RP was induced by stimulation with rhIL-11 in the absence but not the presence of Wortmannin (Fig. 5D). Wortmannin did not influence the state of P90 RSK p44/p42 MAPK and P38 MAPK.

Discussion

A multi-step carcinogenesis has been proposed for CRC, a theory that is generally accepted (30). Our results suggest that IL-11 and IL-11Rα have a role in the lymphatic and venous invasions of colorectal carcinoma, as one of the steps in carcinogenesis and in the development of invasive characteristics. Poorly differentiated types of adenocarcinomas show invasive spreading histologically, and have poorer prognoses than well differentiated types (31). It has been reported that there was a significant correlation between the type of histological differentiation and prognosis (32), but in this study, there was not significant correlation between IL-11 and IL-11Rα expression and histological differentiation of the tumor. Our results suggested that IL-11 and IL-11Rα might play an important role not in the differentiation but in the invasion of CRC.

The p44/p42 MAPK pathway is mostly known to be involved in proliferation, although several groups have stated that in some cell types it could also be involved in migration (23,24,33). The PI3K pathway is said to be an antiapoptotic pathway in myeloma (21), although several groups have found that PI3K plays a key role in migration and gradient-sensing in other cell types (19,20). In our study, Wortmannin, the inhibitor of the PI3K pathway, suppressed the phosphorylation of P70 S6 RP and reduced the invasion activity of CRC cell lines, and PD98059, the inhibitor of the p44/42 MAPK pathway, suppressed the phosphorylation of P90 RSK and reduced the proliferation activity of CRC cell lines. The activation of the PI3K and p44/p42 MAPK pathways by IL-11 may play an important role in the invasion and proliferation of colorectal carcinoma cells.

In clinical specimens, T4 and/or Dukes' D CRC expressed high intensity of IL-11, IL-11Rα and gp130, compared with adenomas and normal colorectal mucosa. In T4 and/or

Dukes' D CRC, phosphorylation of P70 S6 RP, P90 RSK and P38 MAPK were detected. However, other clinical samples also showed faint phosphorylation of these proteins. Moreover, adenomas had slightly higher phosphorylation of these proteins compared with normal colorectal mucosa. IL-11 did not phosphorylate P38 MAPK in CRC cell lines, so the phosphorylation of P38 MAPK in the clinical specimens may be induced by other cytokines and/or stimulations. These data suggest that IL-11/IL-11Rα signaling is needed for invasion and proliferation activity of carcinoma.

In conclusion, we demonstrated that the IL-11/IL-11R system regulated the activation of PI3K and MAPK pathways and up-regulated the invasion and proliferation activity of CRC cells. Further identification of tyrosine kinases and/or other pathways associated with IL-11 is needed to fully understand the mechanisms of the invasion and proliferation of colorectal carcinoma cells associated with this cytokine.

Acknowledgements

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