GROα promotes invasion of colorectal cancer cells

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Abstract. Growth-regulated oncogene α (GROα) is a chemokine that plays a role not only in inflammation, but also in tumorigenesis. Accumulating data suggest that GROα is involved in tumor development and invasion in various malignancies, such as melanoma and bladder cancer. However, the pathophysiological role of GROα in human colorectal cancers (CRCs) is still unknown. We examined the expression of GROα and its pathophysiological significance in human CRCs and investigated whether GROα promotes the invasive potential of colon cancer cells. Specimens of 62 primary CRCs were examined immunohistochemically for GROα, and the relationship between GROα expression and clinicopathological features was investigated. The mRNA expression of *GROα* and its receptor *CXCR2* was examined in ten colon cancer cell lines using RT-PCR. The effect of GROα protein on invasive potential was investigated in DLD-1 and LoVo cells using a Matrigel invasion chamber assay. Forty-nine (79%) of the 62 CRCs showed positive immunoreactivity for GROα. GROα expression was significantly associated with tumor size, tumor stage, depth of invasion, LN metastasis and patient survival (P=0.021, P<0.0001, P=0.0033, P<0.0001, P=0.039, respectively). Expression of *CXCR2* mRNA was detectable in all ten colon cancer cell lines examined, whereas expression of *GROα* mRNA was detectable in six. Treatment with GROα protein significantly increased the number of invasive cells. In conclusion, GROα may play a pivotal role in the invasion of human CRCs.

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Abbreviations: CRC, colorectal cancer; GRO, growth-regulated oncogene; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-polymerase chain reaction

Key words: growth-regulated oncogene α, CXCR2, colorectal cancer, invasion

Introduction

Chemokines are a group of small molecular cytokines that regulate the chemotaxis of leukocytes into inflammatory tissue (1). They are classified into four groups (CXC, CC, C and CX_3C) based on the arrangement of the two N-terminal cysteine residues (2,3). Chemokines play crucial roles in the regulation of inflammation, wound healing, and development (4-6). Recently, accumulating evidence has indicated that they play equally important roles in the proliferation, survival, and migration of tumor cells, suggesting their involvement in tumor development and invasion (7-9).

Growth-regulated oncogene α (GROα) was originally identified by subtractive hybridization between tumorigenic and non-tumorigenic Chinese hamster embryonic fibroblasts (10). GROα belongs to the CXC chemokine subfamily (CXC chemokine ligand 1) and promotes chemoattraction, wound healing and angiogenesis through the seven-transmembrane G-protein-coupled receptor CXCR2 (11-13). GROα protein has also been isolated from human melanoma cells (14,15), and reported to be up-regulated in various tumors, such as glioma (16), and cancers of the breast (17-19), ovary (20), prostate (21,22) and bladder (23). In these tumors, GROα is suggested to be involved in tumor development and invasion as a growth and anti-apoptotic factor, or as a mediator to promote tumor invasion. Although elevated expression of GROα has been reported in a series of human tumors, the role of GROα in human colorectal cancers (CRCs) is poorly understood. Recently, *GROα* was identified as a distinctly overexpressed gene in human CRCs by microarray analyses (24-26), suggesting that GROα is involved in the pathophysiology of human CRCs. Moreover, Wang *et al* have reported that GROα promotes the growth of colon cancer cells in a mouse xenograft tumor model (27). In the present study, therefore, we examined the expression of GROα and its pathophysiological significance in human CRCs, and investigated whether the GROα protein regulates the invasive potential of colon cancer cells.

Materials and methods

Tissue specimens and histological examination. A total of 62 patients with CRC who underwent surgical resection at

Dokkyo University School of Medicine between 2002 and 2004 were enrolled. Patients who had received preoperative treatment such as chemotherapy and radiation therapy were excluded. This study was performed with the approval of the Dokkyo University Surgical Pathology Committee, and informed consent was obtained from all patients.

The resected specimens were fixed in 10% formalin solution and embedded in paraffin. Multiple hematoxylin-eosin-stained sections of all the lesions were examined. The following factors were determined for all patients and lesions: age, gender, tumor size, tumor location, tumor differentiation, tumor invasion, lymph node metastases, and tumor stage. Tumor differentiation and stage were determined according to the WHO and UICC criteria, respectively. All these clinicopathological features are summarized in Table I.

Immunohistochemical staining. Immunohistochemical staining for GROα was performed as described previously (28,29). In brief, the sections (4 μm thick) were deparaffinised, rehydrated, placed in 0.01 M citrate buffer (pH 6.0), and treated by microwave heating for 40 min. The sections were then preincubated with 0.3% H_2O_2 in methanol for 20 min at room temperature to quench endogenous peroxidase activity. Subsequently, the sections were immunostained using an Envison+ System kit (Dako, Carpinteria, CA, USA), according to the manufacturer's instructions. The sections were incubated with 1% bovine serum albumin in phosphate-buffered saline and then incubated with anti-GROα antibody (dilution 1:20; R&D Systems, Minneapolis, MN, USA) for 1 h at room temperature. Then, the sections were incubated with HRP-conjugated secondary antibody for 60 min. After washing with phosphate-buffered saline, the sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 5 min and then counterstained with Carazzi's hematoxylin. To evaluate the immunoreactivity of GROα protein, at least 500 tumor cells were counted in 5 different visual fields for each cancerous tissue sample. A specimen was considered positive for GROα when >20% of the tumor cells were stained.

Cell culture. Human colon cancer cell lines Caco2, HT29, Colo320DM, WiDr, SW403, Colo201, Colo205, and LoVo were cultured in RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum (Sigma, St. Louis, MO, USA). DLD-1 and SW48 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% fetal bovine serum. All cell lines were incubated in a humidified incubator at 37˚C with an atmosphere of 5% CO_2.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from colon cancer cell lines with TRIzol reagent (Invitrogen). Five micrograms of total RNA was reverse-transcribed using oligo-dT primer (Applied Biosystems, Branchburg, NJ, USA) and 200 U of Superscript™ II reverse transcriptase (Invitrogen) in a total volume of 20 μl. For the subsequent PCR, pairs of oligonucleotide primers for human *GROα*, human *CXCR2* (*GROα receptor*), and human *glyceraldehydes-3-phosphate dehydrogenase* (*GAPDH*) were prepared. Human *GROα*, 5'-CCGAAGTCATAGCCACACTCAA-3' (sense), 5'-TGTTGCAGGCTCCTCAGAAATA-3' (antisense); human *CXCR2*, 5'-ATTCTGGGCATCCTTCACAG-3' (sense), 5'-TGCACTTAGGCAGGAGGTCT-3' (antisense); human *GAPDH*, 5'-GGCTGCTTTTAACTCTGGTA-3' (sense), 5'-ATGCCAGTGAGCTTCCCGT-3' (antisense). RT-PCR was performed as described previously (29). In brief, one microliter of RT product (cDNA) was amplified by PCR in a 50-μl reaction volume containing 40 pmol of the above sets

Table I. Clinicopathological features of the patients with colorectal cancers.

Characteristics	No. (%)
Gender	
Male	38 (61.3)
Female	24 (38.7)
Age (Years, mean ± SD)	63.2±10.9 (35-80)
Tumor location	
Colon	33 (53.2)
Rectum	29 (46.8)
Tumor size (cm, mean ± SD)	4.4±1.8 (1.0-8.5)
Differentiation	
Well	21 (33.9)
Moderate	38 (61.3)
Poor	1 (1.6)
Mucinous	2 (3.2)
UICC stage	
I	13 (21.0)
II	11 (17.7)
III	32 (51.6)
IV	6 (9.7)
Depth of invasion	
T1	10 (16.1)
T2	9 (14.5)
T3	40 (64.5)
T4	3 (4.8)
Lymphatic invasion	
None	17 (27.4)
Present	45 (72.6)
Venous invasion	
None	22 (35.5)
Present	40 (64.5)
Lymph node metastasis	
None	24 (38.7)
Present	38 (61.3)

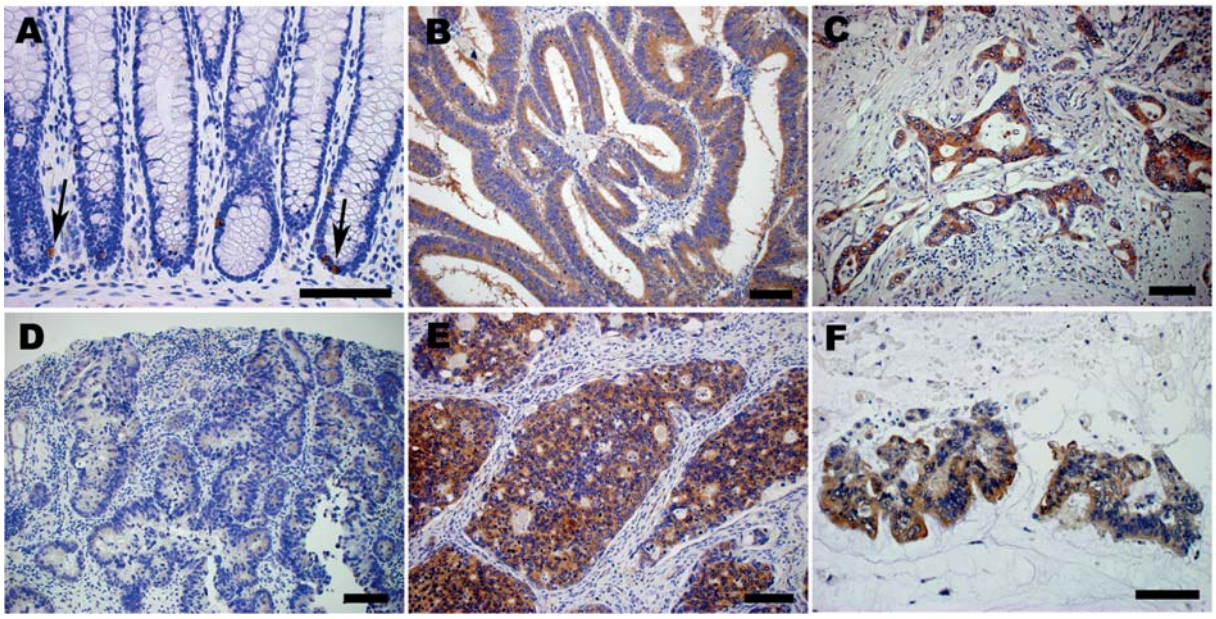

Figure 1. Immunohistochemistry for GROα in normal colon and colorectal cancer tissues. (A) GROα immunoreactivity is observed in a few cells at the base of normal crypts. Arrow indicates an epithelial cell expressing GROα. (B) Presence of GROα immunoreactivity in the cytoplasm of human CRC cells. Strong immunostaining for GROα is evident at the invasive front of CRCs, whereas weak immunostaining is present in the superficial part of the tumor. Representative images of the invasive front (C) and superficial part (D) of CRC. GROα immunoreactivity is also detectable in poorly differentiated adenocarcinoma (E) and mucinous adenocarcinoma (F). Scale bars, 100 μm.

of primers, 1.25 U of Ampli-Taq DNA polymerase (Applied Biosystems), and the final PCR buffer: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM dNTP. The PCR amplification was performed as follows: at 95˚C for 5 min once; 35 cycles at 94˚C for 1 min, at 60˚C for 45 sec, and at 72˚C for 1 min; then at 72˚C for 7 min.

Cell invasion assay. Cell invasion assay was performed using BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA, USA) in accordance with the manufacturer's protocol. Briefly, DLD-1 ($5x10^4$) or LoVo cells ($5x10^4$) were seeded in the insert of the Matrigel-coated invasion chamber (24 wells, 8-μm pore size) filled with serum-free DMEM medium containing different concentrations of GROα (0-100 ng/ml). Then, the cells were incubated with DMEM medium containing 10% FBS in the lower chamber at 37˚C in 5% CO_2. To inhibit the effects of GROα, anti-GROα antibody (20 μg/ml) was also added to the upper chamber. After incubation for 36 h, non-invading cells were removed using a cotton swab and the cells that had invaded into the lower surface of the membrane were fixed with ethanol. The invading cells were then stained with hematoxylin and counted using a microscope in 5 different visual fields (magnification, x200).

Statistical analysis. The χ^2 test was performed to determine correlations among the various parameters, and Fisher's extract test was also used, as necessary. Age, tumor size and the number of invasive cells are expressed as mean ± SEM. Statistical differences between the two groups were assessed by the unpaired two-tailed t-test or by the Mann-Whitney U test when data were not parametric. Cumulative survival rate was evaluated by the Kaplan-Meier method and analyzed by log-rank test. A P-value of <0.05 was considered to indicate statistical significance.

Results

Expression of GROα in normal colon and CRC tissue. In normal colorectal mucosa adjacent to the tumor, immunoreactivity for GROα was observed in the cytoplasm of a few epithelial cells in the basal portion of crypts (Fig. 1A). In the CRC tissue samples, GROα immunoreactivity was detected in the cytoplasm of cancer cells (Fig. 1B). Forty-nine (79.0%) of the 62 CRCs were positive for GROα expression. In the GROα-positive CRCs, cytoplasmic staining for GROα was often more intense at the invasive front (Fig. 1C) than in the superficial part of the tumor (Fig. 1D). Moreover, GROα immunoreactivity was detected in CRCs showing various types of differentiation, including poorly differentiated adenocarcinoma (Fig. 1E) and mucinous adenocarcinoma (Fig. 1F).

Relationship between GROα expression and clinicopathological features in CRCs. To clarify the pathophysiological significance of GROα expression in human CRCs, the differences between the clinicopathological features of patients with GROα-positive CRC and those with GROα-negative CRC were investigated. As shown in Table II, with regard to tumor size, GROα-positive CRCs were significantly larger than GRO-negative CRCs (P=0.021). Moreover, tumor stage, depth of invasion, and prevalence of lymphatic invasion, venous invasion, and LN metastasis were significantly higher in the former than in the latter ($P<0.0001$, P=0.0033, P=0.0005, P=0.0078, $P<0.0001$, respectively) (Table II).

Table II. Comparison of the clinicopathological features between the GROα-positive and GROα-negative CRC patients.

	GROα expression		
	Positive (n=49)	Negative (n=13)	P-value
Gender			
Male	29	9	0.509
Female	20	4	
Age	62.4±10.6	66.2±12.1	0.264
Tumor location			
Colon	26	7	0.960
Rectum	23	6	
Tumor size	4.7±1.7	3.3±2.0	0.021
Differentiation			
Well	16	5	0.675
Moderate	31	7	
Poor	1	0	
Mucinous	1	1	
UICC stage			
I	5	8	<0.0001
II	7	4	
III	31	1	
V	6	0	
Depth of invasion			
T1	4	6	0.0033
T2	6	3	
T3	36	4	
T4	3	0	
Lymphatic invasion			
None	8	9	0.0005
Present	41	4	
Venous invasion			
None	13	9	0.0078
Present	36	4	
Lymph node metastasis			
None	12	12	<0.0001
Present	37	1	

However, none of the other parameters, gender, age, tumor location, or differentiation, had a significant relationship to GROα expression.

Prognostic significance of GROα expression in patients with CRC. Log-rank statistics showed that venous invasion, LN metastasis and tumor stage were significant prognostic factors for overall survival of patients with CRC (P=0.041, P=0.0048, P<0.0001, respectively). No significant correlation was evident between prognosis and other clinicopathological features. To evaluate the prognostic significance of GROα expression in patients with CRC, Kaplan-Meier curves were generated. As shown in Fig. 2, patients with GROα-positive CRC had significantly shorter overall survival than those with GROα-negative CRC (P=0.039).

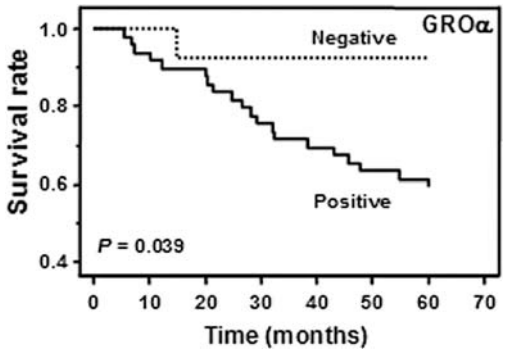

Figure 2. Overall survival in relation to GROα expression in patients with colorectal cancer. Kaplan-Meier survival curves were constructed, and the difference between the GROα-positive (n=49) and GROα-negative (n=13) groups was analyzed by log-rank test.

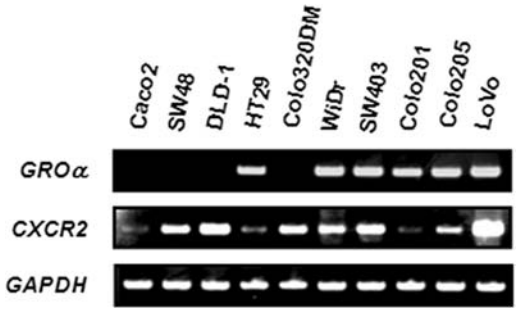

Figure 3. Expression of *GROα* and *CXCR2* mRNA in various human colon cancer cell lines. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Expression of GROα and CXCR2 mRNA in colon cancer cell lines. Expression of mRNA for *GROα* and its receptor *CXCR2* was examined in 10 colon cancer cell lines using the RT-PCR method. As shown in Fig. 3, expression of *GROα* mRNA was detected in 6 (60%) of 10 colon cancer cell lines examined. However, expression of *GROα* mRNA was not detected in the 4 remaining cell lines, Caco2, SW48, DLD-1 and Colo320DM. On the other hand, expression of *CXCR2* mRNA was detected in all 10 of the colon cancer cell lines examined (Fig. 3).

Effect of GROα protein on invasive potential in colon cancer cells. To clarify the pathophysiological role of GROα protein in CRCs, we next examined the invasion ability of colon cancer cells stimulated with GROα protein using a Matrigel invasion assay. When DLD-1 cells were unstimulated, only a few invaded across the membrane during incubation for 36 h

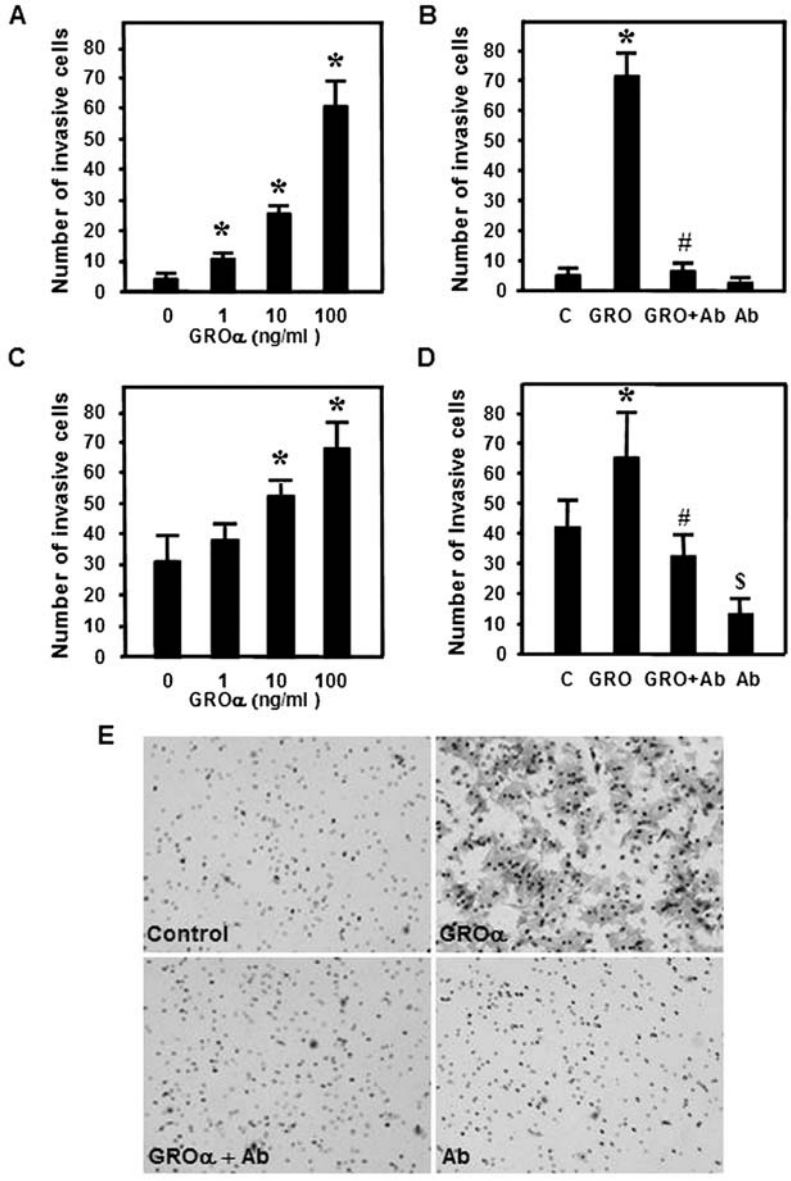

Figure 4. Effect of GROα protein on invasive potential of colon cancer cells. Changes in number of invasive DLD-1 (A) and LoVo cells (C) under GROα treatment were examined. Effect of anti-GROα antibody (Ab, 20 μg/ml) on GROα (100 ng/ml)-induced invasion of DLD-1 (B) and LoVo cells (D) was investigated. (E) Images from a representative experiment show control DLD-1 cells, GROα-treated cells, GROα-treated cells in the presence of neutralizing antibody against GROα, and cells cultured with neutralizing antibody against GROα. All the results in A-D are presented as the mean ± SEM of 4 independent experiments. *Significantly greater than the control cell group (P<0.01). #Significantly lower than the GROα-treated cell group (P<0.01). $Significantly lower than the control cell group (P<0.01).

(Fig. 4A and E). However, when the cells were stimulated with GROα protein, the number that showed invasion increased significantly in a dose-dependent manner (Fig. 4A). We then inhibited the function of GROα protein with a neutralizing antibody. Treatment with GROα protein (100 ng/ml) significantly increased the number of invasive DLD-1 cells, but this effect was decreased by the addition of anti-GROα antibody (Fig. 4B and E). There was no significant difference in the number of invasive cells between non-stimulated cells and cells treated with anti GROα antibody (Fig. 4B and E).

We next examined the effect of GROα protein on invasive potential in LoVo cells which endogenously express GROα When LoVo cells were unstimulated, some cells invaded across the membrane during the incubation for 36 h. The number of invasive cells in unstimulated LoVo cells was more than that in unstimulated DLD-1 cells. When the LoVo cells were stimulated with GROα protein, the number of invasive cells increased in a dose-dependent manner (Fig. 4C). Treatment with GROα protein (100 ng/ml) significantly increased the number of invasive LoVo cells, but this effect was abolished by the concomitant administration of anti-GROα antibody (Fig. 4D). Moreover, the inhibition of endogenous GROα function with a neutralizing antibody in unstimulated LoVo cells led the reduction in number of invasive cells (Fig. 4D).

Discussion

Chemokines and their receptors are implicated in leukocyte migration to inflammatory sites and angiogenesis in various

inflammatory conditions (6,30). Besides their functions in inflammation, accumulating evidence suggests that they also play critical roles in tumor development, invasion and metastasis (7). In support of this idea, our immunohistochemical analyses revealed that a considerable number of human CRCs indeed produce GROα. Moreover, we demonstrated that all of the human colon cancer cell lines we tested expressed *CXCR2* mRNA. Previously, CXCR2 expression has been reported to be elevated in human CRCs (27,31). Thus, although we did not examine the expression of CXCR2 in human CRC tissues in the present study, it appears reasonable to speculate that the GROα/CXCR2 axis is involved in the pathophysiology of human CRCs.

The most important finding of this study was that GROα-positive CRCs showed a significantly larger tumor size, a higher tumor stage and a higher frequency of LN metastasis. We also demonstrated that GROα immunostaining was often more intense at the invasive front of CRCs and GROα protein indeed promoted the invasive potential of colon cancer cells. Recently, GROα protein has been reported to exert a trophic and anti-apoptotic effect on colon cancer cells (26). Moreover, several studies have indicated that GROα promotes the invasive potential of glioma cells (16) and bladder cancer cells (23). These studies, together with our present data, suggest that GROα is involved in the development and invasion of human CRCs, acting as a trophic and anti-apoptotic factor and also a potential factor mediating tumor invasion, in an autocrine/paracrine manner.

One issue of interest is the molecular mechanism by which GROα promotes tumor invasion and metastasis in CRCs. During the process of tumor invasion and metastasis, tumor cells need to acquire the capability to degrade the extracellular matrix and promote neoangiogenesis (32,33). Accumulating evidence suggests that activation of matrix metalloproteinase (MMP) and the promotion of angiogenesis play pivotal roles in chemokine-related tumor invasion and metastasis (34,35). Using a mouse cancer model, Kitamura *et al* demonstrated that activation of MMP-2 and MMP-9 is involved in CCL9/CCR1-related invasion of colon cancer (36). Moreover, in invasive breast cancers, the CXCL12/CXCR4 axis has been shown to play important roles in tumor angiogenesis (37). Recent reports have also indicated that GROα promotes tumor invasion by enhancing the expression of MMP-2 in bladder cancer cells (23) and mediates prostaglandin-induced angiogenesis by accelerating endothelial migration and tube formation in CRC (27). From these data, it can be speculated that, in human CRCs, GROα protein play a role in tumor invasion and metastasis through activation of MMPs and promotion of angiogenesis, although further studies will be needed to test this hypothesis.

Importantly, activation of the chemokine-receptor axis in cancer tissues sometimes affects the prognosis of patients. Previous studies have indicated that activation of the CXCL12/CXCR4 axis is a predictor of poor outcome in patients with various tumors, such as glioma (38), melanoma (39), and cancers of breast (40,41), lung (42), and colon (43,44). In this context, it is noteworthy that patients with GROα-positive CRCs showed a significantly worse outcome than those with GROα-negative CRCs, suggesting that GROα is not only a potential mediator of tumor invasion but also a significant prognostic marker in CRC patients. Recently, Yan and Chen have reported that mutant p53 binds to the promoter region of the GROα gene and exerts its function by inducing *GROα* expression in colon cancer cells (45). Since mutation of *p53* is suggested to be involved in the tumorigenesis of various tumors (46), GROα may play a crucial role in the presence of *p53* mutation. In support of this idea, our present study showed that GROα was up-regulated in a large proportion of human CRCs, and was related to tumor invasion, LN metastasis and subsequent poor outcome. Therefore, inhibition of GROα/CXCR2 signaling on tumor cells may have the potential to prevent invasion and metastasis in a large number of human CRCs. The therapeutic efficacy of targeting chemokine-receptor signaling has been intensively studied in melanoma (47,48), breast (34,49-51), and colon cancer (52,53). Recently, Wang *et al* showed that abolishment of GROα signaling using anti-GROα neutralizing antibodies led to a reduction of colorectal xenograft tumor growth with decreased the formation of microvessels (27). Moreover, Yamamoto *et al* have reported that blockade of the GROα/CXCR2 axis decreased the frequency of liver metastasis from colon cancer (54). Taken together, these data suggest that GROα/CXCR2 targeting could be a potentially useful new strategy for the treatment of human CRC, although more extensive studies will be required.

In summary, we have demonstrated that GROα is up-regulated in a large proportion of human CRCs and closely associated with advanced tumor stage, LN metastasis and poor prognosis. We have also clarified that GROα protein promotes the invasive potential of colon cancer cells. These findings suggest that GROα plays a pivotal role in the invasiveness of human CRCs. Therefore, targeting of GROα as a potential treatment for invasive human CRCs should be examined in future studies.

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