

Therapeutics target of CXCR4 and its downstream in peritoneal carcinomatosis of gastric cancer

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1. ABSTRACT

Patients with advanced gastric carcinoma, especially peritoneal dissemination, have a poor prognosis. Various treatments have been used for peritoneal dissemination of gastric cancer, but there is no effective therapy for this condition. At present, similar properties of chemokines between trafficking of leukocytes during immune and inflammatory reactions and organ selective migration of cancer cells during metastasis are widely recognized. In particular, chemokine CXCL12 and its receptors CXCR4 are now known to play an important role in cancer progression. Recently, we reported for the first time that CXCR4 and its ligand, CXCL12, were involved in the development of peritoneal carcinomatosis of gastric cancer, and additionally, clarified the molecular mechanisms of the cell signaling pathways by which gastric cancer develops metastatic ability via CXCR4 and mTOR. In this review, we focus on the biological functions of chemokine receptors, particularly CXCR4 expressed on gastric cancer cells, and the therapeutic strategies targeting CXCR4-mediated signaling pathways in peritoneal carcinomatosis.

2. INTRODUCTION

Chemotactic cytokines, namely chemokines, are known as the center of cell migration. Chemokines are a family of small (8-14 kDa), mostly basic and heparin-binding cytokines that primarily induce the directed migration of various types of leukocytes; that is, rapid normal leukocyte trafficking is controlled strictly by chemokines and their receptors (1, 2). Chemokines are classified into four subfamilies according to the arrangement of amino-terminal conserved cysteine residues; CXC, CC, C, and CX3C chemokines (3). To date, at least 46 chemokine ligands have been identified in humans. There are also 18 functionally signaling chemokine receptors and two 'decoy' or 'scavenger' receptors (4). Leukocyte trafficking and, to a lesser degree, cancer metastasis have regular behavior, called organ selectivity.

The metaphor of seed and soil coined by Stephen Paget in 1889 commonly holds that metastasis is dependent on both the organ from which the primary tumor originates, and the organs to which the tumor cells travel (5). The

Table 1. Expression of CXCR4 in cancer metastasis

Cancer cell type	Site of metastasis
Breast	Lung, Lymph node
Prostate	Bone
Non-small cell lung cancer	Pleural space
Ovarian	Peritoneum
Pancreas	Liver, Lung
Melanoma	Lymph node
Neuroblastoma	Bone, Bone marrow
Esophageal	Lymph nodes, Bone marrow
Colorectal	Liver
Osteosarcoma	Lung
Renal	Adrenal glands, Bone
Gastric	Peritoneum

cancer metastatic process can be divided into several migration steps. First, cancer cells are released from the primary cancer lesion to the surrounding tissues, enter the vascular or lymphatic vessels, and are transported through circulatory system. Then, the cells arrive in the capillary bed of a distant organ and extravasate from the circulation to organ parenchyma.

Recently, it has been shown that various types of cancer cells express chemokine receptors, suggesting that chemokines may play a role in cancer progression and/or organ-selective metastasis (6) (7) (8) (9). In fact, several therapeutics challenges, which target cancer-chemokine receptors, have been developed and are under clinical trials. For example, in Japan, CC chemokine receptor 4 (CCR4) is regarded as the most promising molecular target for cancer immunotherapy (10, 11). Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell neoplasm with a dismal prognosis, and no therapy is available for most ATLL patients. Ueda *et al.* developed a novel humanized anti-CCR4 monoclonal antibody (mAb) whose Fc region is artificially defucosylated to enhance antibody-dependent cell-mediated cytotoxicity (ADCC) activity by increasing its binding affinity to Fc receptors on effector cells (12), and are now conducting a phase I clinical trial of this anti-CCR4 mAb in patients with CCR4-positive T-cell leukemia/lymphoma (clinical trials gov. identifier: NCT00355472).

With regard to CXC chemokine receptors, CXCR4 has been shown to be overexpressed in many human cancers, including breast cancer, ovarian cancer, melanoma, and prostate cancer, and is recognized as the dominant chemokine receptor controlling metastasis (13).

3. CXCR4 IS A NEW PLAYER IN CANCER METASTASIS

CXCL12 (also called stromal-derived factor-1 α , SDF-1 α) is a CXC chemokine which has unique properties in chemokines. Hematopoietic stem cells proliferate and differentiate into mature hematopoietic cells in the parenchyma of bone marrow, and are essential for the viability of an embryo, B lymphopoiesis, bone marrow hematopoiesis and cardiogenesis. Bone marrow stromal cells are the major constitutive source of CXCL12, and CXCL12 plays an important role as a highly efficient

chemoattractant for lymphocytes, monocytes and CD34-positive hematopoietic precursor cells expressing its receptor CXCR4 (1, 2). CXCR4 was previously noted as an entry co-receptor for T cell line-tropic human immunodeficiency virus (HIV)-1, and a small molecule CXCR4 inhibitor that blocks HIV-1 entry was identified. Moreover, CXCR4 has been revealed to play critical roles in the development of embryos because CXCR4 gene knockout mice are lethal owing to defects in hematopoietic stem cell homing, the heart, and central nervous system (14).

Muller *et al.* reported the landmark finding that the chemokine receptor CXCR4 is highly expressed in human breast cancer cells, and that its respective ligands play critical roles in determining the metastatic destination of breast cancer (6). Later studies have supported their findings that CXCR4 plays an important role in the metastatic behavior and destination of solid cancers. Table 1 lists CXCR4 expression in various types of cancer cells: breast (15), prostate (16), lung (17), ovarian (18), pancreatic (19), melanoma (20), neuroblastoma (21), esophageal (22), colorectal (23), osteosarcoma (24) and renal (25). In addition, high-level expression of CXCR4 results in poor prognosis in lung cancer (26), melanoma (20), pancreatic (19), ovarian (18), colorectal (23) and breast cancers (6, 15, 27).

4. TARGETING CXCR4 IN PERITONEAL CARCINOMATOSIS OF GASTRIC CANCER

The reported 5-year survival rate remains only 2% in the patients with intraperitoneal cancer cells even if they do not have macroscopic peritoneal carcinomatosis (28). Peritoneal carcinomatosis, often related to malignant ascites, frequently causes death in patients with advanced gastric cancer, and no available regimens for this condition can be found at present (29-31). To overcome this difficulty and design a novel effective treatment for peritoneal carcinomatosis, it is most important to understand the molecular mechanisms that promote the development of peritoneal carcinomatosis.

We previously revealed the relationships between the development and CXCR4 expression in gastric cancer (7). Table 2 lists all 49 cases of CXCR4 in 49 primary tumors of stage IV gastric carcinoma. These samples were derived from 33 patients with peritoneal metastases and 16

Table 2. Significant correlations between CXCR4 expression in primary gastric cancer and peritoneal carcinomatosis

Peritoneal Carcinomatosis	CXCR4	
	+ (n=26)	- (n=23)
Positive	22*	11
Negative	4	12

Immunohistochemical staining of CXCR4 was done in 46 samples of primary gastric cancer with (n=26) or without (n=23) peritoneal carcinomatosis. (*, $P < 0.01$; χ^2 test, 2-sided)

patients with distant metastases but not peritoneal metastases. Among the 33 primary gastric cancers with peritoneal metastasis, 22 tumor cells scored positive for CXCR4 expression (67%). On the other hand, only 4 out of 16 primary tumors with other distant metastases scored positive for CXCR4 (25%). Furthermore, 22 out of 26 CXCR4-expressing primary tumors developed peritoneal metastases (85%), while 11 out of 23 CXCR4-negative primary tumors developed peritoneal metastases (48%).

To clear the critical role of CXCR4 in promotion of peritoneal carcinomatosis by gastric carcinoma, we next investigated human clinical samples. We have shown that peritoneal mesothelial cells strongly express its ligand CXCL12. In addition, we have shown that malignant ascitic fluids from patients with peritoneal carcinomatosis of gastric cancer contained CXCL12 at concentrations much higher than normal fluids in the peritoneal cavity. In contrast, CXCL12 is barely expressed in liver or lymph nodes, the other main sites of distant gastric cancer metastasis. Collectively, these results strongly suggest that CXCR4-expressing gastric carcinoma cells are preferentially attracted to the peritoneum cavity where its ligand CXCL12 is abundantly produced.

The administration of anti-CXCR4 mAb in experimental metastasis models resulted in significant inhibition of lung metastasis of breast cancer (6) and melanoma (32), and bone metastasis of prostate cancer (33), suggesting that the inhibitors of CXCL12-CXCR4 signaling may improve the outcomes of cancer patients in advanced stages.

A therapeutic approach using antagonists of CXCR4 is thus thought to be reasonable for cancer metastasis. Chemokine works through interactions with its receptor, which is a group of seven transmembrane, G protein-coupled receptors (GPCRs). GPCRs mediate biological effects such as cell migration. Most targets of the current drugs are GPCRs, and new drugs targeting GPCRs continue to be discovered and developed as novel therapeutic targets in several diseases.

AMD3100 was developed as a new type of low-molecular-weight molecules that have a protective effect against human immunodeficiency virus (HIV) infection through specific blockade of CXCR4 (34). Phase I clinical trials of AMD3100 against HIV were unfortunately halted because of an unexpected side effect, i.e., an increased white blood cell count. Interestingly, a specific increase of CD34-positive hematopoietic stem cells in the peripheral blood was clearly observed, with the result that AMD3100 is currently used for transplantation in patients with hematological malignancies, such as non-Hodgkin's lymphoma or multiple myeloma (35).

Given the significant correlation between CXCR4 expression on biopsy of primary gastric cancers and peritoneal carcinomatosis, we performed a therapeutic experiment applying AMD3100 to a xenograft model of human gastric cancer, NUGC4 cells which develop severe peritoneal carcinomatosis in nude mice (7). AMD3100 significantly decreased the sizes of disseminated tumors in the greater omentum and mesenterium in the abdominal cavity, and also significantly decreased the volume of ascitic fluid; therefore, usage of CXCR4 antagonist may be an effective therapy for gastric cancers with peritoneal dissemination.

The US Food and Drug Administration approved AMD3100 (Mozobil TM ; Genzyme Corp.), a new small-molecule inhibitor of the CXCR4 chemokine receptor, for use in combination with granulocyte colony-stimulating factor (G-CSF) to mobilize hematopoietic stem cells (HSC) to the peripheral blood for collection and subsequent autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma. As the results of the safety and efficacy of AMD3100 were demonstrated by 2 multicenter, randomized, placebo-controlled studies, no serious adverse effects were observed. Common adverse reactions included diarrhea, nausea, vomiting, flatulence, injection site reactions, fatigue, arthralgia, headache, dizziness, and insomnia (36). However, it is important to pay attention that AMD3100 might accelerate tumor angiogenesis and premetastatic niche formation because of its profound effect of mobilizing HSC.

5. GOING DOWNSTREAM OF CXCR4 IN PERITONEAL CARCINOMATOSIS OF GASTRIC CANCER

As noted above, we first reported that chemokine CXCL12 developed peritoneal carcinomatosis from gastric cancer via its receptor CXCR4. However, the signaling pathway of CXCR4 and some other important cytokines/chemokines which induce peritoneal carcinomatosis has not been well-defined in gastrointestinal malignancies.

In the development of peritoneal carcinomatosis of gastric cancer, cancer cell proliferation is facilitated by several cytokines such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF). Furthermore, vascular endothelial growth factor (VEGF) enhances the permeability of blood vessels in the accumulation of malignant ascitic fluid (37-40). The protein kinase Akt is included in a significant signaling pathway of diverse environmental cues to control a plethora of cancer cellular processes, such as initiation, progression and metastasis (41).

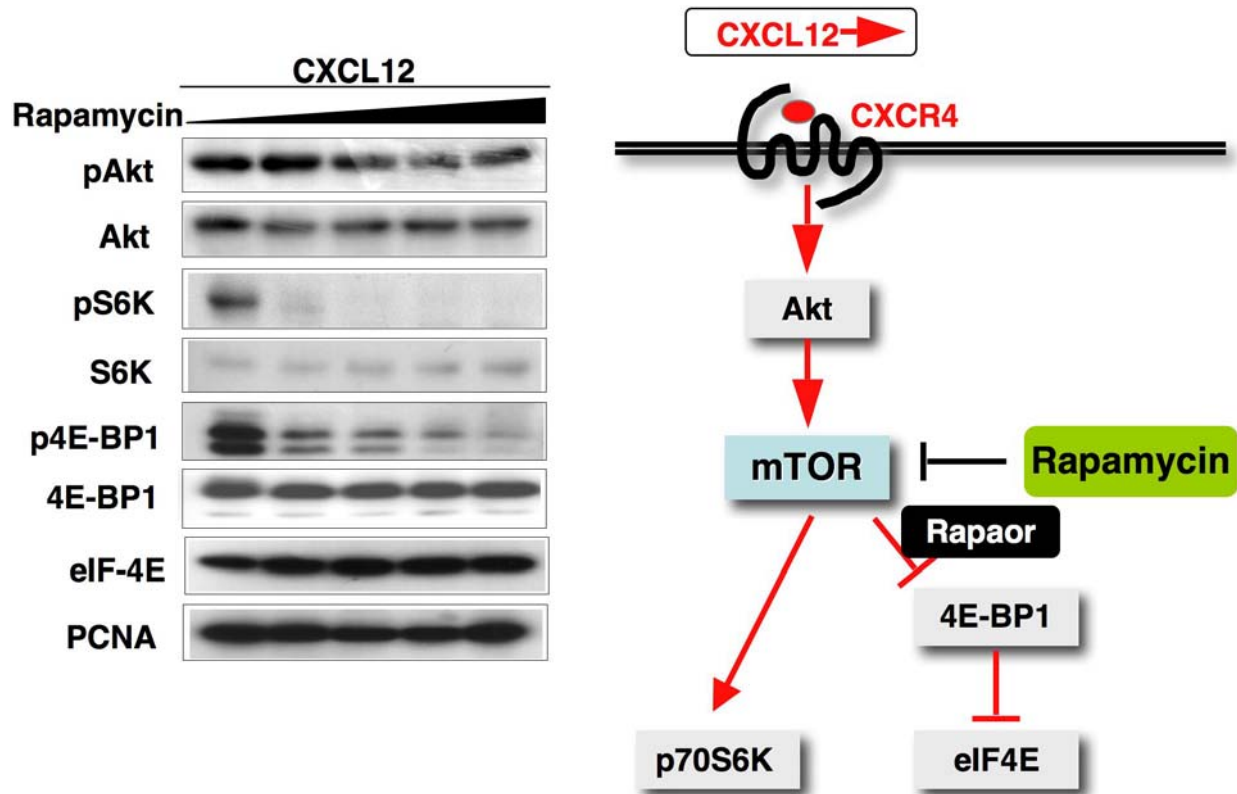


Figure 1. Activation of mTOR signaling by chemokine CXCL12 in peritoneal disseminated gastric cancer cell. The accumulation of phosphorylated Akt (pAkt), pS6 and p4E-BP1 were strongly induced by CXCL12 in NUGC4 cells. Constitutively pS6 and p4E-BP1 were inhibited by rapamycin.

We investigated the signaling cascade activated in peritoneal carcinomatosis, and found that CXCL12 (Figure 1) and these cytokines rapidly induced the intense phosphorylation of Akt, and subsequently phosphorylated mTOR signaling pathway components (S6K and 4E-BP1) in disseminated gastric cancer NUGC4 cells (42). The phosphorylation of S6K and 4E-BP1, which was induced by CXCL12 in NUGC4, was inhibited by rapamycin, an mTOR inhibitor. These results strongly suggested that the development of peritoneal carcinomatosis of gastric cancer was mediated by the CXCL12 signaling pathway via CXCR4 / Akt / mTOR.

Cell proliferation is a separate event in mammalian cell growth and cycle progression. mTOR- and PI3K-dependent signals maintain the proper cell size for cell growth (40). Rapamycin, also called sirolimus, is a macrocyclic antibiotic derived from the bacterium *Streptomyces hygroscopicus*, discovered in the soil of Easter Island. Rapamycin was originally found as an antifungal agent, and unexpectedly, it also exhibited an immunosuppressive property. Fortunately, rapamycin was available and was developed as a useful drug for cancer patients in various clinical stages. Rapamycin and another immunosuppressive drug FK506 (tacrolimus) have similar chemical structures, and they bind to the same intracellular receptor, FKBP12. FK506 blocks the Ca^{2+} /calcineurin-

dependent transcriptional activation of genes for growth to inhibit T cell proliferation selectively, whereas rapamycin interrupts growth-promoting cytokine signaling (43).

Clinical trials of the mTOR inhibitor rapamycin and its derivatives CCI-779, RAD001, and AP23573, are just getting underway in renal cancer, breast cancer, lymphoma, glioblastoma, sarcoma and several other solid cancers, and good results are being obtained (44).

We therefore investigated whether rapamycin could inhibit the metastatic properties induced by CXCL12 in peritoneal disseminated NUGC4 cells. As a result, rapamycin also suppressed migration and the production of matrix metalloproteinase (MMP), which is the driving force of cancer cell invasion, and triggered cell death by autophagy (42). In this study, we have focused on the association of peritoneal dissemination with the mTOR signaling pathway induced by chemokine CXCL12 alone; however, we also observed that mTOR activation was induced not only by CXCL12 but also by some other cytokines (EGF, HGF, VEGF and tumor necrosis factor- α [TNF- α]) related with peritoneal carcinomatosis (37-40).

Therefore, blocking the mTOR signaling pathway may also be an attractive therapeutic target of

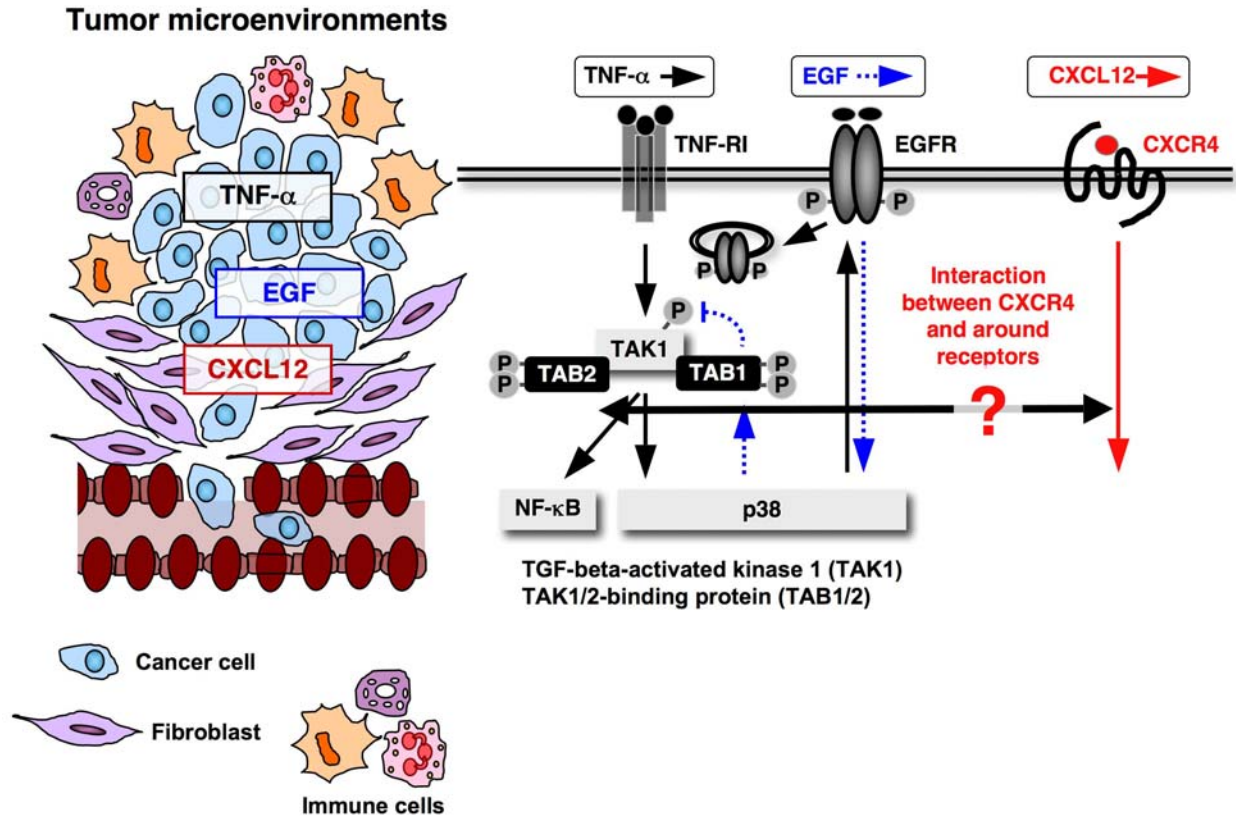


Figure 2. Cancer cells are co-stimulated with a variety of protein ligands secreted in tumor microenvironments. A possible schematic diagram for cross-talk between TNF- α , EGF and CXCL12 signaling pathways are shown. EGFR is phosphorylated at Ser-1046/7 through p38, which leads to receptor endocytosis (black arrow). The EGFR-mediated signal prevents TNF- α -induced phosphorylation of TAK1 through p38-mediated phosphorylation of TAB1 (blue arrow). CXCR4, a member of the GPCR family, uses different signaling modules from TNF- α and EGF receptors. Functional interaction of the CXCR4 signaling pathway (red arrow) with its around receptors will provide new insights into the CXCL12-CXCR4 axis.

peritoneal disseminated gastric cancer that is activated by various types of growth factors, cytokines and chemokine receptors including CXCR4.

6. CONCLUSION - AROUND CXCR4 IN CANCER CELLS

Given that CXCR4 has become a promising target in cancer metastasis, we should go on the next stage from “the biology of chemokine receptors in cancer metastasis” to “the development of new therapeutic strategies based on chemokine receptors, especially CXCR4, for cancer metastasis in patients”. CXCR4 is here highlighted as the headquarters of the receptor team in peritoneal carcinomatosis of gastric cancer.

However, to explain cancer metastasis behavior by only CXCL12-CXCR4 axis is not practical. CXCL12 is also abundant in the bone. While there is no report with correlation of CXCR4 expression and bone metastasis in gastric cancer, Taichman et al, firstly reported that prostate cancer cells were also observed migrating across bone marrow endothelial cell monolayers in response to CXCL12. Invasion of the cancer cell lines through basement membranes was also supported by CXCL12 and inhibited by antibody to CXCR4 (16). Therefore,

it would therefore be valuable to discuss the cooperation between CXCR4 and other receptors expressed around CXCR4 in cancer cells. Multiple kinds of extracellular ligands present in tumor microenvironments coordinately regulate tumor progression.

We have recently found a novel intracellular communication network between the TNF- α and EGF signaling pathways, where intracellular signals triggered by one receptor interfere with signals from the other receptor to predominantly incorporate the earlier signals (45, 46). These findings raise the possibility that cellular responses to more than one ligand differ in their order of stimulation. It has been demonstrated that CXCR4 can cross-talk with growth factor receptors, including EGFR, HER2/ErbB2 and VEGFR, in cancer cells and vascular endothelial cells. HER2, for instance, induced the CXCR4 expression required for the lung metastasis of breast cancer in a mouse model. Moreover, a significant correlation between HER2 and CXCR4 expression was observed and CXCR4 expression was also associated with poor patient overall survivals in human breast cancer (27). This evidence encourages a comprehensive study of the cross-talk between CXCR4 and other receptors to provide information from different viewpoints to develop a new strategy for CXCR4-targeted therapy (Figure 2).

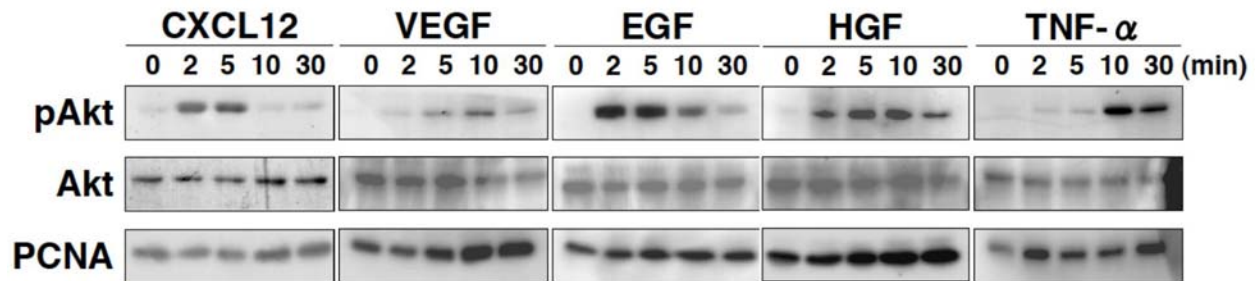


Figure 3. Western blot analysis for phosphorylation of Akt. Serum-deprived NUGC4 cells were stimulated with several growth factors and chemokine (CXCL12, VEGF, EGF, HGF and TNF- α). These factors induced intense phosphorylation of Akt in disseminated gastric cancer cells, NUGC4 cells

Finally, the following two points must be considered. The first is whether rapamycin has therapeutic effects on peritoneal dissemination of gastric cancer. The authors conducted treatment tests with rapamycin using the above-mentioned mouse model of peritoneal dissemination of human gastric cancer cells. Unfortunately, however, increased ascites retention due to the side effect of rapamycin was observed, and therefore the study was discontinued. In fact, edema has also been reported as a side effect of rapamycin in humans. Second- and third-generation mTOR inhibitors have already been developed and clinical indications have actually begun to lessen the side effect (47). Therefore, the treatment of peritoneal dissemination of gastric cancer with these next-generation inhibitors is expected to be available in the near future. The second is whether the mTOR signaling cascades are involved only in the chemokine CXCL12-induced formation of peritoneal dissemination of gastric cancer. As is well known, it has been reported that many cytokines and growth factors other than CXCL12 are involved in the formation of peritoneal dissemination of gastric cancer (37-40). The authors have also found that stimulating NUGC4 with these factors (CXCL12, VEGF, EGF, HGF and TNF- α) leads to the activation of mTOR signaling cascades (Figure 3); therefore, it is thought that mTOR inhibitors may broaden treatment options for peritoneal dissemination of gastric cancer.

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