TGF-β1 signaling mediated lymphangiogenesis in gastric cancer

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TGF-β1 signaling mediated lymphangiogenesis in gastric cancer

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ABSTRACT

TGF-β1 signaling-mediated lymphangiogenesis in gastric cancer

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Background

Recent studies have shown that TGF-β1 may have an important role in gastric cancer progression and metastasis. However, the role of TGF-β1 in lymph node metastasis and lymphangiogenesis, one of the most important steps of gastric cancer dissemination, is largely unknown. The goal of this study was to investigate the role of TGF-β1 signaling in gastric cancer and the molecular mechanisms involved in lymphangiogenesis.

Methods

Two gastric cell line models, MKN45 and KATOIII, were selected for this study. The potential role of TGF-β1 signaling in *in vitro* lymphangiogenesis was investigated. The expression of TGF-β1 pathway molecules and VEGF-C, a representative prolymphangiogenic factor, was examined by RT-PCR, western blot, or ELISA in response to the TGF-β1 and TGF-β1 receptor I inhibitor treatment. To elucidate whether Smad3 binds to the specific DNA sequences in the *VEGFC* promoter, we performed an electrophoretic mobility shift assay (EMSA). The cell line-specific effects of the Smad-dependent and Smad-independent pathways on lymphangiogenesis were also examined. Tube formation of lymphatic endothelial cells was assayed on a matrigel to evaluate TGF-β1-activated tumor cell-stimulated lymphangiogenesis.

Results

Two gastric cell line models, MKN45 and KATOIII, showed functional regulation of TGF-β1 signaling pathway. Conditioned media of both cells were able to induce the

TGF-β1 signaling pathway in a cell line which only expresses TGF-β receptor II. TGF-β1 induced activation of Smad2/3 and Smad pathway-modulated VEGF-C expression. Phosphorylated and activated Smad3 in the nucleus bound to the promoter of *VEGFC* in KATO III cells. Of note, in MKN45 cells, the Smad-independent AKT pathway was also activated in response to TGF-β1 and induced VEGF-C expression. Inhibition of TGF-β1 signaling down-regulated the expression of VEGF-C and blocked tube formation of lymphatic endothelial cells *in vitro*.

Conclusion

TGF-β1 signaling may promote *in vitro* lymphangiogenesis through VEGF-C production in gastric cancer cells. Cell line-specific Smad-dependent and -independent pathways were able to induce the expression of VEGF-C and enhance tube formation of lymphatic endothelial cells.

Key words: TGF-β1, lymphangiogenesis, gastric cancer

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I. INTRODUCTION

1. Gastric cancer

In 2012, gastric cancer was responsible for 723,000 deaths and was ranked as the world's third leading cause of cancer mortality.[1] Gastric cancer is also the second most common malignancy in Korea.[2] Regional lymph nodes are the most common site of tumor spread, and lymph node metastasis is a major prognostic factor for gastric carcinomas. Thus, understanding the mechanism of lymphatic metastasis is crucial toward bringing a new therapeutic strategy to the treatment of gastric cancer. Although conventional chemotherapy has improved the overall prognosis of gastric cancer, the survival rate of patients with advanced cancer still falls short of expectations. With recent advances in our understanding of the molecular basis of this deadly disease, deregulated cellular pathways have been identified and targeted, providing new therapeutic options beyond conventional chemotherapies. Indeed, human epidermal growth factor receptor 2 (HER2) and vascular endothelial growth factor receptor 2 (VEGFR2) have been evaluated as therapeutic targets and are now available as treatment regimens in metastatic gastric cancer.[3,4]

2. Lymph node metastasis and lymphangiogenesis

Recent studies suggest that lymphangiogenesis, the formation of new lymphatic vessels induced by tumors, is directly correlated with lymph node metastasis in gastric cancer.[5-7] The most studied lymphangiogenic signaling system is the vascular endothelial growth factor-C (VEGF-C)/VEGF-D and VEGF receptor 3 (VEGF-R3) signaling axis, which play a central role in lymphangigoenesis in

animal models. Elevated expression of VEGF-C and VEGF-D has been observed in gastric cancer.[8-12]

3. The importance of TGF-β1 in gastric cancer biology

Data from one multicenter transcriptome study[13] and The Cancer Genome Atlas[14] have established the significance of transforming growth factor beta 1 (TGF)-β1 signaling on gastric cancer progression. These support its role as an emerging candidate biomarker for gastric cancer. In line with these pivotal studies, others also have shown the relation between high expression of TGF-β1 and unfavorable prognosis of gastric cancer patient.[15-18] Our pilot study of mRNA transcriptome microarray data also showed that gastric cancer patients with a higher level of TGF-β receptor 2 (TβRII) experience a poorer survival rate (Figure 1).

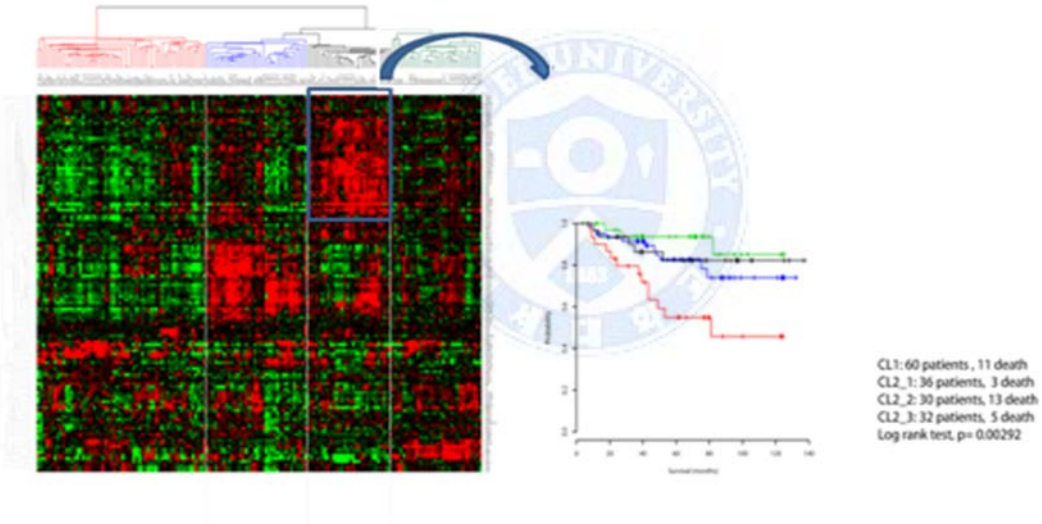


Figure 1. 158 mRNA microarray data of gastric cancer. Gastric cancer patients with higher TβRII expression (CL2_2, red line) show a poor prognosis compared to those with lower expression. TβRII, TGF-β receptor 2.

4. Transforming growth factor-β (TGF-β) signaling

TGF-β signals through a complex network of transduction pathways and has important roles in embryonic development, cell proliferation, differentiation, angiogenesis, and wound healing. TGF-β belongs to a family of dimeric peptide growth factors that induces bone morphogenetic proteins

(BMPs), activins, and inhibins.[19] The isoforms of TGF-β ligand are TGF-β1, TGF-β2, and TGF-β3. TGF-β1 is expressed in epithelial, endothelial, hematopoietic, and connective tissue cells. TGF-β2 is expressed in epithelial and neuronal cells. TGF-β3 is expressed primarily in mesenchymal cells.[20] There is 70%-80% homology among TGF-β ligand isoforms, which have different binding affinities to their receptors in a tissue-specific manner.[21] TGF-β ligand is synthesized as a large precursor molecule that contains a latent TGF-β binding protein region, which is stored in the extracellular matrix. Latent TGF-β can be activated by thrombospondin-1[22] and the integrin αvβ6[23] to release the mature TGF-β protein, which can bind TGF-β receptors and stimulate a response.

The TGF-β ligand signals through type I and II TGF-β receptors (TβRI and TβRII, respectively). There are seven TβRIs and 5 TβRIIs that provide a receptor system for the whole family of TGF-β ligands.[24] These receptors contain a serine/threonine kinase domain within the cytoplasmic domain. TGF-β ligand signals through TβRII, which recruits and phosphorylates the TβRI kinase domain. The membrane-bound TβRIII (β-glycan) assists TGF-β direct ligation to TβRII by forming a high affinity ternary complex.[25] This results in recruitment and phosphorylation of the downstream mediators Smad2 and Smad3. Phosphorylated Smad2 and Smad3 combine with Smad4 and enter the nucleus. This Smad complex recruit coactivators, repressors, and chromatin remodeling factors to regulatory regions of target genes in specific cells in a context-dependent fashion. Samd7 is a negative regulator of the Smad signaling pathway.[26]

In addition to Smad-dependent signaling, the binding of TGF-β to its receptors activates many non-canonical signaling pathways, such as the phosphoinositol-3 kinase (PI3K), mitogen-activated protein kinase, and small guanosine triphosphatase pathways.[27] Theses pathways most often are implicated in tumor cell motility and migration.[28]

5. TGF-β1 and lymphangiogenesis

TGF-β1 is known to play a critical role in the malignant progression of various tumors. Tumor-stimulated lymphangiogenesis is required for regional lymph node metastasis of cancer cells. It is believed that the interaction between tumor cells and their microenvironment is important in lymphangiogenesis, as it similarly is in angiogenesis. Recent studies suggest that TGF-β1 signaling in tumor cells may promote lymphangiogenesis by producing a key prolymphangiogenic growth factor, VEGF-C.[29] Although the precise mechanisms of TGF-β signaling in lymphangiogenesis in various tumor types need to be further elucidated, there are controversial results suggesting dual or contradictory roles in lymphangiogenesis.[30-33] TGF-β signaling may be involved in the expression of

VEGF-C in certain types of cells thereby promoting tumor lymphagiogenesis.[29,34] In contrast, TGF-β has been reported to downregulate VEGFR-3, the cognate receptor of VEGF-C, in lymphatic endothelial cells leading to the suppression of lymphangiogenesis.[30] Given that TGF-β1 is secreted and instigates TGF-β1-induced signaling pathways both in tumor cells and lymphatic endothelial cells, it remains unclear what would be the collective effects of TGF-β on tumor lymphangiogenesis.

6. The overarching goal of this study

Although the role of TGF-β in angiogenesis has been known to some extent,[35,36] its role on lymphangiogenesis has not been well elucidated,[30,32,33] especially in the field of gastric cancer. We hypothesized that TGF-β signaling in cancer cells plays an important role in lymphangiogenesis via expression of prolymphangiogenic factor VEGF-C, which enhances lymphatic tube formation. The aim of this study was to investigate the role of TGF-β1 signaling on lymphangiogenesis and its molecular mechanisms in gastric cancer cell line models.

II. MATERIALS AND METHODS

1. Cell culture

The human gastric cancer cell lines AGS, MKN28, MKN45, NCI-N87, SK4, KATOIII, HS746T, and YCCs were cultured in RPMI 1640 medium (Hyclone, South Logan, Utah) containing 10% fetal bovine serum, 100 U/ml of penicillin sodium, and 100 ㎍/ml of streptomycin sulfate at 37°C in a humidified incubator containing 5% CO2. Cultured human lymphatic endothelial cells (HLECs) were purchased from Promo Cell (Promo Cell, Heidelberg, Germany). This cell line was maintained in complete medium (Endothelial cell growth medium 1; Promo Cell, Heidelberg, Germany) on gelatin-coated dishes. HLECs were used between passages 5 and 8.

2. Western blot analysis

Gastric cancer cells were collected and lysed on ice for 30 min in lysis buffer [1% Triton X-100, 50 mmol/L HEPES (Ph 7.5), 150 mmol/L NaCl, 25mmol/L β-glycerophosphate, 25 mmol/L NaF, 5 mmol/L EGTA, and 1 mmol/L EDTA as a protease inhibitor cocktail (Roche Diagnostic, Indianapolis,

IN)]. The lysates were clarified by centrifugation for 13,000 rpm for 30 min. Equal amounts of protein were loaded onto a sodium dodecyl sulfate-polyacrylamide gel (12% polyacrylamide) followed by electrophoresis at 100 V for 3 h and transferred to polyvinylidene floride membrane at 100 V for 1 h. Subsequently, the PVDF membrane was incubated in TBS-T with 5% skim milk (blocking solution) for 1 h at room temperature. The PVDF membrane was subsequently incubated 4°C overnight with the target primary antibody. Anti-TGFRII, Smad3, Erk, Akt, TAK1, VEGF-C (dilution 1:1000; cell signaling Technology, Massachusetts, USA) and anti-β-actin (dilution 1:1000; Sigma-Aldrich, USA) antibodies were diluted in TBS-T (TBS/Tween 20: 2% skin milk). The appropriate secondary antibodies were applied (1:5000, horseradish peroxidase-conjugated anti-rabbit and anti-mouse) at room temperature for 1 h. Labeled bands were detected by enhanced chemiluminescence (ECL; ThermoScientific, USA).

3. Electrophoretic mobility shift assay (EMSA)

The DNA binding activity of Smad3 against the *VEGFC* promoter was investigated using a 32P-labeled oligonucleotide encoding the Smad3 transcription factor binding sites found in the *VEGFC* promoter region. Double-stranded oligonucleotides containing the consensus-binding site for Smad3 sense-(GTCGGCCAGCCACTCGCATTGTGACTAC), anti-sense-(ATAGAGTGCTGCCCCGTTAGTCTCCGAC) were 5′ end-labeled using polynucleotide kinase and ɣ-32P-dATP. Nuclear extracts (5.0 μg) were incubated with 1 μl of labeled oligonucleotide (20000 c.p.m.) in 20 μl incubation buffer (10 mM Tris-HCl, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, and 2 μg poly dI-dC) for 20 min at room temperature.

4. Tube formation assay

HLECs (1×10^5) were cultured in a 24-well plate coated with 150 μl of Growth factor-reduced Matrigel in MVI1 medium for cell attachment for 1 h. The MVI medium was replaced with conditional medium and continuous cell culture for 24 h. Tube length was quantified after 8 h by measuring the total cumulative tube length in three random microscopic fields with a computer-assisted microscope using NIH ImageJ1.44 image analysis software, which is available at http://rsb.nih.gov/nih-image. The original magnification used was x100.

5. Enzyme-linked immunosorbent assay (ELISA)

Protein content in culture medium was determined using the Quantikine Immunoassay systems for human VEGF-C ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. ELISA data analysis (target protein content in culture medium) was expressed as the quantity of protein secreted from 10,000 cells for 24 h.

6. Statistical analysis

Values are expressed as means ± S.D. Mann-Whitney test was used to evaluate the data. Differences were considered to be statistically significant at $P<0.05$. All analyses were performed using SPSS 21 software (SPSS, Chicago, IL).

III. RESULTS

1. Selection of model cell lines

To select model cell lines suitable for testing the hypothesis, we examined the expression of TGF-β1, TGF-β1 receptor II, and VEGF-C in a panel of gastric cancer cell lines (AGS, MKN28, MKN45, NCI-N87, SK4, KATOIII, HS746T) by western blot analysis. TGF-β1 and TGF-β1 receptor II were expressed in all seven gastric cancer cell lines examined (Figure 2A), whereas VEGF-C was expressed only in MKN45 and KATOIII (Figure 2B) cell lines. Based on these results, we selected MKN45 and KATOIII as model cell lines for downstream experiments investigating the role of TGF-β1 signaling on lymphangiogenesis.

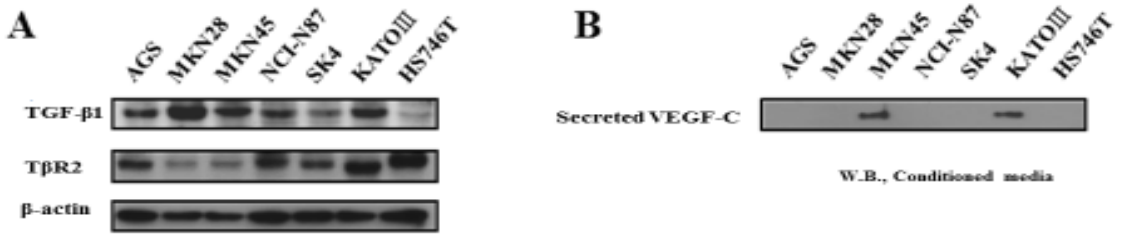


Figure 2. TGF-β1, TGF-β1 receptor II, and VEGF-C expression in gastric cancer cells. Among gastric cancer cell lines, MNK45 and KATOIII were selected as a model for the regulation of TGF-β1 on lymphangiogenesis. These two cell lines expressed TGF-β1, TGF-β receptor II (A), and VEGF-C (B). TβR2, TGF-β receptor 2

2. The paracrine regulation of TGF-β1 in gastric cancer cells.

We further investigated additional gastric cancer cell lines and found that YCC2 and YCC3 only express a significant level of TGF-β1 receptor II, but not TGF-β1 (Figure 3A). We ascertained the existence of TGF-β1 in conditioned media of MKN45 and KATOIII but not in YCC2 (Figure 3B). We noticed that Smad2 and Smad3, the receptor activated Smads (R-Smads), were phosphorylated and activated in YCC2 cells when treated with the conditioned media of MKN45 and KATOIII (Figure 3C). Therefore, we confirmed the paracrine effect of TGF-β1 signaling in certain gastric cancer cell lines.

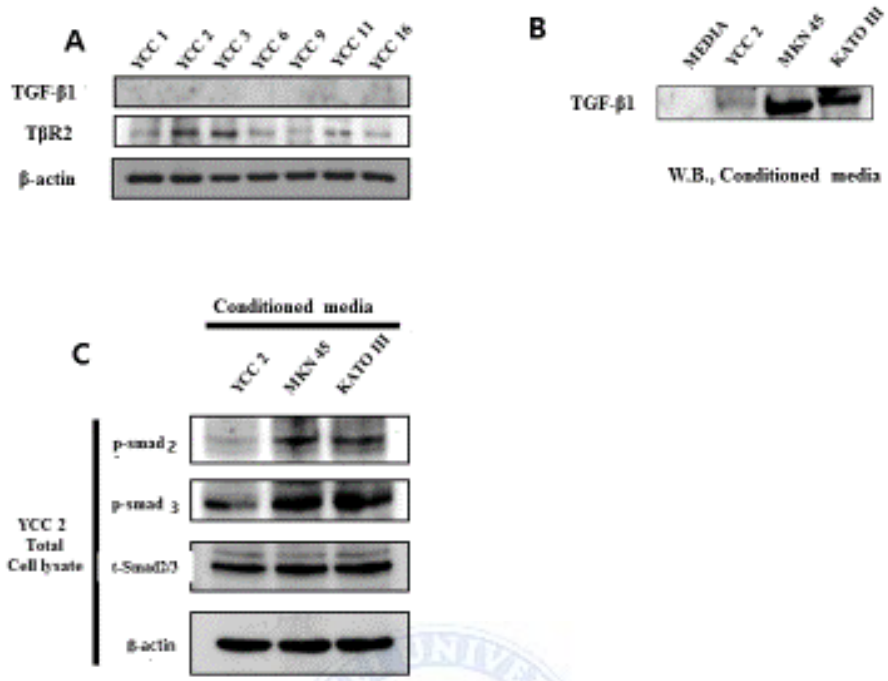


Figure 3. Paracrine regulation of TGF-β1. YCC2 was selected as a paracrine model of TGF-β1 production (A, B). Smads were detected only in the total cell lysate of YCC2, which was cultured in the conditioned media of MKN45 and KATOIII cells (C).

3. The TGF-β1 signaling pathway in gastric cancer cells

To confirm the validity of these model cell lines for TGF-β1 responsiveness, MKN45 and KATOIII cells were treated with TGF-β1 (10 ng/ml) and were analyzed for target gene expression. As shown in Figure 4A, the mRNA expression of *twist1*, a well-established target gene of the TGF-β1 signaling pathway, was significantly induced by TGF-β1 treatment in both cell lines. In addition, the corresponding increase of phosphorylation of Smad3 was evident in TGF-β1-treated cells (Figure 4B). Further, the treatment of TGF-β1 receptor inhibitor (LY2157299) abrogated the effect of TGF-β1-induced phosphorylation of Smad3, confirming intact and functioning TGF-β1 signaling in these cell lines (Figure 4C).

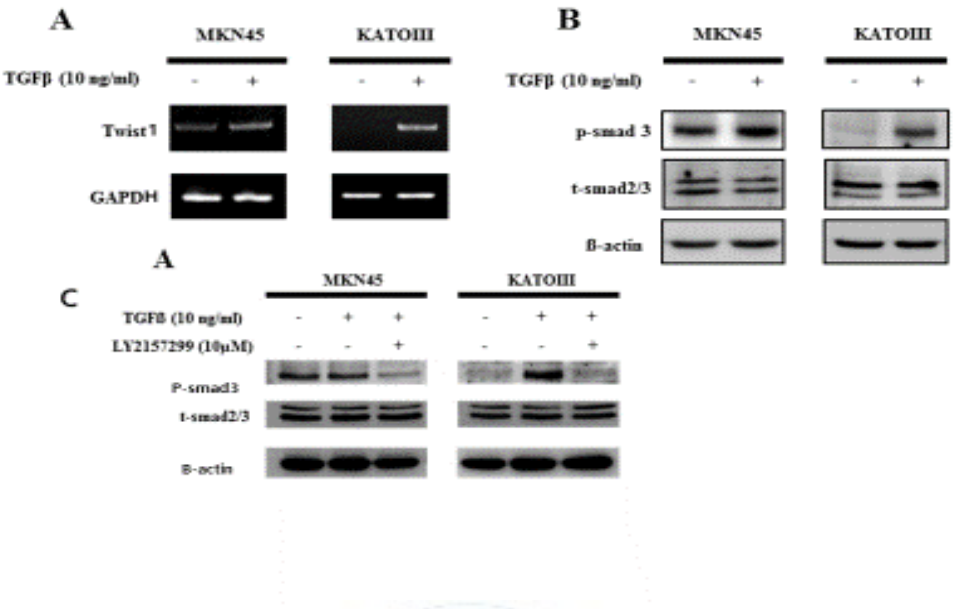


Figure 4. Activated TGF-β1 signaling in MKN45 and KATOIII gastric cancer cells. The expression of twist I (A) and p-Smad3 (B) were enhanced in responding to TGF-β1, but was decreased at TGF-β1 receptor inhibitor (LY2157299) (C).

4. The downstream signaling of TGF-β1 in gastric cancer cells

To investigate the effect of TGF-β1 on the expression of VEGF-C, we treated KATO III cells with TGF-β1 and/or TGF-β1 receptor I inhibitor (LY2157299), as indicated. In KATOIII cells, the expression of phosphorylated form Smad3 (p-Smad3), not p-Smad2, was down-regulated in response to TGF-β1 receptor I inhibitor (LY2157299). The inhibitory response was correlated positively in a dose-related manner (Figure 5A). In addition, to elucidate the nuclear localization of activated Smad3 in response to TGF-β1, we examined the expression level of both p-Smad3 and total form Smad3 (t-Smad3) in nucleus and cytosol according to the treatment of TGF-β1 and LY2157299. The expression of p-Smad3 in the nucleus was increased with TGF-β1, while it was decreased with LY2157299 (Figure 5B).

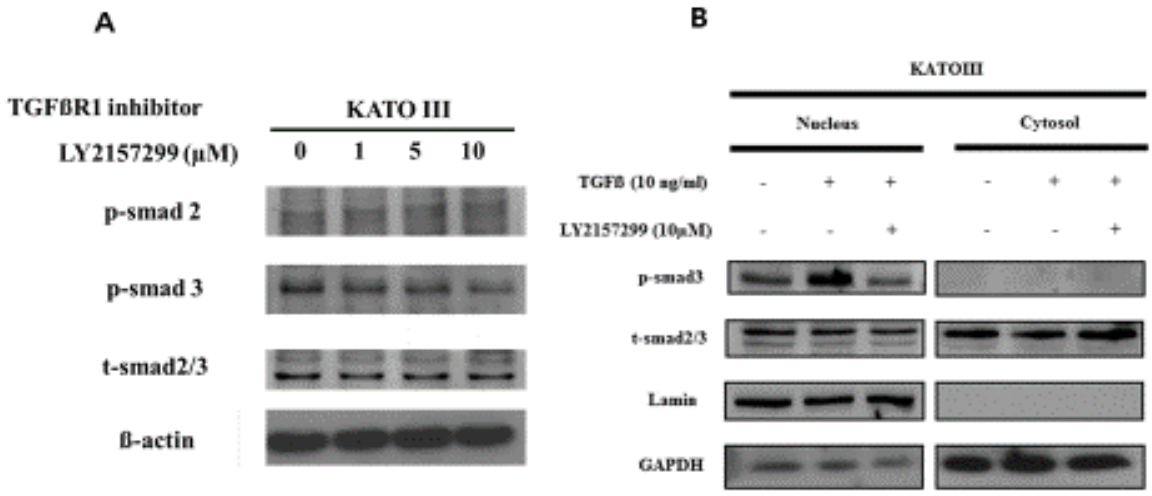


Figure 5. Smad-dependent pathway of TGF-β1 signaling. KATOIII cells showed decreased expression of p-Smad3 with an increasing dose of LY2157299 (A). The change in p-Smad3 expression in response to TGF-β1 in the cytosol and nucleus. In KATOIII cells, the amount of nucleus p-Smad was increased in response to TGF-β1, while decreased in response to LY2157299. Lamin and GAPDH were used as a loading controls in the nucleus and cytosol, respectively (B).

5. The binding interaction between Smad3 and the *VEGFC* promoter

To elucidate whether TGF-β1 signaling-induced lymphangiogenesis is mediated through increased transcription of VEGF-C, we first investigated the interaction between transcription regulator Smad3 and the promoter region of *VEGFC*. A few Smad3 binding sites have been identified in the *VEGFC* promoter region.[29,37,38] We carried out an EMSA with a ^{32}P-labeled oligonucleotide containing Smad3 binding sites found in the *VEGFC* promoter (Figure 6A). Labeled Smad3 probe-nuclear extract (from the MKN45 and KATO III cells) complexes produced two bands. The specificity of the EMSA result was confirmed by complete inhibition of Smad3 DNA binding by excess labeled and unlabeled Smad3 (lane 1, Figure 6B). In addition, a similar amount of mutated Smad3 probe also failed to bind to the Smad3 transcription complex (lane 2, Figure 6B).

In KATOIII cells (lane 4 and 6, Figure 6B) Smad3 binding activity to the *VEGFC* promoter region was significantly increased compared to that in MKN45 cells (lane 3 and 5, Figure 6B). The binding signals were increased with five times more nuclear extract alone (lane 7 and 8, Figure 6B). To assure the binding interaction between Smad3 and the promoter of *VEGFC*, a super-shift assay with anti-Smad3 antibody was performed and showed more potentiated signals (lane 9 and 10, Figure 6B). Furthermore, that response was promoted with more concentrated nucleic extract (lane 11 and 12, Figure 6B). Together, these results demonstrate that Smad3 can bind to the promoter of the *VEGFC* gene.

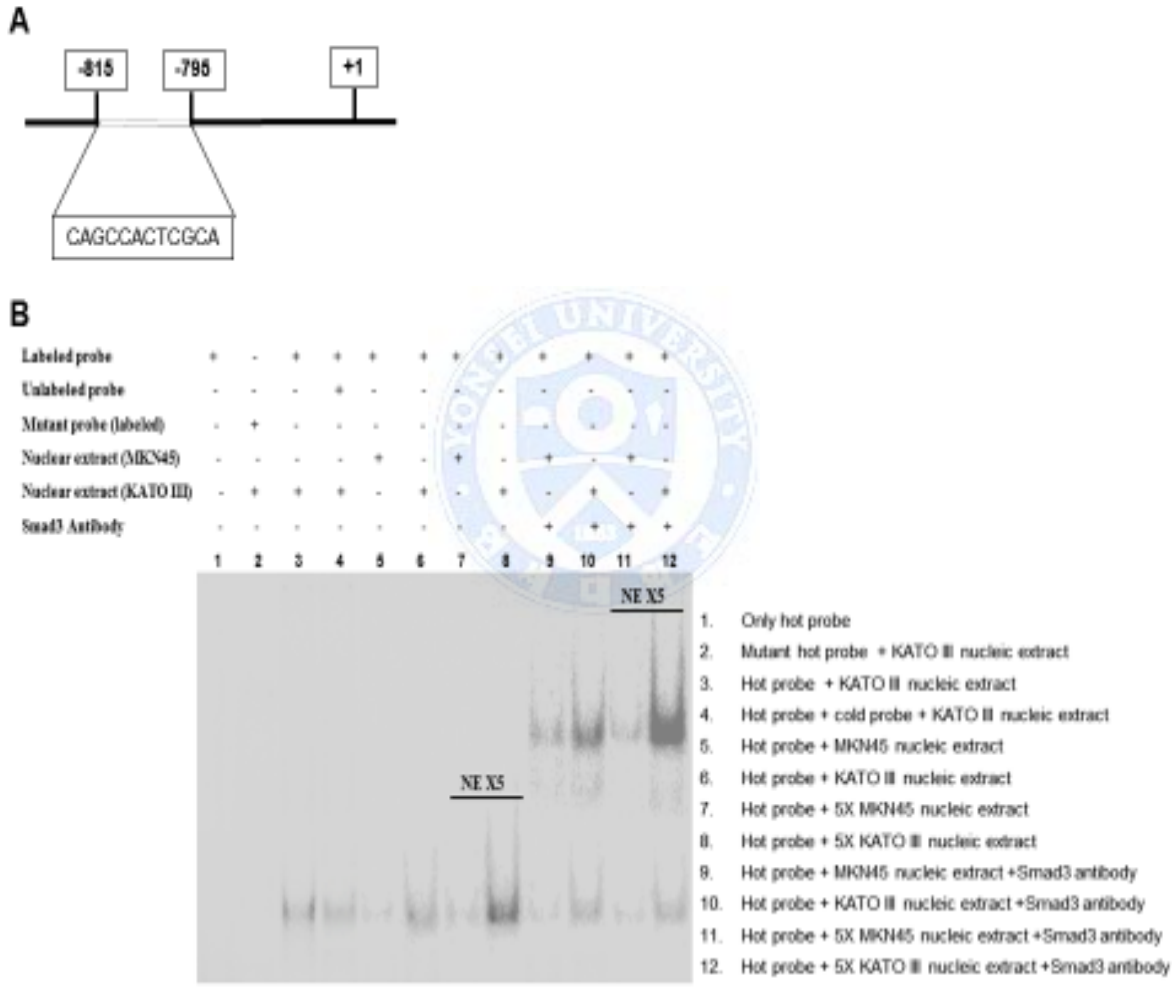


Figure 6. EMSA between Smad3 and *VEGFC*. The binding site of Smad3 to the promoter of *VEGFC* (A). KATOIII cells (lane 4 and 6) showed significantly increased binding activity between Smad3 and the *VEGFC* promoter region compared to MKN45 cells (lane 3 and 5, B). NE, nuclear extract.

6. Inhibition of TGF-β1 signaling suppresses VEGF-C expression

Next, we examined whether the inhibition of TGF-β1 signaling in model cell lines suppresses VEGF-C expression. By western blot analysis of conditioned media of KATO III and MKN45 cells, we demonstrated that the protein level of VEGF-C is decreased when treated with LY2157299 (Figure 7).

Based on these results, we confirmed that TGF-β1 signaling promotes VEGF-C expression in gastric cancer cells.

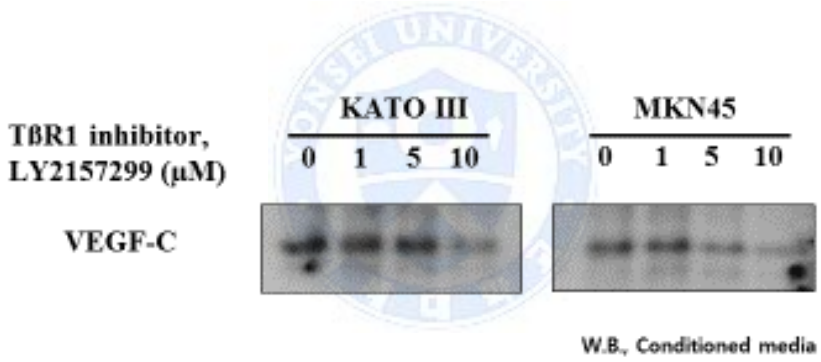


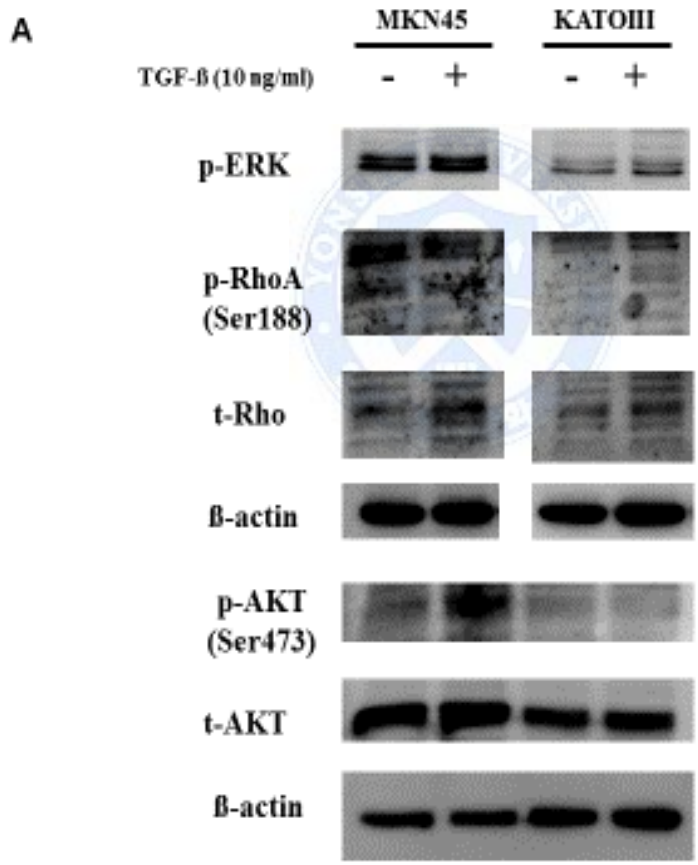
Figure 7. The expression of VEGF-C in response to TGF-β1 receptor I inhibitor. TβR1 inhibitor, TGF-β receptor 1 inhibitor.

7. Smad-independent pathway of TGF-β1 in gastric cancer cells

According to the EMSA results, the binding interaction between Smad3 and the *VEGFC* promoter region is lower in MKN45 than KATOIII cells. These findings may suggest the existence of other

TGF-β1 signaling pathways for VEGF-C activation, which are different from the Smad-dependent pathway. Therefore, we examined Smad-independent signaling pathway molecules. Among them, phosphorylated Akt (p-Akt) was remarkably induced in response to TGF-β1 treatment only in MKN45 cells (Figure 8A). The expression of p-Akt was increased when treated with TGF-β1, but decreased when treated with its inhibitor LY2157299 in MKN45 cells (Figure 8B).

Taken together, TGF-β1 signals may be transduced through Smad-dependent and Smad-independent pathways in accordance with gastric cancer cell line characteristics.



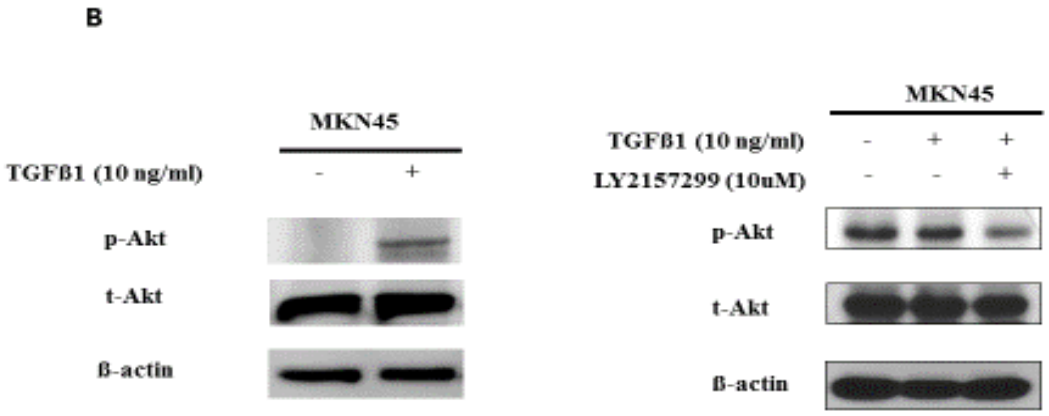


Figure 8. Smad-independent pathway of TGF-β1 signaling. Only p-Akt was remarkably increased in response to TGF-β1 in MKN45 but not KATOIII cells (A). The level of p-Akt was increased in response to TGF-β1 but decreased in response to LY2157299 (B).

8. Lymphatic endothelial cell tube formation

Next, we validated the effect of TGF-β1 signaling on lymphangiogenesis by conducting a lymphatic endothelial cell tube formation assay. HLECs were cultured in the conditioned media of MKN45 and KATOIII cells. After 18 h of culture, we noticed the formation of a tubular structure of HLECs in MKN45- and KATOIII-conditioned media compared to HLECs alone or in YCC2-conditioned media (Figure 9). In addition, the tubule forming ability of HLECs was decreased when treated with the conditioned media of tumor cells treated with TGF-β receptor 1 inhibitor (LY2157299). Quantitation of the results was confirmed by measuring the length of tube formation of HLECs (Figure 10). The expression level of secreted VEGF-C in YCC2-, MKN45-, and KATOIII-conditioned media according to the treatment of TGF-β1 and LY2157299 was also investigated by ELISA analysis. The level of VEGF-C was increased for TGF-β1, whereas it was decreased for LY2157299 (Figure 11).

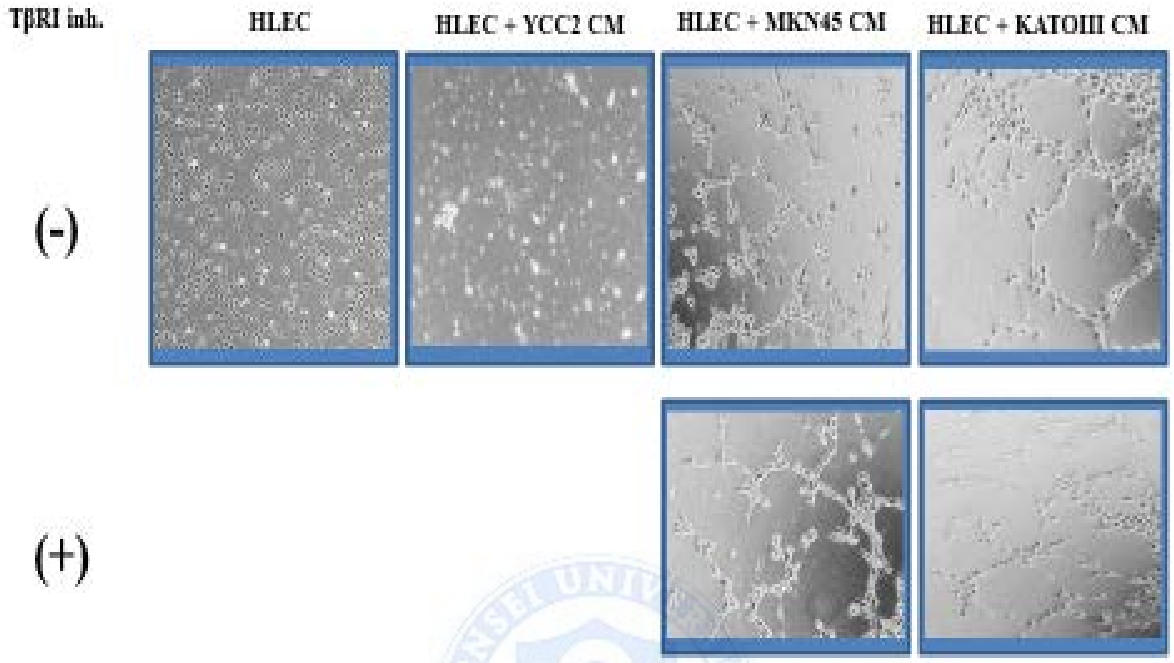


Figure 9. Lymphatic endothelial cell (HLEC) growth in the conditioned media of gastric cancer cells. The growth of HLEC in MKN45- and KATOIII-conditioned media was increased compared to HLECs alone or with YCC2-conditioned media. However, tube formation was decreased in responding to TGF receptor I inhibitor. All photos were taken after 18 h of culture. CM, conditioned media; TβR1 inh., TGF-β receptor 1 inhibitor.

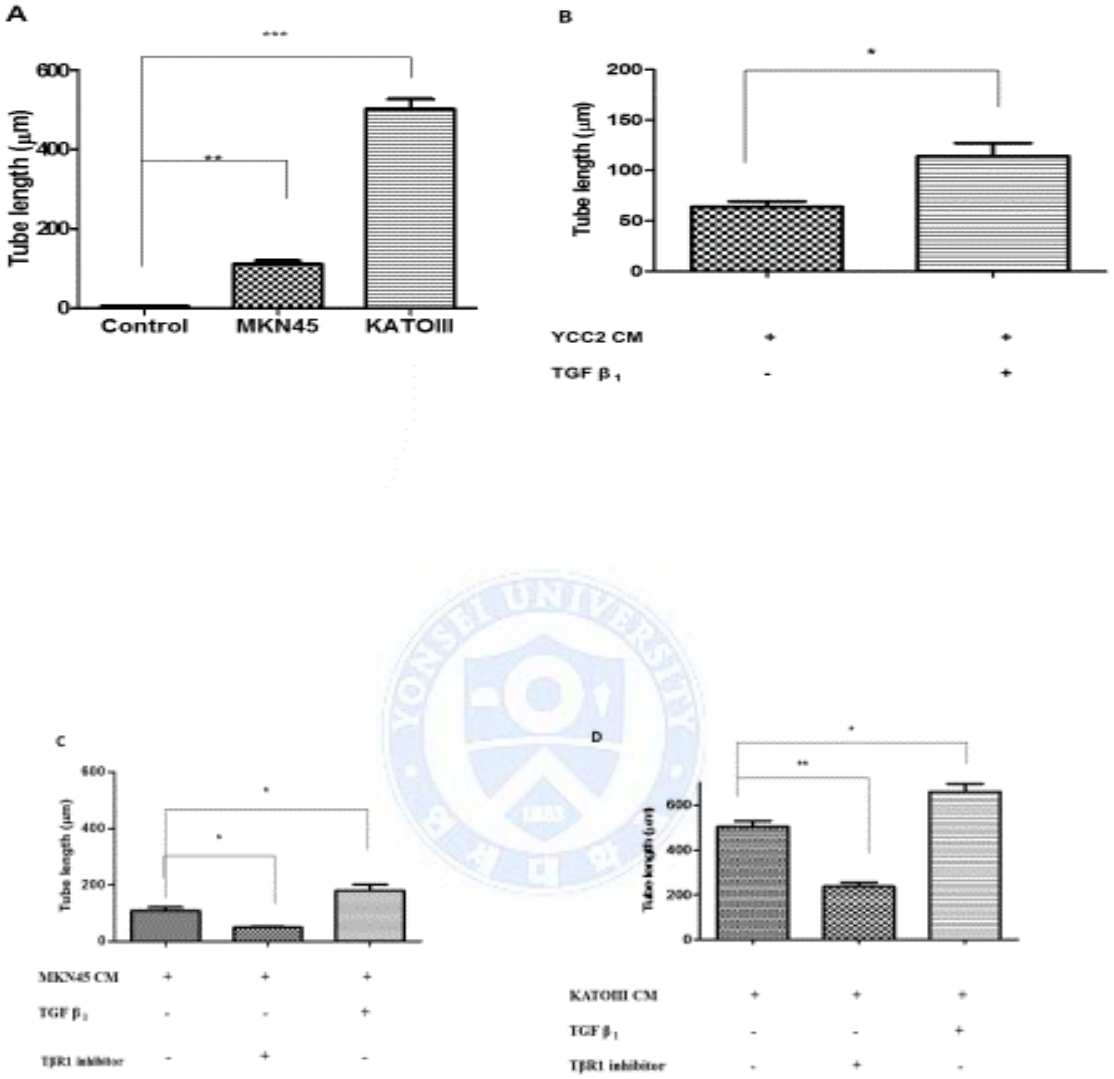


Figure 10. The quantitation of tube formation of HLECs. The length of its tube was longer in MKN45- and KATOIII-conditioned media than with HLECs (A). With YCC2-conditioned media, only TGF receptor-expressing cell line showed increased tube length with TGF-β1 (B). With MKN45 and KATOIII-conditioned media, the tube length was increased for TGF-β1 and deceased with TβR1 inhibitor (C, D). TβR1, TGF-β receptor 1. *P < 0.05, **P < 0.01, ***P < 0.001, Mann-Whitney test)

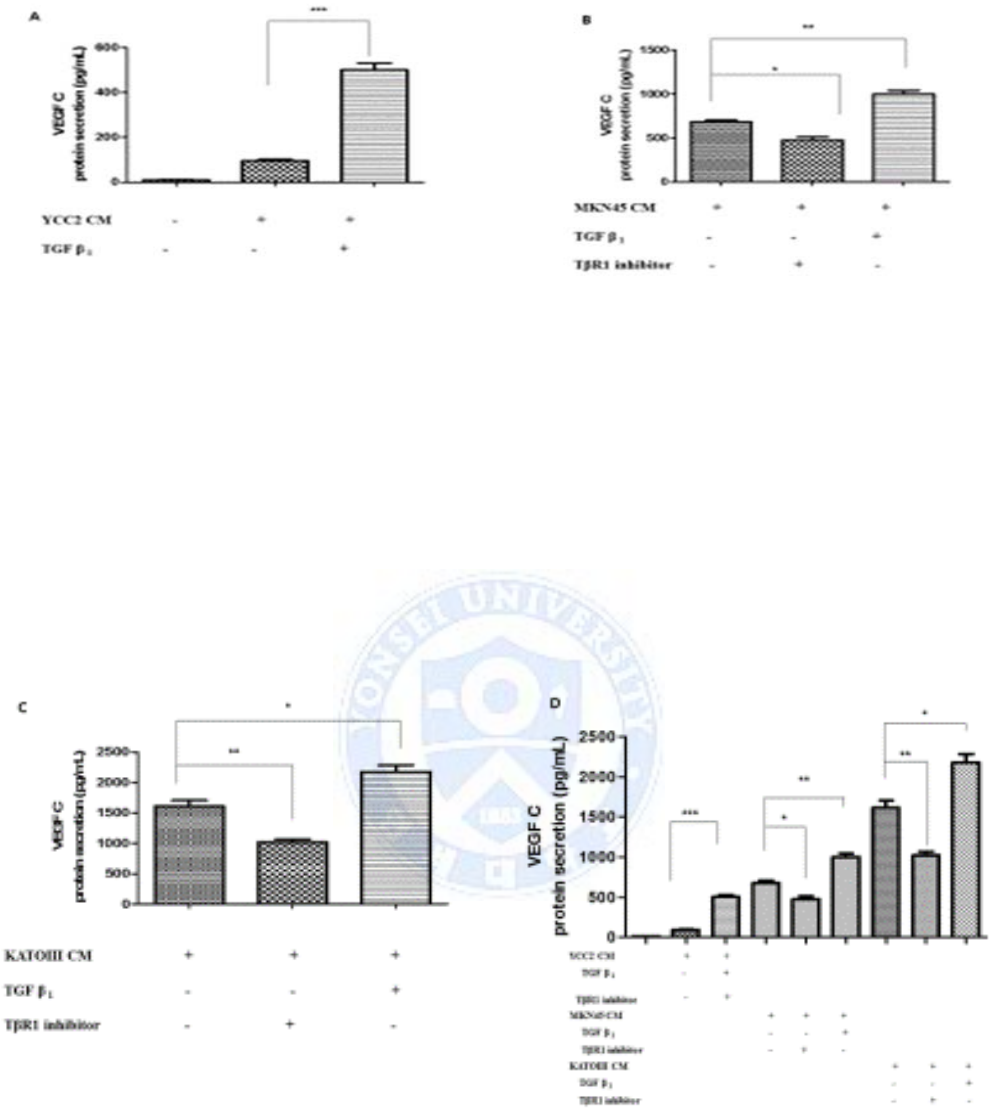


Figure 11. The level of VEGF-C in the conditioned media of gastric cancer cell lines. YCC 2-conditioned media resulted in an increased level of VEGF-C with TGF-β1 (A). The level of VEGF-C was increased with TGF-β1, but decreased with TβR1 inhibitor in MKN45- and KATOIII-conditioned media treatments (B, C, D). TβR1, TGF-β receptor 1. *P < 0.05, **P < 0.01, ***P < 0.001, Mann-Whitney test)

IV. DISCUSSION

Our study shows that TGF-β1 signaling activated in gastric cancer cells can transcriptionally induce VEGF-C expression, which leads to enhanced tube formation of HLECs *in vitro*. In addition, the affecting signaling pathway of TGF-β1 for VEGF-C regulation can be diverse based on different types of gastric cancer cells, potentially reflecting a tumor heterogeneity. Our results are confirmed also by a HLEC tube formation assay and by measuring the level of secreted VEGF-C in the conditioned media of gastric cancer cells via ELISA. This study is the first report to demonstrate the mechanism of how TGF-β1 affects lymphangiogenesis in gastric cancer cell models, suggesting both Smad-dependent and Smad-independent pathways.

Recent studies in gastric cancer[13,14] and expected favorable results of a clinical trial for hepatocellular carcinoma[39] have intensified an interest in the role of TGF-β1 in gastric cancer biology. TGF-β1 has been known to have a distinct biphasic role in tumor progression. It functions as a tumor suppressor in early stages of cancer, while a tumor promoter in late stages.[24] This dual role of TGF-β1 on tumor biology makes it difficult to understand its mechanism and to apply it therapeutically to a clinical setting. In addition, the reciprocal relation between cancer and surrounding stromal cells, such as lymphocytes, macrophage, fibroblast, etc., makes the situation more complex and complicated. Among many other oncogenic effects of TGF-β1, the role for Epithelial-mesenchymal transition (EMT) has been studied most thouroughly.[24] However, the role of TGF-β1 on lymphangigoenesis is largely unknown, despite the fact that lymphangiogenesis and lymph node metastasis is the most important prognostic factor in gastric cancer. Although the relation between TGF-β1 and tumor lymphangiogenesis has not been well elucidated, recent studies suggest that TGF-β1 might upregulate VEGF-C expression in some types of cells, including tumor cells, implying that TGF-β1 signaling might contribute to tumor lymphangiogenesis.[29] Conversely, TGF-β1 has been found to downregulate VEGF receptor-3 (VEGFR3) in HLECs and suppress HLEC properties, thus inhibiting lymphangiogenesis.[34] So far the effect of TGF-β1 on HLECs and the final effect of TGF-β1 on tumor lymphangiogenesis remains unclear, especially for gastric cancer. Therefore, it is important to elucidate the role of TGF-β1 for lymphangiogenesis in gastric cancer. In this regard, our study may contribute to providing potential evidence for the role of TGF-β1 signaling in lymphangiogenesis regulation in gastric cancer cell models. The data in this study suggest that TGF-β1 signaling can upregulate VEGF-C expression, which leads to lymphangiogenesis in gastric cancer. The signals of TGF-β1 to transcriptional induction of VEGF-C can be mediated via canonical Smad3 in some gastric cancer cells, while it can also be conveyed via the non-canonical Smad-independent AKT pathway. Of

note, LY2157299 which is used in this study as a TβR1 small molecule inhibitor might inhibit both Smad-dependent and Smad-independent pathways, because both pathways are downstream of the common upstream complex of TGF-β1 and TβR1.[24] Thus, the results signify that LY2157299 can be a new therapeutic agent against gastric cancer progression and metastasis regardless of which pathways being activated. This has been tested in a phase II clinical trial of a target therapy for hepatocellular carcinoma (NCT01246986).[40]

In MKN45 cells, but not in KATOIII cells, TGF-β1 signaling increases the expression of p-Akt, while inhibition of TGF-β1 signaling decreases it. Our previous results showed that activated p-Akt inhibits FOXO3a, a transcriptional repressor of VEGF-C, thereby resulting in increased transcription of VEGF-C.[32] Based on these results, TGF-β1 signaling increases VEGF-C transcription via different pathways according to individual cancer cell characteristics in the context of tumor heterogeneity. These results imply that VEGF-C transcription is induced in gastric cancer cells through Smad-dependent (in KATO III cells) or Smad-independent (especially the PI3K-Akt pathway in MKN45 cells) pathways, both of which are triggered by the TGF-β1 signaling and instigate lymphatic cells to a form tubular network. The physiological relevance of TGF-β1 signaling-induced lymphangiogenesis needs to be validated in animal tumor models.

V. CONCLUSION

The activation of TGF-β1 signaling in gastric cancer cells instigates lymphangiogenesis in HLECs through induction of VEGF-C production in gastric cancer cells. Also, downstream pathways may be Smad-dependent or Smad-independent in different types of gastric cancer cells.

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ASTRACT (IN KOREAN)

위암에서의 TGF-β1 신호를 매개로 한 림프관신생

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배경

최근 연구들은 TGF-β1이 위암의 진행과 전이과정에서 중요한 역할을 한다는 증거들을 보여주고 있다. 그러나, 위암 환자의 예후에서 중요한 림프절전이나 림프관신생에 대해서는 잘 알려져 있지않다. 위암에서 림프관신생에 대한 TGF-β1에 대한 역할과 그 분자적 기작을 규명하는 것이 이번 연구의 목적이다.

방법

우리는 먼저 이 번 실험 목적에 맞는 두 종류의 위암세포주 (MKN45와 KATOIII)를 선택하였다. TGF-β1과 그 억제제를 위암세포주에 처리하였을 때 TGF-β1 신호물질과 대표적인 림프관신생 촉진물질인 VEGF-C의 변화를 western blotting으로 확인하였다. Smad3와 VEGF-C promoter와 결합이 일어나는지 보기 위하여 electrophoretic mobility shift assay (EMSA) 를 시행하였다. Smad-의존 경로와 Smad-비의존 경로에 대하여 모두 분석하였으며, 림프관 내피세포의 tube forming assay를 실시하여 형태학적 변화 및 종양세포 배양 조건배지의 VEGF-C의 양을 측정하여 확인하였다.

결과

TGF-β1의 분자적 신호는 Smad를 거쳐서 VEGF-C 발현 증가로 나타났다. TGF-β1의 신호에 대하여 Smad3는 세포질에서 핵 내로 이동한 후 *VEGFC* promoter에 결합한다는 것을 EMSA로 확인할 수 있었다. EMSA 결과를 바탕으로, KATOIII에서 MKN45에 비하여 Smad3와 *VEGF-C* promoter와 결합이 강하다는 것을 확인하였고, MKN45에서는 Smad-비의존 경로인 AKT 경로를 통하여 TGF-β1 신호가 VEGF-C로 전달될 수 있다는 것을 또한 확인할 수 있었다. 실제로 두 위암세포주의 조건배지에서 자란 림프관내피세포는 더 효율적인 tubular network를 형성한다는 것을 알 수 있었으며, 실제 TGF-β1에 의해 활성화된 종양세포로부터 분비된 VEGF-C 양이 높았다.

결론

TGF-β1은 위암세포주에서 Smad3를 매개로 하여 VEGF-C를 통하여 림프관신생을 촉진하였다. 이러한 과정은 위암세포주 종류에 따라 Smad-비의존 경로를 통해서도 이루어 질 수 있다.

핵심되는 말: TGF-β1, 림프관신생, 위암