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Connection between inflammation and carcinogenesis in gastrointestinal tract: Focus on TGF-β signaling

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Abstract

Inflammation is a primary defense process against various extracellular stimuli, such as viruses, pathogens, foods, and environmental pollutants. When cells respond to stimuli for short periods of time, it results in acute or physiological inflammation. However, if the stimulation is sustained for longer time or a pathological state occurs, it is known as chronic or pathological inflammation. Several studies have shown that tumorigenesis in the gastrointestinal (GI) tract is closely associated with chronic inflammation, for which abnormal cellular alterations that accompany chronic inflammation such as oxidative stresses, gene mutations, epigenetic changes, and inflammatory cytokines, are shared with carcinogenic processes, which forms a critical cross-link between chronic inflammation and carcinogenesis. Transforming growth factor (TGF)-β is a multi-potent cytokine that plays an important role in regulation of cell growth, apoptosis and differentiation. Most importantly, TGF-β is a strong anti-inflammatory cytokine that regulates the development of effector cells. TGF-β has a suppressive effect on carcinogenesis under normal conditions by inhibiting abnormal cell growth, but on the other hand, many GI cancers originate from uncontrolled cell growth and differentiation by genetic loss of TGF-β signaling molecules or perturbation of TGF-β adaptors. Once a tumor has developed, TGF-β exerts a promoting effect on the tumor itself and stromal cells to enhance cell growth, alter the responsiveness of tumor cells to stimulate invasion and metastasis, and inhibited immune surveillance. Therefore, novel development of therapeutic agents to inhibit TGF-β-induced progression of tumor and to retain its growth inhibitory activities, in addition to anti-inflammatory actions, could be useful in oncology. In this review, we discuss the role of TGF-β in inflammation and carcinogenesis of the GI tract related to abnormal TGF-β signaling.

TRANSFORMING GROWTH FACTOR-β IN HOMEOSTASIS AND INFLAMMATION OF GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract is composed of the esophagus, stomach, small intestine and large intestine; it is the longest and largest surface among human organs, and is the first contact place for various pathogens and food antigens. Therefore, the GI tract should have many lymphoid organs to control the untoward or harmful immune reactions. The immune system of the GI tract can induce a series of host defensive reactions such as recruitment of immune cells, activation of anti-inflammatory enzymes, and secretion of anti-inflammatory cytokines[1]. To prevent pathological inflammation against various antigens exposed to the GI tract, the human body has developed various regulatory mechanisms: the development of regulatory T (Treg) cells, the maintenance of the gut barrier, and immune reactions. Since the inflammatory process in the GI tract is started by food antigens, extracellular pollutants and pathogenic infections, disruption of the intestinal barrier results in intestinal inflammation by inducing the pro-inflammatory reactions of immune cells[2].

Therefore, in order to maintain homeostasis of the GI tract, tolerance is a prerequisite to maintain the balance between pro-inflammation and anti-inflammation. Although many types of regulatory immune cells perform their suppressive functions in the GI tract to acquire tolerance, this activity is mediated by a relatively small number of materials including interleukin (IL)-10 and transforming growth factor (TGF)-β, which are well known regulators of immune reactions in the GI tract[3,4].

TGF-β is a multifunctional cytokine with critical roles in many cellular pathways including cell growth, apoptosis, differentiation and immune reactions[5,6]. TGF-β is secreted as a latent inactive protein and needs to be activated *via* conformational change or protein cleavage by protease or thrombospondins. Since TGF-β1 knockout mice show a dramatic phenotype and develop severe autoimmunity that leads to death within 2 wk after birth[7,8], and T-cell specific disruption of TGF-β signaling results in serious inflammatory changes through constitutively activated T cells in the gut and lung, as a similar phenotype to whole TGF-β knockout mice[9], TGF-β is thought to be a strong anti-inflammatory cytokine. In the regulation of intestinal inflammation, TGF-β inhibits T-cell proliferation as well as blocking differentiation of CD4+ and CD8+ naïve T cells into helper T cells by inhibiting expression of the transcription factors such as T-bet, STAT4 and GATA-3[10]. Furthermore, TGF-β suppresses immune reactions through the induction of Treg cells. In fact, TGF-β has been shown to be essential for the induction and maintenance of peripheral CD4+CD25+ Treg cells by activation of Foxp3 expression[11].

However, TGF-β also has pro-inflammatory effects through the differentiation of Th17 cells by induction of retinoic-acid-receptor-related orphan nuclear receptor γt, a Th17-specific transcription factor[12]. Conclusively, cross-talk with surrounding tissues may be important for activity of TGF-β in GI tract inflammation. TGF-β has a critical role in regulation of inflammatory processes, therefore, it should be tightly regulated by various mechanisms. Dysregulated or attenuated TGF-β signaling has been suspected in the pathogenesis of various inflammation-related diseases including chronic inflammatory disorders and cancer. T-cell specific deficiency of furin, which activates latent TGF-β, leads to spontaneous autoimmune disease such as colitis and intestinal inflammation in murine models[13]. Similarly, overexpression of mutant TGF-β, which has a defect in binding activity with integrins, shows similar phenotypes to TGF-β null mice, such as vascular defects, multi-organ inflammation, and lack of Langerhans cells[14].

Disruption of Smad3 in mice also shows defects in mucosal immunity and results in early death after birth. Smad3 mutant mice exhibit large amounts of infiltration of T cells and bacterial abscess formation in the GI tract[15]. Activation of Smad3 in patients with inflammatory bowel disease (IBD) is diminished compared to unaffected persons. Isolated cells from IBD patients do not respond to treatment with TGF-β, and do not activate phosphorylation of Smad3, even in the presence of high concentrations of TGF-β[16,17]. In addition, interaction between Smad3 and Smad4 is markedly decreased in lamina propria mononuclear cells of IBD patients. All of these pathogenic phenotypes seem likely to originate from overexpression of Smad7, which is inhibitory for TGF-β signaling. The anti-inflammatory activity of TGF-β comes from inhibition of nuclear factor (NF)-κB activation, but it is lost in the intestine of IBD patients due to high levels of Smad7[18]. An *in vitro* mouse model of colitis treated with trinitrobenzene sulfonic acid or oxazolone shows similar results to those in humans[19]. When anti-sense oligonucleotide against Smad7 is given to mice to reduce the level of Smad7, TGF-β-induced phosphorylation of Smad3 is markedly increased. Conversely, the inflammatory phenotypes of the colon, such as weight loss and microscopic changes, are recovered to normal states after treatment with Smad7 antisense nucleotides. These data indicate that precise regulation of TGF-β signaling, including activation of Smad3 and level of Smad7, is important for homeostasis during inflammation of the GI tract. In contrast to these data, Smad7 also has anti-inflammatory activity against tumor necrosis factor (TNF) signaling and mediates the suppressive activity of TGF-β in primary macrophages and mouse skin[20]. This discrepancy might originate from

differences in cell lines, mouse strains, and experimental systems.

TGF-β LIGANDS AND THEIR SIGNALS

TGF-β sends signals through the heterodimeric complex formation of type I and type II serine/threonine kinase receptors. When the ligands interact with receptor II, type I receptor is activated by phosphorylation of serine residues. Activated receptor complexes recruit the adaptor proteins, receptor-activated Smad proteins (R-Smad). For example, Smad2 and Smad3 are R-Smad proteins for TGF-β and Smad1, Smad5 and Smad8 are R-Smad proteins for bone morphogenic protein signaling. R-Smad proteins have two conserved domains that are separated by a middle linker domain. The MH1 domain of the N-terminal region functions as a DNA-binding domain and the C-terminal of the MH2 region regulates the nuclear localization and transcriptional activity of R-Smad proteins. Receptor-mediated phosphorylation of C-terminal serine residues induces activation and nuclear translocation of R-Smad proteins. Activated R-Smad proteins form a complex with common Smad (co-Smad) protein, Smad4. The interaction between R-Smad and co-Smad proteins is mediated through the MH2 domain and induces the translocation into the nucleus of Smad complexes. In the nucleus, Smad complexes interact with Smad binding elements (SBEs) of target genes and regulate the transcriptional activity. TGF-β upregulates or downregulates the expression of target genes, which depend on Smad-interacting partners, coactivators or corepressors[6].

CROSS-LINKING BETWEEN INFLAMMATION AND GI CARCINOGENESIS

Relationship between chronic inflammation and carcinogenesis was originally suggested by Virchow, who first hypothesized in 1893 that the lymphoreticular infiltrate may be the origin of tumor at sites of chronic inflammation. Over 100 years later, his hypothesis was cited as evidenced that chronic inflammatory conditions definitely promote progression of malignant tumors. Inflammatory cells and cytokines act as a tumor promoter that affects cell survival, proliferation, invasion, angiogenesis and chemo-resistance[21-23]. Almost 15% of cancers are reported to occur through chronic inflammation-related processes (Table 1). We have established animal models of GI tumors, all of which were based on the concepts that inflammation leads to tumorigenesis, Barrett's esophagus originates through repeated reflux-induced inflammation in the esophagus, colitic tumors arise from repeated bouts of colitis, and gastric cancer is associated with *Helicobacter pylori* (*H. pylori*) infection.

Reflux esophagitis and esophageal cancer

More than 500 000 patients are diagnosed with esophageal cancer annually, which has the second highest mortality after pancreatic cancer among GI cancers worldwide. The most typical type of esophageal cancer is, in

Table 1 GI tumorigenesis associated with chronic inflammation

	Tumor	Causes
Infection-related		
Chronic gastritis	Gastric cancer	*H. pylori*
Chronic gastritis	Gastric cancer	Cytomegalovirus
Prostate inflammatory atrophy	Prostate cancer	Pathogens
Chronic hepatitis	Hepatocellular cancer	HBV and HCV
Infection-unrelated		
Esophagitis	Esophageal cancer	Gastric acid, alcohol, tobacco
Pancreatitis	Pancreatic cancer	Alcohol, tobacco
IBD	Colorectal cancer	Crohn's disease, UC
Cholecystitis	Gall bladder cancer	Gall bladder stone

GI: Gastrointestinal; *H. pylori*: *Helicobacter pylori*; HBV: Hepatitis B virus; HCV: Hepatitis C virus; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

histological terms, squamous epithelial carcinoma, and Asians have a greater prevalence of this histological type of carcinoma than adenocarcinoma. On the other hand, Caucasians have just as many cases of adenocarcinoma as squamous carcinoma[24,25]. Many reasons to explain such differences can be put forward, including lifestyle and genetic factors, but in particular, Barrett's esophagus results in a great discrepancy in incidence between Western and Asian populations. Since the numbers of reflux esophagitis patients are rapidly increasing in Korea, as lifestyle as well as living environment have changed to more western styles, in the near future, it is expected that esophageal adenocarcinoma will be more prevalent among Koreans. Esophageal adenocarcinoma is closely related to Barrett's esophagus, therefore, carcinogenesis has the order of chronic reflux esophagitis followed by Barrett's esophagus and esophageal adenocarcinoma. Reflux esophagitis leads to chronic esophagitis and an increase in Barrett's esophagus, which directly creates a precancerous lesion, for which we have demonstrated that chronic exposure to gastroduodenal contents through duodenoesophageal anastomosis leads to metaplastic changes in the squamous epithelium (Figure 1). Molecular factors related to reflux esophagitis are as follows: increase in cyclooxygenase-2 (COX-2), IL-8, IL-1β, IL-10 and TNF-α; loss of TGF-β signaling; and activation of NF-κB. All of these contribute to development of Barrett's esophagus. This mediates chronic inflammation that increases expression of CDX-like homeobox family genes and the caudal gene family, which results in intestinal metaplasia of squamous epithelial cells. Intestinal metaplasia is the direct cause of esophageal adenocarcinoma and because it shares similar molecular mediators with chronic esophagitis, it is thought to be involved in carcinogenesis[26,27]. Barrett's esophagus is thought to be a precancerous lesion because of the appearance of the following changes: augmentation of the cell cycle and proliferation, increased angiogenesis and aneuploidy, decreased anti-proliferative signaling and apoptosis, accompanied by the histological changes involved in the progression from chronic esophagus to Barrett's esophagus.
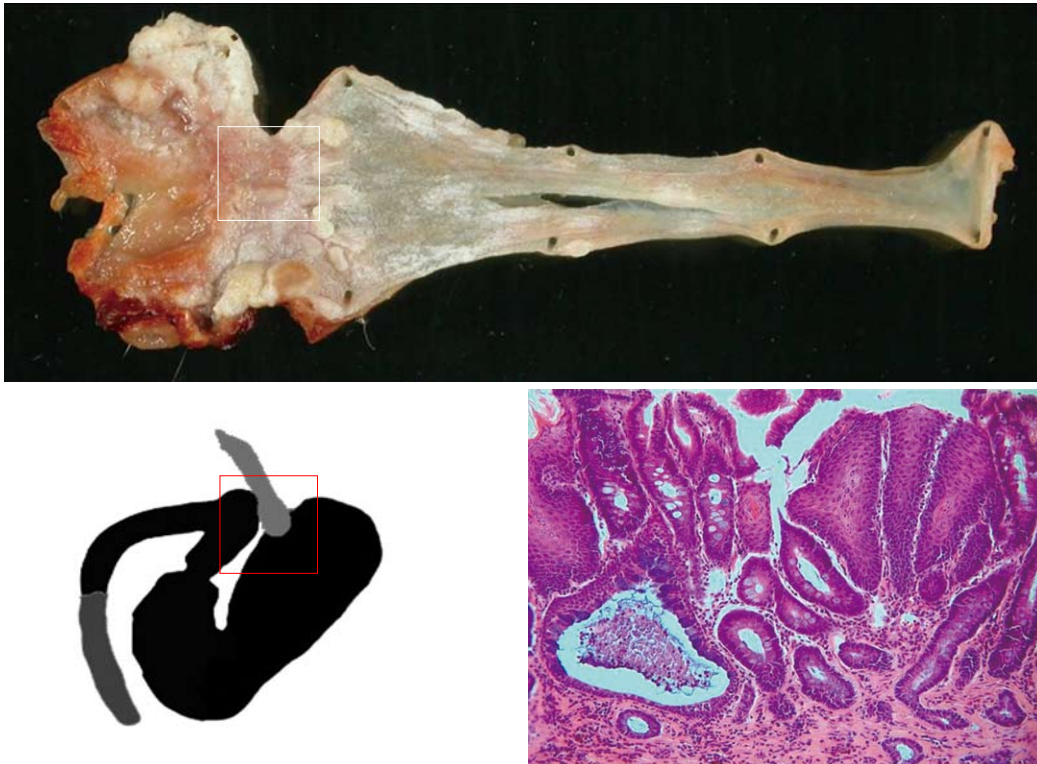
Figure 1 Connection between inflammation and carcinogenesis as demonstrated in a rat model of Barrett's esophagus. With the application of surgical bypass, esophagoduodenostomy (red boxed), overt discolored lesions (white boxed) were noted at the esophagogastric junction, which showed the existence of intestinal metaplasia against a background of squamous epithelium. These animal models provided evidence of the cross-linking between inflammation and premalignant lesions.

Chronic pancreatitis and pancreatic cancer

Pancreatic cancer has the poorest prognosis among GI cancers because patients are diagnosed at a fully progressed stage, and the tumor cells are very aggressive, so that metastasis is very frequent and they do not respond to anti-cancer therapy. Chronic pancreatitis patients have a 3.8-18.5 times higher chance of developing pancreatic cancer[28]. However, severe chronic pancreatitis cannot be discriminated from pancreatic cancer, especially in cases with a history of smoking and alcohol consumption, and a family history. Continuation of chronic pancreatitis for long periods can create a cancer-like molecular environment, such as activation of COX-2, NF-κB and inducible nitric oxide synthetase, production of cytokines such as IL-1, IL-6, IL-8 and TNF-α, and free radical oxygen formation[29,30]. TGF-β plays such an important role in regulating onset of chronic pancreatitis, and we have shown that lack of TGF-β in pancreatic acinar cells leads to increased sensitivity to acute pancreatitis, as well as a favorable environment for chronic pancreatitis.

Chronic atrophic gastritis and gastric cancer

Although there has been a tendency towards a decrease in gastric cancer for the past 80 years, it is still the second highest cause of death following lung cancer. In Korea, about 25-30 people out of every 100 000 develop gastric cancer regardless of their sex, and its mortality rate is ranked as the highest. However, the discovery of a causative pathogen of gastric cancer, *H. pylori*, has raised hopes for preventing gastric cancer through eradication or regulation of the bacterium. The International Agency for Research on Cancer in 1994 defined *H. pylori* as an imminent carcinogenic pathogen, based on epidemiological research and animal studies. This definition is based on epidemiological evidence, and several animal experiments have shown that infection with *H. pylori* causes infiltration of inflammatory cells, oxidative damage and gene mutations. In resected specimens from gastric cancer, the intestinal metaplastic lesions are easily identified around the cancer lesions, with which *H. pylori* infection is closely associated. Although *H. pylori* is a class I carcinogen, gastric cancer is not prevented by *H. pylori* eradication in all patients. This can be explained by the relationship between gastritis, metaplasia and gastric cancer, whereby prevention of *H. pylori*-associated carcinogenesis only benefits those in whom the malignant process has not begun[31]. That is, even though *H. pylori* is completely eradicated, gastric inflammation remains, thus it seems that amelioration of gastric inflammation itself seems to be far more essential in achieving cancer prevention than eradication of the pathogen. This emphasizes that chronic gastritis has a greater chance of causing onset of gastric cancer than the presence of *H. pylori* itself. Furthermore, genetic factors, toxicity of the pathogen, and environmental factors are intertwined in the development of *H. pylori*-induced gastric cancer from inflamma-
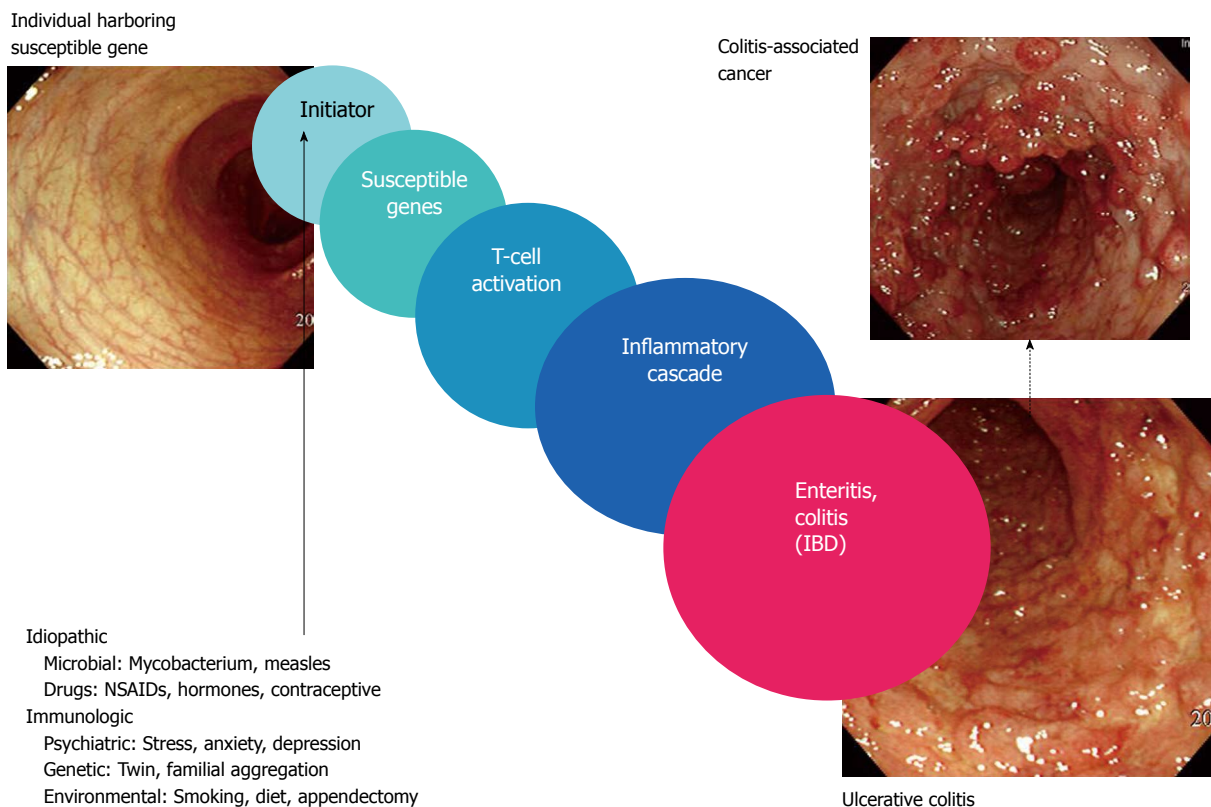
Figure 2 Inflammatory bowel disease (IBD) and colitis-associated cancer. The cause of IBD is idiopathic because several etiologies have been suggested including microorganisms, drugs, psychiatric illness, genetic abnormality, and environmental factors. However, when individuals that harbor IBD susceptibility genes are exposed to the above-mentioned etiological factors, they experience T cell activation, after which perpetual inflammatory cascades lead to enteritis or colitis. Repeated bouts of chronic colitis alone provoke colitis-associated cancer, which emphasizes the cross-linking between inflammation and carcinogenesis. NSAIDs: Nonsteroidal antiinflammatory drugs.

tion, thus alleviation or treatment of inflammation could form the basis of prevention of gastric cancer[32,33]. The contribution of TGF-β to *H. pylori*-associated gastric carcinogenesis has been investigated using pS2-dnRⅡ mice, which are defective in TGF-β signaling due to expression of the dominant negative RⅡ receptor (TGF-β-dnRⅡ) in the stomach, using gastric specific promoter pS2 (gastric trefoil peptide). pS2-dnRⅡ mice developed gastric adenocarcinoma with *H. pylori* alone compared to their wild-type littermates, which suggests that the development of gastric cancer after *H. pylori* infection might be influenced by host genetic conditions[34].

IBD and colitis-associated cancer

The most common cause of colorectal cancer is sporadic. However, 10%-15% of these patients continually suffer from chronic IBD for 10 years, which later develops into colitis-associated cancer, namely, colitic cancer. Ulcerative colitis (UC) is a form of chronic IBD that usually has a clinical course of repeated exacerbation and remission, and less commonly presents with an unremitting, fulminating course. Another feature of the clinical course of UC is an increase risk for the development of colitic cancer (Figure 2). Although sporadic colorectal cancer and colitic cancer arise from dysplastic precursor lesions and share several molecular alterations, the nature of the

dysplasia and the frequency and timing of several of the key molecular changes differ sufficiently to support our hypothesis that colitic cancer can be prevented by early intervention with strong anti-inflammatory agents or cancer surveillance with strict biomarkers. Etiologically, in the case of sporadic colorectal cancer, most cases are preceded by adenomatous polyps, namely adenoma, followed by the so-called "adenoma-carcinoma" sequence[35]. On the other hand, colitic cancer is contracted at a relatively young age and the crucial factor is the range, extent and period of inflammation, which implies the "inflammation-dysplasia-carcinoma" sequence. Considering the fact that the number of IBD patients is increasing, in the forthcoming 10 years, inflammation-related cancer is expected to increase, thus we should be focusing on cancer prevention through control of inflammation. We have studied colorectal cancer prevention based on an animal model of inflammation-related cancer, and are working on how the control of inflammation itself prevents colorectal cancer. We have performed another similar animal model with repeated bouts of colitic cancer and have shown that potent anti-inflammatory drugs inhibit cancer formation. This suggests that colitis is involved at the beginning as well as the progression of cancer, and control of inflammation is one of the methods for preventing inflammation-related colorectal cancer.
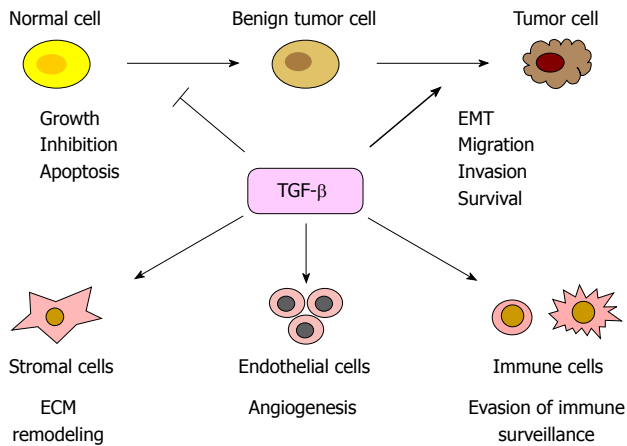
Figure 3 Role of transforming growth factor (TGF)-β in cancer progression. TGF-β is a secreted polypeptide that signals *via* receptor serine/threonine kinases and intracellular Smad effectors. TGF-β inhibits proliferation and induces apoptosis in various cell types. Accumulation of loss-of-function mutations in the TGF-β receptor or Smad genes classifies the pathway as a tumor suppressor in humans. In addition, various oncogenic pathways directly inactivate the TGF-β receptor-Smad pathway, thus favoring tumor growth. On the other hand, all human tumors overproduce TGF-β whose autocrine and paracrine actions promote tumor cell invasiveness and metastasis. Accordingly, TGF-β induces epithelial to mesenchymal transition (EMT).

TGF-β IN GI CARCINOGENESIS

TGF-β signaling is a crucial regulator of intestinal homeostasis and inflammation, therefore, dysregulation of this pathway could be associated with inflammation-related carcinogenesis of the GI tract (Figure 3). Altered signaling pathways can disturb the regulatory mechanism of TGF-β to block chronic inflammation followed by carcinogenesis. As a tumor suppressor gene, TGF-β can inhibit initiation of cell transformation, through growth inhibition and apoptosis in normal epithelium. During changes in the genetic and epigenetic context of transforming cells, the responsiveness of cells to TGF-β is decreased, but the expression and activation of TGF-β are markedly increased[36].

Mouse models have been used to investigate the role of TGF-β in GI inflammation and tumorigenesis. Heterozygotic TGF-β null mice express only 10%-30% of wild-type TGF-β1 protein level, and show increased cell turnover with chemical carcinogens, which results in enhanced tumorigenesis compared with wild-type mice[37]. Furthermore, TGF-β1/Rag2 double knockout mice can live for a long time and develop colon cancer after developing inflammatory lesions[38]. Mutations of TGF-β receptor Ⅱ (TGF-β-R Ⅱ) are frequently found in colon and gastric cancer with microsatellite instability[39]. Conditional transgenic mice using TGF-β-dnR Ⅱ with pS2/TFF1 promoter were created and infected with *H. pylori*, and they developed dysplasia as well as adenocarcinoma, which suggests that disruption of TGF-β signaling is related to gastric cancer formation[34]. The findings that TGF-β-dnR Ⅱ mice have higher levels of gastritis than their wild-type littermates, suggests that TGF-β1 plays an important role in prevention of gastric inflammation

as well as cancer. An experiment for inducing IBD was performed on mice that overexpressed TGF-β-dnR Ⅱ in the small/large intestine using the promoter of ITF (intestinal trefoil factor)/TFF3. TGF-β-dnR Ⅱ mice showed much more severe colitis than their wild-type littermates when induced with the same concentration of dextran sulfate sodium[40].

Some pancreatic and biliary adenocarcinomas originate from mutation of TGF-β receptor Ⅰ (TGF-β-R Ⅰ) with very low frequency[41]. In a mouse model, mutation or deletion of bone morphogenetic protein receptor, which is another member of the TGF-β superfamily, also induces chronic inflammatory phenotypes and then precancerous lesions[42].

Deletion of Smad proteins also leads to loss of the regulatory role of immune suppression and induces malignant cell proliferation. Deficiency of Smad4 in mutant mice causes gastric polyps and progression to gastric tumor in old age[43]. In humans, deletion of Smad4 is found in juvenile polyposis syndrome and is a strong risk factor for GI cancer[44]. In addition, T-cell specific deletion of Smad4 results in spontaneous epithelial cancer of the oral cavity, stomach, colon, rectum and duodenum in mice[45]. Smad3 knockout mice also develop metastatic colorectal cancer through dysregulation of cell proliferation. The expression of Smad3 shows low to undetectable levels in 40% of human gastric cancer tissues[46]. Reintroduction of Smad3 into gastric cancer cells restored their responsiveness to TGF-β and blocks tumor formation in nude mice. These data support the suggestion that Smad-mediated TGF-β signaling regulates homeostasis and inflammation in the GI tract.

Mutations in fine tuning of regulatory circuits cause the loss of normal communication within target organs, and defects in regulating stromal and epithelial interactions. In addition to Smad proteins, cofactors of TGF-β signaling can also affect the biological roles of TGF-β and the progress of GI carcinogenesis. Based on mouse model studies, mutations of these cofactors, such as embryonic liver fodrin (ELF), promyelocytic leukemia tumor suppressor, Smad anchor for receptor activation, and filamin lead to malformation of intestinal structures and induce the formation of liver, gastric and colon cancers. In case of ELF, 40%-70% of heterozygotic null mice develop hepatocellular cancer within 15 mo through abnormal angiogenesis or cyclin D1 activation[47,48].

In contrast to its tumor suppressive activity, expression of TGF-β1 is markedly increased in colon, esophageal, gastric, hepatocellular and pancreatic cancer[49]. High levels of TGF-β expression are correlated with tumor progression, metastasis and angiogenesis, which results in poor prognostic outcome. In addition to its oncogenic nature related to TGF-β mutation, TGF-β, which is secreted from tumor cells, can control tumor progression by suppressing cytotoxic immune reactions, stimulating expression of cell survival factors, or regulating autonomous signaling of tumor cells, depending on cell type and context. At the late stage of tumor develop-
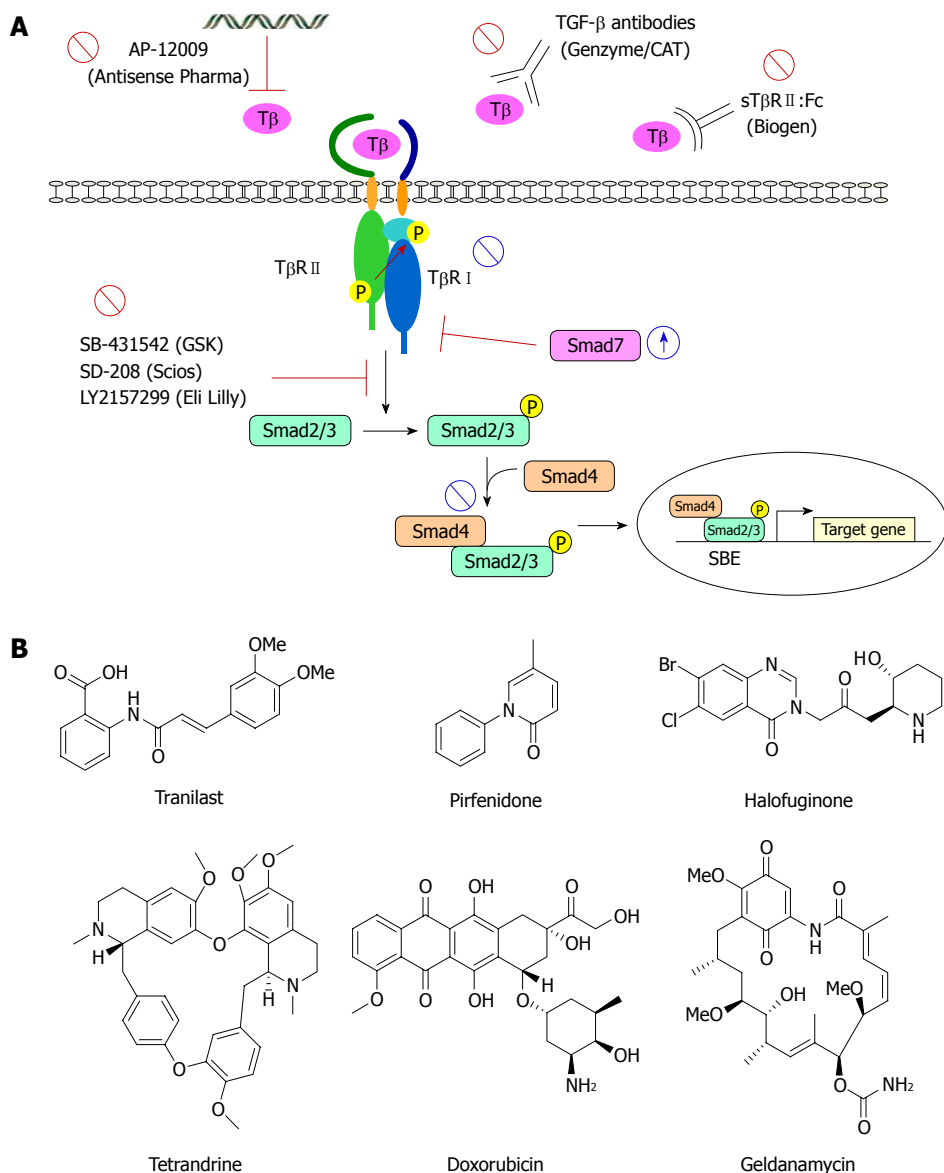
Figure 4 Therapeutic target of TGF-β signaling. A: TGF-β signaling and points of therapeutic intervention; B: Structures of multi-action TGF-β signal inhibitors. SBE: Smad binding element.

ment, invasive and metastatic potentials are important properties, and TGF-β signaling is a crucial pathway for tumor cell invasion and metastasis by inducing epithelial to mesenchymal transition (EMT)[50,51]. EMT progresses by decreasing the expression of E-cadherin to inhibit cell-cell adhesion and by increasing the expression of laminin-5, vimentin, integrins, and fibulin-5 that are involved in cell-extracellular matrix associations[52,53].

THERAPEUTIC TARGET OF TGF-β SIGNALING

Until now, the most successful target-aimed anti-cancer treatment has focused on the suppression of oncogenes that are abnormally activated in cancer cells. As examples, Gleevec® (imatinib), which is a *Bcr-abl* kinase inhibitors, and Herceptin (trastuzumab), which is a monoclonal antibody that interferes with the *Erb*B2/*Neu* receptor, are good examples of target-aimed molecular cancer treatments[54]. However, regarding TGF-β, the development of TGF-β inhibitors for the purpose of cancer treatment has shown no apparent progress so far in spite of TGF-β being known as a potent growth inhibitory factor against epithelial and blood cells. Instead, TGF-β signal transduction has been recognized as one of the representative targets of cancer treatment, aided by recent clinical results and cancer biology with regard to TGF-β. TGF-β ligands, as well as various downstream signal transducers can be a target in the therapeutic regulation of TGF-β signaling (Figure 4A). For instance, a potential target for the therapeutic intervention can be unveiled by inhibiting TGF-β synthesis, suppressing TGF-β activity at the cell surface, removing or neutralizing synthesized TGF-β, or regulating receptors and Smads involved in intracellular signaling pathways. By using EMT6 murine mammary tumor cell line that stably expresses antisense TGF-β transduced with a retroviral vector, it has been

demonstrated that the decreased production of TGF-β inhibited tumor growth[55]. In particular, the decreased secretion level of TGF-β by cancer cells induced tumor regression by increasing the activity of anti-tumor cytotoxic T cells. Currently, the inhibition of TGF-β production by using anti-sense oligonucleotides is one of the most potent methods in clinical cancer treatment. For example, AP-12009, which is synthesized by Antisense Pharma and is especially selective for TGF-β2, has prominent clinical effects on malignant neuroglial tumor patients[56,57]. It is now in phase Ⅱ clinical trials for the treatment of neuroglioma and pancreatic and skin cancer. The suppression of latent TGF-β activation can also be useful for regulating TGF-β activity. It has been reported that the furin protease inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (*dec-RVKR-cmk*), participates in TGF-β activity and inhibits the release of mature bioactive TGF-β molecules *in vitro*[58]. However, furin is not considered as a good selective inhibitor for TGF-β signaling because it is known to be involved in the activity of other growth factors, growth factor receptors, integrin, and matrix metalloproteinase.

Agents that promote TGF-β signaling

Restoration or augmentation of TGF-β signal transduction can be a good strategy for cancer prevention and treatment. For instance, chemical compounds such as retinoid and deltanoid (vitamin D), which are related to the expression or regulation of TGF-β signaling, have been used for chemoprevention of cancer[59,60]. Based on tumor suppressor activity of TGF-β, various attempts have been made to screen compounds that may activate TGF-β signaling. The first attempt, which was made by Seto's group, was to screen small molecular chemical compounds that have similar activities to TGF-β[61,62]. By searching for agents that increased the transcriptional activity of plasminogen activator inhibitor-1 (PAI-1), which is one of target genes of TGF-β, they finally isolated diheteropeptin and spiruchostatin A and B from microbial metabolites. These compounds turned out to increase the expression of PAI-1 and p21 and suppress the growth of lung epithelial cells in nanomolar concentrations. In addition, they had an inhibitory activity against histone deacetylase (HDAC). Lee *et al*[63] have isolated onnamide A and theopederin B from marine sponge, which promotes the transcriptional activity of TGF-β.

Onnamide A and theopederin B induce expression of PAI-1 gene and apoptosis through activation of mitogen-activated protein kinases (MAPKs) such as p38 and JNK, which suggests that they have strong anti-tumor activity. Moses's group[64] identified one TGF-β mimicking agent, A-161906, by performing high-throughput screening using mink lung epithelial cells stably transfected with PAI-1 promoter reporter. As an HDAC inhibitor, A-161906 demonstrated cellular effects similar to those of TGF-β. A-161906 not only inhibited cell proliferation, but also induced growth arrest at the G1-S checkpoint,

and expression of the cyclin-dependent kinase inhibitor p21. In addition, based on the fact that Smad4 (DPC4) mutation, which is frequently found in pancreatic cancer, plays a crucial role in the loss of TGF-β-mediated growth inhibition, Sohn's group[65] performed high-throughput screening. Using a stably transfected reporter gene that has six copies of SBE (p6SBE-Luc) in a pancreatic cancer cell line, PANC-1, they screened a library of approximately 16 000 compounds from ChemBridge DIVERSet, and finally identified scriptaid compound, which increased Smad transcriptional activity.

It is well known that expression of TGF-β receptors plays an important role in TGF-β-mediated cell growth inhibition. Therefore, increasing expression levels of TGF-β receptors in cancer cell lines, in which TGF-β receptors are mutated or their expression levels are attenuated, may be a good therapeutic target in cancer treatment. In fact, it has been reported that HDAC inhibitors, such as MS-275 and suberoylanilide hydroxamic acid (SAHA), increase response to TGF-β by inducing the expression of TGF-β-RⅡ and TGF-β-RⅠ, respectively, in breast cancer cell lines[66,67]. Besides, it has been found that the expression level of TGF-β-RⅡ is increased by captopril (angiotensin-converting enzyme inhibitor), FTI-27742 (farnesyl transferase inhibitor), and 5-aza-2'-deoxycytidine (DNA methyltransferase inhibitor)[68-70]. Thus, these compounds may be used as anti-cancer drugs or for combination treatments when treating colon or small cell lung cancer that commonly displays decreased expression levels of TGF-β-RⅡ.

Intriguingly, the screened chemical compounds including A-161906 and scriptaid that were identified by high-throughput screening, diheteropeptin, and spiruchostatins are strong HDAC inhibitors. This indicates that the activity of HDAC may play a key role in the regulation of TGF-β signaling. Recently, the FDA has approved SAHA (Vorinostat® Zoniza) as a treatment for cutaneous T-cell lymphoma (CTCL). It has been reported that SAHA activates p21 and induces apoptosis of T cells in CTCL patients[71].

Many transport molecules of TGF-β signaling as well as TGF-β receptors are regulated by epigenetic mechanisms. Therefore, even though HDAC inhibitors do not induce selective activation during TGF-β signal transduction, it is considered that HDAC inhibitors and various chemical compounds similar to or increasing TGF-β activity can be good targets in the prevention and treatment of cancer. Since the most frequent genetic alteration found in cancer is the loss-of-function in tumor suppressor genes, this alteration is very specific to cancer cells and it may be a good therapeutic target in the development of anti-cancer drugs. In order to identify the agents selectively targeting cancer cells with loss-of-function mutations, Wang *et al*[72] have demonstrated a novel screening technique, named pharmacologic synthetic lethal screening, and have identified UA62001, which shows selectivity against DPC4-deficient cell line BxPC-3, and by investigation of

downstream target genes of UA62001, they have found that UA62001 arrests cells in G2/M phase by inhibiting cyclin B/CDC2 expression. In addition, Park *et al*[73] have screened the Maybridge Diversity Set chemical library using a cell-based assay to measure nuclear translocation of Smad proteins. Through high-throughput screening, DAM-1976, which enhances TGF-β signaling, has been identified. DAM-1976 increases sensitivity to TGF-β by prolonging Smad2/3 activation in the nucleus in a TGF-β-independent manner.

Professor Sporn MB, who discovered TGF-β and has been in the vanguard of cancer chemoprevention research, and his colleagues[74] have made many modifications of triterpenoids that possess anti-inflammatory and anti-tumor activity. They have screened derivatives of two known triterpenoids, oleanolic acid, and ursolic acid, and synthesized 2-cyano-3,12-dioxooleana-1, 9-dien-29-oic acid (CDDO), which has the most potent anti-inflammatory activity and low toxicity. It has been reported also that CDDO not only promotes Smad signal transduction, but also displays various biological activities similar to that of TGF-β[75]. Moreover, CDDO is currently in phase II clinical trials for the treatment of leukemia and solid tumors, based on its anti-inflammatory and anti-tumor activities, cellular protection, and suppression of tumorigenesis. Therefore, CDDO is considered to be a promising chemopreventive and chemotherapeutic agent in cancer treatment.

TGF-β antibody and soluble TGF-β receptors

The monoclonal antibody that neutralizes TGF-β ligand is one of most developed inhibitors for the clinical application of suppressing TGF-β activity. The human monoclonal antibodies CAT-152 (lerdelimumab), CAT-192 (metelimumab) and GC1008, and the mouse monoclonal antibodies 1D11 and 2G7 against TGF-β have been generated to date. CAT-152 and CAT-192 are the human IgG4 monoclonal antibodies that neutralize TGF-β2 and TGF-β1, respectively. CAT-152 and CAT-192 (Cambridge Antibody Technology) have entered phase III and II clinical trials for the treatment of wounds after glaucoma filtration surgery and pachyderma, respectively. However, the trials were discontinued in 2004 due to problems concerning their effectiveness[76]. GC1008, developed by Genzyme Corporation, is a pan-TGF-β selective monoclonal antibody that works against all TGF-β isomers. Clinical trials for GC1008 were conducted in idiopathic pulmonary fibrosis patients. Also, it is currently in a clinical trial for the treatment of metastatic skin cancer and renal cancer. The anti-tumor effectiveness of 1D11 and 2G7 has been studied in various animal tumor models. According to the report of Arteaga *et al*[77], intraperitoneal injection of 2G7 suppresses not only tumor growth of MDA-MB-231 breast cancer cell lines, but also lung metastasis. Moreover, 2G7 partially inhibits angiogenesis by reducing the levels of vascular endothelial growth factor in blood plasma. On the other hand, suppression of TGF-β activity can be achieved by using macromolecular proteins such as soluble TGF-β receptors that are able to inhibit competitively interactions between the ligand and the receptor. In particular, soluble TGF-β-R II has been extensively studied in preclinical tumor models. A soluble chimeric protein, composed of the extracellular domains of the TGF-β-R II and the Fc portion of IgG1, has been shown to block TGF-β activity[78]. When tested in a transgenic mouse model that expressed a soluble TGF-β-R II receptor: Fc fusion protein (Fc:TGF-β-R II), metastatic capacity was suppressed without any increase of tumor growth, which indicated that this fusion protein selectively inhibited metastasis-associated TGF-β activity[79]. In addition to soluble TGF-β-R II, soluble TGF-β-R III also suppressed tumor growth and lung metastasis in an MDA-MB-231 xenograft tumor model by antagonizing the tumor-promoting activity of TGF-β[80].

TGF-β receptor kinase inhibitors

TGF-β receptor kinases are not only key molecules in intracellular TGF-β signal transduction, but potential therapeutic targets that can be easily regulated by small molecular compounds. Owing to the success of Gleevec®, many projects, focused on TGF-β receptor kinases, have been carried out along with large pharmaceutical companies. In particular, numerous studies concerning TGF-β-R I have been performed to elucidate the domain structures, biochemical activity, and biological functions of TGF-β-R I [81]. Thus, TGF-β-R I serves as an attractive target for drug development. The development of protein kinase inhibitors can be achieved in several ways, such as target hopping, novel screening and virtual screening.

TGF-β-R I kinase inhibitors with various structures have been developed by drug companies. Their various effects including anti-tumor activity confirmed in animal models, relations with TGF-β-induced Smad phosphorylation, activation of reporter genes, cell cycle arrest, and EMT, have been reported. SB-431542, SB-505124 (Glaxo Smith Kline; GSK), LY364947, LY2157299 (Eli Lilly), SD-208 (Scios), and IN-1130 (In2gen) are well known TGF-β-R I kinase inhibitors. SB-431542 and SB-505124 compounds from GSK were developed by target hopping based on the structures of the kinases. Phosphorylation of TGF-β-R I is inhibited by SB203580, a p38 kinase inhibitor, because TGF-β-R I has a gatekeeper residue equivalent to that of p38 kinase, which results in structural similarity to p38 kinase[82]. SB-431542 was synthesized having SB-203580 as a lead compound. It has shown greater selectivity for TGF-β-R I compared with other serine/threonine kinases (protein kinase C, extracellular signal-regulated kinase, JNK, p38 MAPK). However, it also has displayed strong inhibitory activity against activin receptor (ALK4) and nodal receptor (ALK7)[83]. In addition, SB-431542 inhibits proliferation of glioma, TGF-β-mediated morphological changes, and cell motility, which suggests that small molecule inhibitors of TGF-β receptor kinases have potential to suppress tumor progression[84].

Based on the growth inhibitory characteristics of TGF-β in epithelial cells, researchers at Eli Lilly (Indianapolis, MN, USA) have identified the heteroarylpyrazole structure of LY364947 through high-throughput screening[85]. After they confirmed that TGF-β-R I and TGF-β-R II kinase were the molecular targets of LY364947, the latter became a lead compound in the development of inducers. LY364947 also turned out to be identical to HTS-466274, which was identified by Biogen Inc. through computer modeling of a compound database that contained over 200 000 compounds. The result suggested shape-based virtual screening as a powerful tool to discover and develop new TGF-β-R I kinase antagonists. Furthermore, studies on the relationship between structure and activation, as well as optimization of lead compounds have led to the development of more advanced compounds, such as LY2157299. In particular, LY2157299 entered a phase I clinical trial for the first time as a TGF-β-R I kinase antagonist. Also, studies on its pharmacotoxicity, pharmacokinetics, and pharmacodynamics are currently in progress[86].

Oral bioavailability of SD-208, another type of TGF-β-R I kinase inhibitor under development by Scios Inc., has been verified by demonstrating its ability to inhibit infiltration of glioma cells and metastasis of a highly metastatic breast cancer cell line[87]. Besides, A-83-01 has been found to be more potent in the inhibition of TGF-β-R I than SB-431542, and GW788388 can be administered orally, with improved pharmacokinetics[88,89]. IN-1130, recently developed in Korea as a small molecular inhibitor of TGF-β-R I, has shown kinase selectivity similar to that of SB-431542. IN-1130 also inhibits tumor growth and promotes activation of cytotoxic T cells in xenograft prostate tumor models, which suggests that it might restore TGF-β-induced immunosuppression in cancer cells[90].

Non-selective agents that suppress TGF-β signaling
Besides the selective inhibitory substances, including antibodies against the ligand and receptor kinase inhibitors, non-selective chemical compounds may provide strategies for the regulation of TGF-β signaling. Such agents include compounds that suppress the expression level of TGF-β, increase the expression level of Smad7, an endogenous inhibitory protein, or regulate the stability of TGF-β receptors (Figure 4B). Natural or synthesized agents that suppress TGF-β signaling through these mechanisms generally demonstrate anti-inflammatory, anti-fibrosis, and various biological activities. Among these, tranilast (Rizaben®) has been used as an anti-allergic drug for the treatment of asthma and atopy. Also, it has been known that tranilast has anti-fibrosis and anti-tumor effects. According to animal experiments recently carried out with the highly metastatic murine 4T1 breast cancer cell line, tranilast effectively suppressed metastasis by activating Smad proteins, inhibiting EMT, and strongly suppressing the production of TGF-β and IL-17 that were secreted by immune cells[91]. The suppressive effect

of tranilast on metastasis turned out to be greater than that of TGF-β antibody 1D11 or paclitaxel.

In addition, pirfenidone, which is in a clinical trial for idiopathic pulmonary fibrosis, due to its anti-fibrosis effects, suppresses proliferation of neuroglioma by inhibiting expression and activation of TGF-β[92]. Halofuginone, a plant alkaloid and inducer of febrifugine, is a representative small molecular weight molecule in the suppression of collagen synthesis. It was first isolated from *Dichroa febrifuga*, approved by the FDA, and has been used for the treatment of scleroderma. Recently, clinical trials have been conducted on Kaposi's sarcoma to examine its inflammatory activity. Halofuginone has been reported to inhibit TGF-β signaling by decreasing TGF-β-R II expression and elevating inhibitory Smad7 expression[93].

Moreover, tetrandrine, another plant alkaloid, and doxorubicin have demonstrated increased expression of Smad7[94,95]. While screening for agents that suppress TGF-β signal transduction, geldanamycin, which is also known as a heat shock protein 90 (Hsp90) inhibitor, was identified. Geldanamycin inhibits TGF-β-induced target gene expression and Smad phosphorylation even at the subnanomolar concentrations. Analysis of its mechanism of action revealed that geldanamycin suppresses TGF-β signaling *via* complex processes. For instance, geldanamycin regulates Hsp90 chaperone-associated stability of TGF-β receptors, suppresses receptor complex formation between TGF-β-R II and TGF-β-R I, and induces interaction between Hsp70 and its receptors[96]. In general, Hsp90 inhibitors are widely recognized as a promising therapeutic target for anti-cancer drug development because of their ability to suppress tumor growth by inactivating various proteins that are related to tumorigenesis, such as Bcr-abl, ErbB2 and Raf-1. Various Hsp90 inhibitors, including geldanamycin inducer and 17-(allylamino)-17-demethoxygeldanamycin (17-AGG) are currently undergoing clinical trials. In fact, 17-AGG has completed a phase III clinical trial for the treatment of multiple myeloma[97].

CONCLUSION AND FUTURE DIRECTIONS
Although TGF-β signaling has been shown to have some paradoxical actions in tumorigenesis, many factors activate TGF-β signaling for tumor suppression, such as HDAC inhibitor, SAHA, synthetic terpenoid, CDDO, in addition to inhibitors of TGF-β signaling. It is a good strategy to block the initiation of inflammation-associated tumorigenesis through the development of TGF-β mimics in order to achieve chemoprevention. Activators of TGF-β signaling inhibit initiation of tumorigenesis and invasion and metastasis of advanced-stage tumors. This sort of approach will be very helpful for TGF-β-responsive tumor therapy, so-called tumor vaccination. In terms of chemoprevention, the difficulty in applying TGF-β activators to normal people is due to immunosuppressive action and difficulty in detecting the tumor
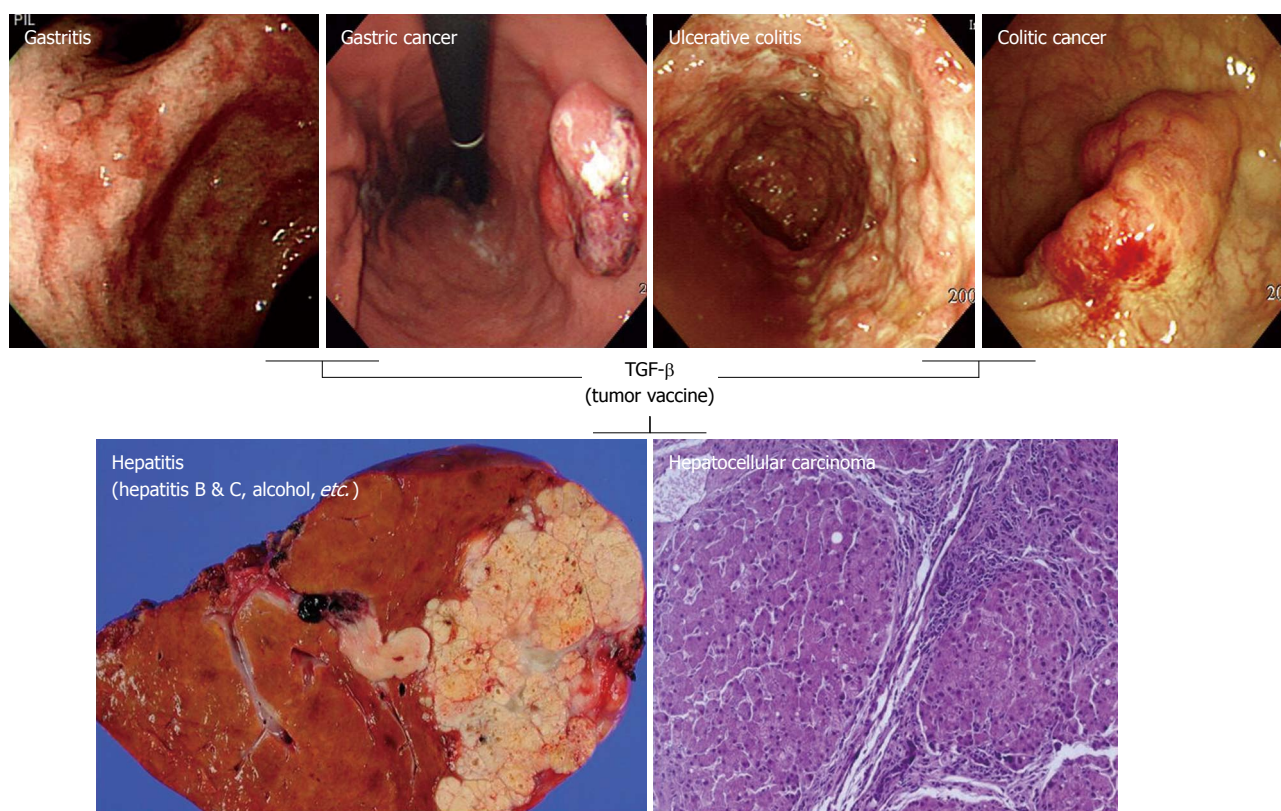
Figure 5 TGF-β1 as pivotal tumor vaccine based on anti-inflammatory actions that block progression from inflammatory lesions to precancerous lesions/ cancer. Chronic inflammation induced by biological, chemical, microbial and physical factors is associated with increased risk of human cancer at various sites. Inflammation facilitates initiation of normal cell transformation, growth, and progression to malignancy through production of pro-inflammatory cytokines and diverse reactive oxygen species. In this pathway, TGF-β plays a key role in chemoprevention through anti-inflammatory and anti-mutagenic actions.

at an early stage. Blocking inflammation-associated carcinogenesis with the anti-inflammatory activity of TGF-β signaling can be used as a chemopreventive strategy in patients at high risk of cancer (Figure 5). Even though TGF-β may regulate the inflammatory process by modulating the differentiation of many kinds of T cells, we still do not know the exact role of TGF-β in T-cell biology. Further study of the role of TGF-β in development of GI tract immune cells can lead to future therapeutic approaches for inflammation and tumorigenesis. To date, tumor chemotherapy by suppression of TGF-β signaling has been tried, to enhance the tumor-specific immune reaction, inhibit angiogenesis of the tumor microenvironment, and block invasion and metastasis of tumor cells. However, TGF-β has different effects depending on the type or context of the tumor, and has an anti-inflammatory effect on various immune cells. For these reasons, it is important to select the right patients, time of intervention, therapeutic reagents to combine, and exact targets, to minimize the side effects and maximize successful treatment. Recently, many low molecular size inhibitors of TGF-β receptor kinase have been tested in clinical trials of tumor chemotherapy. Also, cross-talk between TGF-β signaling and other signaling pathways has been studied in detail, and new candidates have been screened and developed to regulate these signaling pathways. Based on results of cooperative activity of TGF-β

with oncogenes, combination therapy with conventional anti-tumor reagents and TGF-β signaling inhibitors can enhance the therapeutic effects. Since inflammation-associated carcinogenesis is caused by complex cross-talk between many different cytokine signaling pathways, by collaboration with systems biologists, we can understand in more detail the mechanisms and networks of signaling pathways for inflammation and tumorigenesis. Through this approach, we can design and develop more efficient therapeutic drugs for treatment of GI cancer based on the biological action of TGF-β.

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