Japanese Journal of Gastroenterology and Hepatology

Review Article

ISSN: 2435-1210 | Volume 9

Role of TGF-B in Colorectal Cancer: Mechanisms, Diagnosis and Therapies

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Received: 07 Aug 2022
Accepted: 19 Aug 2022
Published: 24 Aug 2022
J Short Name: JJGH

Keywords:

Colorectal cancer; Transforming growth factor beta (TGF-β) signaling; target therapy; Extracellular vesicles; Nanoparticles

Citation:

Deng J. Role of TGF-B in Colorectal Cancer: Mechanisms, Diagnosis and Therapies. J Gstro Hepato. V9(6): 1-8

1. Abstract

Colorectal cancer (CRC) is a substantial public health challenge across the globe. Transforming growth factor-β (TGF-β) is a critical pathway in CRC that regulate numerous cellular processes, including immune-suppression, growth inhibition, cell migration, invasion, epithelial-to-mesenchymal transition and extracellular matrix remodeling. Metastasis is the major cause of death in patients with colorectal cancer. TGF-β participates in this process by regulating its downstream SMAD and non-SMAD signaling pathway. Here we summarize the application of extracellular vesicles in CRC diagnosis, and therapies of CRC with targeted anti TGF-β and extracellular vesicles. Finally, we analyzed the difficulties faced by targeted anti TGF-β therapies, and discussed the prospects and limitations of new diagnosis and therapies methods of CRC.

2. Introduction

Colorectal cancer (CRC), as the second leading cause of death, is proved to be a substantial public health challenge across the globe [1]. The unhealthy modern lifestyles including obesity, alcohol, poor diet and physical inactivity, become hotbeds of CRC [2,3], so that the prevalence of CRC is still on the rise. The common cancer treatment strategies mainly concentrated on the tumor surgical removal, along with physical and chemical treatment such as radiotherapy and chemotherapy [4]. Recently, combinational treatment with targeted therapies, such as vascular endothelial growth factor (VEGF), or epidermal growth factor receptor (EGFR) inhibitors, has been proved to be quite effective in patients with specific CRC subtypes [5]. Therefore, analyzing the relevant targets and signaling pathways involved in CRC is necessary to elucidate the mechanisms of CRC and enrich the clinical treatment methods.

The progression and metastasis of CRC is highly sophisticated. It involved several targeted genes and signaling pathways, including Wnt/β-catenin [6-8], p53[9-12], NF-κB [13,17], TGF-β/SMAD, etc. Transforming growth factor beta (TGF-β) signaling plays critical roles in growth, development, inflammation, repair and host immunity [18,19]. TGF-β family of cytokine genes has 33 human members that encode for homodimeric or heterodimeric secreted cytokines [19,20]. It is closely associated with the growth and development of CRC, due to its involvement in proliferation [21,22], epithelial-to-mesenchymal transition (EMT) [23-28] and angiogenesis [29-33]. This review focused on the mechanisms of TGF-β signaling pathway in CRC growth and metastasis.

3. Transforming Growth Factor-Beta (TGF-β) Signaling

Transforming growth factor-β (TGF-β) represents an evolutionarily conserved family of secreted polypeptide factors [34,35] that regulate numerous cellular processes, including immune-suppression, growth inhibition, cell migration, invasion, EMT and extracellular matrix (ECM) remodeling [36]. This superfamily comprises TGF-βs, activins, inhibins, nodal, growth and differentiation factors (GDFs), bone morphogenetic proteins (BMPs) and other subfamilies [37-39]. The protein structures of these family members are first synthesized in the form of precursors and then divided through secretory pathway to produce mature dimer ligands usually bound by a disulfide bond [37-40]. The activation of TGF-β signaling starts from the binding of active TGF-β ligand to Type II receptor (TβRII), which trigger the recruitment of the TGF-β Type I receptor (TβRI) subsequently. In the receptor complex, TβRII phosphorylates and activates TβRI. The activated receptor complexes initiate the TGF-β signaling through SMAD and non-SMAD signaling proteins, which regulate context-specific gene responses and thus control diverse cel-

lular processes [41-44]. SMAD proteins are the major effector molecules in the TGF-β signaling pathway [45,46]. Upon ligand binding, TGFβRI activates SMAD2 and SMAD3 through phosphorylation of its C-terminal specific ser residues. Subsequently, SMAD7 competes with R-SMADs for interacting with TβRI, thus preventing R-SMAD activation and proper propagation of the signaling. These R-SMADs associate with the common mediator SMAD4 protein and form trimeric complexes, which are then transported to the nucleus. In the nucleus, the trimeric complex binds to high-affinity DNA binding transcription factors (TF) and chromatin remodeling proteins (CR) in order to regulate the expression of TGF-β-responsive genes positively or negatively. The non-SMAD signaling pathways include several branches: the mitogen-activated protein kinase (MAPK) pathways, extracellular signal-regulated kinases (Erks), c-Jun amino terminal kinase (JNK), p38 MAPK, IκB kinase (IKK), phosphatidylinositol-3 kinase (PI3K)/Akt, and Rho family GTPases [47-49]. A Study proposed that non-SMAD signaling pathway has three mechanisms in the TGF-β signaling(Moustakas and Heldin, 2005): (1) non-SMAD signaling pathways directly modify (e.g. phosphorylate) the SMADs and thus modulate the activity of the central effectors; (2) SMADs directly interact and modulate the activity of other signaling proteins (e.g. kinases), thus transmitting signals to other pathways; and (3) the TGF-β receptors directly interact with or phosphorylate non-SMAD proteins, thus initiating parallel signaling that cooperates with the SMAD pathway in eliciting physiological responses [50,51]. This reflects the diverse regulatory mechanisms and biological effects of interaction between SMAD and non-SMAD signaling pathway in the TGF-β signaling. The TGF-β has been reported as a critical factor in cancer growth, development and metastasis. TGF-beta can play two different and opposite roles in the process of tumor progression. In the early stage of tumors, TGF-β is a potent tumor suppressor and mediate the actions of chemoprevention agents. However, it turns into a promoting factor that boosts tumor progression by inducing EMT and tumor metastasis in the advanced stage [52-56]. Due to its dual character, TGF is not considered as an anticancer target traditionally. However, an increasing number of metastatic model studies of CRC and other cancers clearly show that TGF signaling can reduce metastasis [57-58].

Regulation of TGF-β in Metastasis Colorectal Cancer (mCRC) Metastasis is the major cause of death in patients with colorectal cancer [59]. The most common sites of metastasis are the liver and the peritoneum. Metastatic colorectal cancer (mCRC) is a heterogeneous disease with diverse clinical responses and poor prognosis [60]. The tumor metastasis is a complex biological process. It is usually termed the invasion-metastasis cascade and consists of five steps [61]: local invasion of tumor cells into surrounding matrix, intravasation of tumor cells into circulatory system, systemic transportation of tumor cells, extravasation of tumor cells into parenchyma of distant tissue sites and colonization of distant organs, and establishment of macroscopic tumors [62]. The invasion-metastasis cascade of mCRC starts from adverse mutations of intestinal stem cells, which depends on a series of genetic events. The epithelium of the normal colon is constantly renewed. At the base of crypts, a pool of rapidly diving intestinal stem cells (ISCs) keeps the homoeostatic regeneration of the epithelium. The high division rate of ISCs will increase the probability of mutations during DNA replication [63]. The genetic instability of CRC increases with time and it can be classified into chromosomal instability, microsatellite instability (MSI) and CpG methylation phenotypes [64]. The first type is characterized by the activation of the K-RAS oncogene. Inactivation of the tumor suppressor gene APC or TP53 and the loss of heterozygosity of the long arm of chromosome 18 can activate this event. The key tumor suppressors on the long arm of chromosome 18 are SMAD2, SMAD4 and DDC [65]. In detail, APC will trigger the constitutive activation of WNT signaling and imposes a continuous stemlike self - renewing state, thus lead to benign outgrowths of the epithelium known as adenomas [66]. The above genetic alternation will make a small number of adenomas gradually become invasive by acquiring additional driver mutations. It mainly affects the other three downstream signal pathways [67-69]: MAPK pathway (providing cell autonomous mitogenic and survival stimulation to cancer cells), p53 pathway (facilitating acquisition of genomic instability) and TGF-β pathway. Loss of function mutations in TGF-βRII, SMAD4, SMAD2 or SMAD3 are key genetic mutation events that lead to the silencing of TGF-β pathway and make it unable to play a tumor suppressive role. An international expert consortium proposed four consensus molecular subtypes (CMS) of CRC based on several gene expression datasets [70]. It is the most robust classification system currently available for CRC [71,72]. CMS1 (MSI-immune, 14 %) includes almost all hypermutated MSI cancers. The MSS cancers subcategorised into three groups of CMS2 (canonical, 37 %), CMS3 (metabolic, 13 %) and CMS4 (mesenchymal, 23 %), with a residual unclassified group (mixed features, 13 %). CMS4 has a mesenchymal phenotype with transforming growth factor-β activation and a high rate of stromal and immune cell infiltration [73,74]. As mentioned above, SMAD4 and TGF-βRII mutations can silence the TGF-β pathway, which loss the ability of inhibiting the growth of normal epithelial cells, so as to promote tumorigenesis.

4. SMAD and Non-SMAD pathway in CRC Progression

Drosophila protein, mothers against decapentaplegic homolog 4 (SMAD4) as an essential mediator in the TGFβ signaling pathway, located on chromosome 18q21[75]. SMAD4 mutations that lead to decreased SMAD4 protein expression have been reported to occur in approximately 5.0–24.2 % of patients with CRC [76-78]. LOH is the main cause of SMAD4 mutation in CRC, but other mechanisms may also lead to SMAD4 mutation in post-transcriptional and post-translational regulation, including ubiquitination, sumoylation and mircoRNA interference [79,80]. Previously, we mentioned that SMAD4 directly regulates target genes by forming trimers with R-SMAD components as transcription factors. Additionally, R-SMAD-SMAD4 complexes can also associate with DNA-binding partners to act as a transcription co-factor.

SMAD signaling plays a crucial role in differentiation of epithelial cells, angiogenesis in the tumor microenvironment, chemoresistance, tumor invasion and metastasisand [81]. The mutations and deletions of SMAD4 mainly influence four downstream pathways. (1) Loss of SMAD4 promotes β-catenin expression and Wnt activation in the intestinal epithelium, which triggers the acquisition of stem cell properties, thus leading to de-differentiation and rapid adenoma formation in the differentiated intestinal epithelium of the Cre-driven conditional mouse model [82,83]. (2) Disruption of SMAD4 can upregulate the VEGFs to promote angiogenesis and lymphangiogenesis in CRC [84,85]. (3) SMAD4 deficiency activates PI3K/Akt/cell-division cycle 2 (CDC2)/survivin pathway to attenuate G1/2 cell cycle arrest, resulting in resistance to 5-FU-based chemotherapy [86,87]. (4) Loss of SMAD4 also activate the upregulation of CCL15-CCR1 and CXCL1/8-CXCR2, which causes both cancer cell phenotype and tumor microenvironment more aggressive [88-90]. Besides, loss of Smad4 usually leads to aberrant activation of STAT3, which is linked to upregulation of ZEB1 expression, reduced E-cadherin, and enhanced N-cadherin and vimentin expression [91]. TGF-β type II receptor (TGF-βRII) is the receptor that TGF-β binds directly, and thus it serves as a gatekeeper for the activation of downstream signaling. TGF-βRII mutations are one of the most common alterations in MSI CRC [92,93]. It is estimated to occur in approximately 30% of colorectal carcinomas [94,95]. Almost 80-90% of colorectal tumors with MSI have TGF-βRII mutations [96]. Non-SMAD signaling pathways also modulate key cellular process in CRC progression and metastasis. The mutations of SMAD4 can not only block the typical TGF- β Pathway, which can also promote colorectal cancer metastasis through Non-SMAD pathway, such as activating Rho/ROCK [97,98], MEK/ERK [99], Ras-MAPK and PI3K-AKT [100,101] pathway, leading to EMT, migration and invasion.

5. Diagnosis and Therapies

5.1. Extracellular Vesicles in CRC Diagnosis

Liquid biopsy is an innovative, promising noninvasive method which enables real-time monitoring of tumor's genetic heterogeneity [102]. In the clinical application of colorectal cancer, it can detect the disease early, assist in staging, monitor the treatment response, and predict relapse and metastasis [103]. Exosomes as biomarkers of liquid biopsies in CRC, participate in growth and metastasis of tumors by regulating the immune response, blocking the EMT, and promoting angiogenesis [104,105]. Exosomes are small extracellular vesicles with sizes between 30-150 nm that mediate intercellular communication. They contain different molecules, such as DNA, RNA, lipid, and protein. They have been proved to play a potential role in regulating different signaling pathways in CRC, including Wnt/β-catenin [106,107], KRAS [108], ERK [109], NF-ϰB [110.111], TGF-β [112-114], PI3K/AKT/mTOR [115], etc. Cancer Exosomes can reprogram fibroblasts through TGF - β on the surface of extracellular vesicles inducing SMAD signaling pathway 116]. Shelke investigated that exosome released by human mast cells can promote human mesenchymal stem cells migration and phenotypic changes via endosomal TGFβ-1 signaling [11,12]. Demonstrated that bladder cancer cells trigger the differentiation of fibroblasts to cancer-associated fibroblasts by exosomes-mediated TGFβ transfer and SMAD pathway activation [113]. In addition, protumor genic TGF-β inside the vesicle contributes 53.4% to 86.3% of the total TGFβ present in the cancer cell supernatant [110]. Anquan Shang et al. found that the circPACRGL enhanced CRC cell proliferation, migration and invasion, as well as differentiation of N1 to N2 neutrophils via miR-142-3p/miR-506-3p-TGF-β1 axis [110]. Extracellular vesicles are also related to TGF-βRII mutations, which are the one of the most common genetic alternation microsatellite instable CRC. Fricke found that TGF-βRII deficiency caused upregulation of several extracellular vesicles' proteins related to the extracellular matrix and nucleosome as well as downregulation of proteasome-associated proteins [101]. Meanwhile, it is worth noting that due to TGF-β is a highly context-dependent tumor suppressor, so it may be challenging to apply extracellular vesicles as a general prognostic tumor marker [112,114]. Extracellular vesicles can also be used as natural delivery vehicles to transport therapeutic drugs, antibodies or RNA to modify gene expression [104]. Compared with other nano drug delivery systems such as liposomes or polymeric nanoparticles, EV has biocompatibility and biodegradability, so it has lower toxicity and immunogenicity [105]. Collectively, extracellular vesicles represented by exosomes is considered to be a promising strategy in cancer diagnosis and therapy [26,27].

5.2. Targeted Anti TGF-β and Nanoparticles Therapies of CRC

The four therapies to inhibiting TGF-β or its pathway components include antisense oligonucleotides (ASOs), anti-integrins, ligand traps, and kinase inhibitors [28-30]. Antisense oligonucleotides represent a new and highly promising class of drugs for personalized medicine [113]. ASOs are single-stranded synthetic RNA or DNA molecules with a mean length of 12 to 25 nucleotides that downregulate TGF-β synthesis by targeting and interfering with mRNA function. Their sequences complement the target to ensure specificity. Trabederson AP 12009 is a phosphorothioate oligodeoxynucleotide designed to be complimentary to TGF-β2 ligand mRNA. It has successfully completed the phase IIB study of high-grade gliomas and demonstrated its good tolerance [112]. The overall survival time of patients treated is longer compared with historical cohorts. Currently, a large phase III trial for AP 12009 in high-grade gliomas has been initiated, and more phase I trials have been initiated in melanoma, pancreatic, and colorectal carcinomas [113,110]. Integrins promote cell adhesion and migration while controlling local activation of latent TGF-β contained in extracellular matrix or cell-surface reservoirs [35,36]. The expression of integrins is related to the increased availability of activated TGF and consequent increase of EMT, migration and invasion of many cancers cell lines in vitro [38-40]. Integrin antagonists currently being studied in clinical trials include RGD peptide mimetics and monoclonal antibodies (mAb). Bevacizumab

(Avastin, LM609, Genentech) is a mouse anti-human integrin αvβ3 and anti-VEGF mAb [113]. Avastin shows good efficacy and tolerance in all of the clinical trials and thus has been approved by the U.S. Food and Drug Administration (FDA) as a first-line treatment for metastatic colorectal cancer as part of a combination chemotherapy scheme. In addition, there are other antibodies, such as Intetumumab (CNTO 95, Centocor), Abciximab (c7E3), Vitaxin (MEDI-532) and Volociximab (M200), have been put into clinical trials and therapies [98]. Synthetic peptides mimic the organization of the natural ligands of integrin. The drugs for the trial period include Cilengitide (EMD 12), ATN-161, HM-3 and AP25. In order to prevent active cytokines from binding to surface receptors to inhibit the activation of TGF-β signaling pathway, two methods have been developed, namely, administration of antibodies against ligand or its receptors and the use of soluble TGF-β receptors II/III or receptors fused to immunoglobulins as ligand sequesters [95]. For instance, Dalantercept (ACE-04), a chimeric protein that acts as a ligand trap and impairs receptor activation, has been shown to inhibit angiogenesis in mouse models of renal cell carcinoma by preventing the TGF-β superfamily proteins, BMP9 and BMP10 from binding to the activin-receptor-like kinase, which is expressed on actively proliferating endothelial cells [67,76]. Mice models treated with the TGF-βRII/III showed a reduced number of metastases in different organs.

Another therapeutic method intends to inhibit the kinase activity of the TGF-β receptors, thereby arresting downstream SMAD and non-SMAD signaling pathway. Galunisertib (LY2157299), the first small molecule TGF-β kinase inhibitor that has been studied in clinical trials, can inhibit TGF-βRI kinase activity and blocks SMAD2 phosphorylation, arresting the activation of the SMAD pathway [100,101]. LY2109761, a dual inhibitor of TGF-βRI/II decreases liver metastasis in mouse models of colorectal cancer [96,98]. IN1130 is a small molecule that blocks the TGF-β/SMAD signaling pathway, influencing cell migration, invasion, EMT and lung metastasis in breast cancer models [50]. Anti TGF-β cancer vaccines can transfect TGF-β antisense molecules into cancer cell lines to reverse the immune suppression effect in host cells and improve anti-tumor immunity. Belagenpumatucel-L is an allogenic TGF-β2 antisense gene-modified vaccine that has been tested in non-small cell lung cancer patients, and the results have no significant difference compared with those who received the placebo in phase III clinical trials [42]. Anti PD-1/PD-L1 interaction is a new line of research for treatment of CRC patients. Increasing literatures recognize the importance of anti-PD-1 in the treatment of MSI tumors in CRC subtypes [76]. Bintrafusp Alfa consists of an IgG1 targeted PD-L1 part that is fused to the extracellular domain of two TGF-βRII molecules through peptide linkers to capture TGF-β in TME. This drug can bring TGF-β trap to TME through its anti-PD-L1 component, so as to attack immunosuppressive PD-L1 and ABC entities at the same time [77]. In a clinical trial, Bintrafusp Alfa showed similar safety to anti-PD-1/PD-L1 monotherapies [79]. The primary tumor of 19 subject patients included pancreatic cancer, cervical cancer, colorectal cancer, etc. Targeted nano-drug delivery to the colon is advantageous for CRC because nanoparticles can accumulate in diseased parts, improve the efficacies of therapeutics, and enable localized treatments, which reduces systemic toxicity [80]. For instance, degradable polyelectrolyte multilayers capsules can be enzymatically degraded upon cell interaction under physiological conditions, and TGF-β inhibitors can be incorporated inside them. This novel carrier can maximize drug administration and improve antimetastatic activity of TGF-β inhibitors in Hepatocellular Carcinoma [88]. Clinical therapeutic effect of similar nano carrier in CRC is also worth waiting in hope. It cannot be ignored that some nanoparticles in daily life may promote the occurrence and development of cancer [89]. Found that titanium dioxide nanoparticles (nano-TiO2), a common food additive, is able to activate TGF - β/MAPK and Wnt pathways, thus driving the EMT process [99].

6. Conclusion

TGF-β signaling plays a key role in CRC progression, enabling cancer cells to escape immune surveillance, proliferation, invasion and metastasis [34,80]. Targeted anti TGF-β therapies have shown promise in extensive clinical trials, but it is undeniable that these therapies still face some difficulties that need to be overcome. Collectively, an excellent anti TGF-β therapies should consider the following four aspects: the dual roles of TGF-β signaling in cancer metastasis, dynamic signaling, functional differences of TGF-β free in solution vs in exosomes, and the regulatory effects of TME [83]. Similarly, nano drug delivery system has a lot to improve, including distinguishing disease sites from healthy tissues, targeting specific cells, and releasing an on-demand dose of therapeutic agents [87]. Liquid biopsies are a potential method of CRC diagnosis, for the real-time examination of tumor clonal development, medication response and acquired resistance. As a potential source of liquid biopsy, extracellular vesicles become the focus of research. Nowadays, the transformation research of extracellular vesicles should focus on the standardization of its classification and detection technology, and further implement a large number of clinical trials to verify its effectiveness. At last, with the further exploration and transformation of TGF-β signaling pathway, as well as the development of tumor diagnosis technology and targeted drug therapy, it is expected to formulate a more personalized and precise treatment plan for each CRC patient.

7. Funding

This work was supported by the grant from National Natural Science Foundation of China (82170395 to DJL).

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