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Mini-review

TGF-beta signalling in colon carcinogenesis

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ABSTRACT

Colorectal cancer remains the most common cancer and the second leading cause of cancer mortality in Europe. There are a number of pathways that have been implicated in colorectal carcinogenesis, including TGF-beta (TGF-β)/Smad signalling pathway. The TGF-β pathway is involved in several biological processes, including cell proliferation, differentiation, migration and apoptosis. Here we review the role of TGF-β signalling cascade in colorectal carcinogenesis and provide some new molecular insights that may aid efforts towards targeted antitumor therapies.

1. Introduction

Colorectar cancer (CRC) is a common and fatal disease. It is the second leading cause of cancer-related mortality in Western countries [1]. CRC is an ideal model of research of the molecular pathogenesis of cancer, due to the ease of obtaining biopsy material and the understanding of the development of invasive carcinoma, from normal epithelium to polyps, and carcinoma. Lately, it has become appreciated that CRC develops by genetic alterations. Mutations on basic genetic material constitute the base of hereditary syndromes, while sporadic cancer derives from somatic mutations. There are a number of pathways that have been implicated in colorectal carcinogenesis, including chromosomal instability pathway, microsatellite instability pathway, serrated pathway, epigenetic mechanisms of colon carcinogenesis, different genes related to invasion and metastasis, and finally transforming growth factor-β (TGF-β)/Smad signalling pathway.

2. TGF-β signalling pathway

TGF-βs are 25-kDa cytokines that play a unique and pivotal role in homeostasis, wound healing, fibrosis, angiogenesis, carcinogenesis and differentiation of the cell [2,3]. TGF-β erroneously obtained its name from its ability to stimulate fibroblast growth in soft agar [4]; however it became apparent that its name was not only misleading but also restrictive, as it is a potent inhibitor of epithelial cell proliferation, and has a multifunctional biological activity [3,5]. There are five isoforms of TGF-βs, namely TGF-β1, TGF-β2, TGF-β3 ligands in mammals, and TGF-β4, TGF-β5, which belong to a large superfamily including activins, inhibins, bone morphogenetic proteins (BMPs), and myostatin among others [5]. Each TGF-β molecule derives from its precursor, a large propeptide, in addition to the TGF-β. Each of the TGF-βs is encoded by different genes,

Abbreviations: CRC, colorectal cancer; TGF-β, transforming growth factor-β; BMP, bone morphogenetic proteins; LTBPs, latent TGF-β binding proteins; CDK, cyclin-dependent kinase; rhTGF-β, recombinant human TGF-β; NO, nitric oxide; Ach, acetylcholine; MSI, microsatellite instability; DPC, deleted in pancreatic cancer; EMT, epithelial to mesenchymal transition.

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although they act through the same receptor signalling cascade. It is stored in the extracellular matrix, attached to latent TGF-β binding proteins (LTBPs). This attachment prevents the binding of the molecule to its receptor [6]. Signalling from TGF-β1, through its receptor, which is a transmembrane serine-threonine kinase, plays an important but ambiguous role in carcinogenesis.

TGF-β1 interacts with TGF-βRII (type II receptor), which in turn attracts and activates TGF-βRI (type I receptor). Smad 2 and Smad 3 are phosphorylated at the carboxyl-terminal serines by the activated TGF-βRI receptor and form heteromeric complexes with Smad 4. Ultimately, Smad 2/3/4 complex translocates into the nucleus and binds to specific regulatory elements on target genes [7,8]. Smad 4 can translocate into the nucleus only when complexed with the R-Smads, whereas Smad 2 and Smad 3 can do so in a Smad 4-independent manner [9]. This implies that Smad 4 has a regulatory role rather than a simple signal transmission from the cytoplasm to the nucleus (Fig. 1). Smad 2/3/4 complex once in the nucleus, induces among others, a cyclin-dependent kinase (CDK) inhibitor p21, leading to growth arrest.

Protein p21 is the product of *waf/cip1* gene, an inhibitor of CDK, and is a powerful and reversible inhibitor of the propagation of cell cycle at G1 and G2 activated upon DNA damage. The p21 protein interacts with complexes of CDK2 and cyclin A or cyclin E and thereby inhibits CDK2 activity, preventing progression of the cell cycle [10]. Furthermore, it has been shown that treatment with TGF-β stimulates p21 at the transcriptional level through a p53-independent pathway as well as at the posttranslational level. Posttranslational control is dependent on treatment with TGF-β in early G1 phase; whereas TGF-β mediated transcriptional control does not appear to be a function of the time of TGF-β treatment during cell cycle progression [11].

3. TGF-β and its role in colon carcinogenesis

TGF-β and its signalling effectors influence cancer biological behavior. The TGF-β signalling pathway has been considered as both a tumor suppressor and a cancer promoter [12]. Many questions have been raised regarding the point at which this switch from tumor suppression to tumor propagation occurs. Moreover, what are the factors that influence this catastrophic change? TGF-β1 switches from an inhibitor of tumor cell growth to a stimulator of growth and invasion during human colon carcinoma progression. It has been observed that metastatic colon

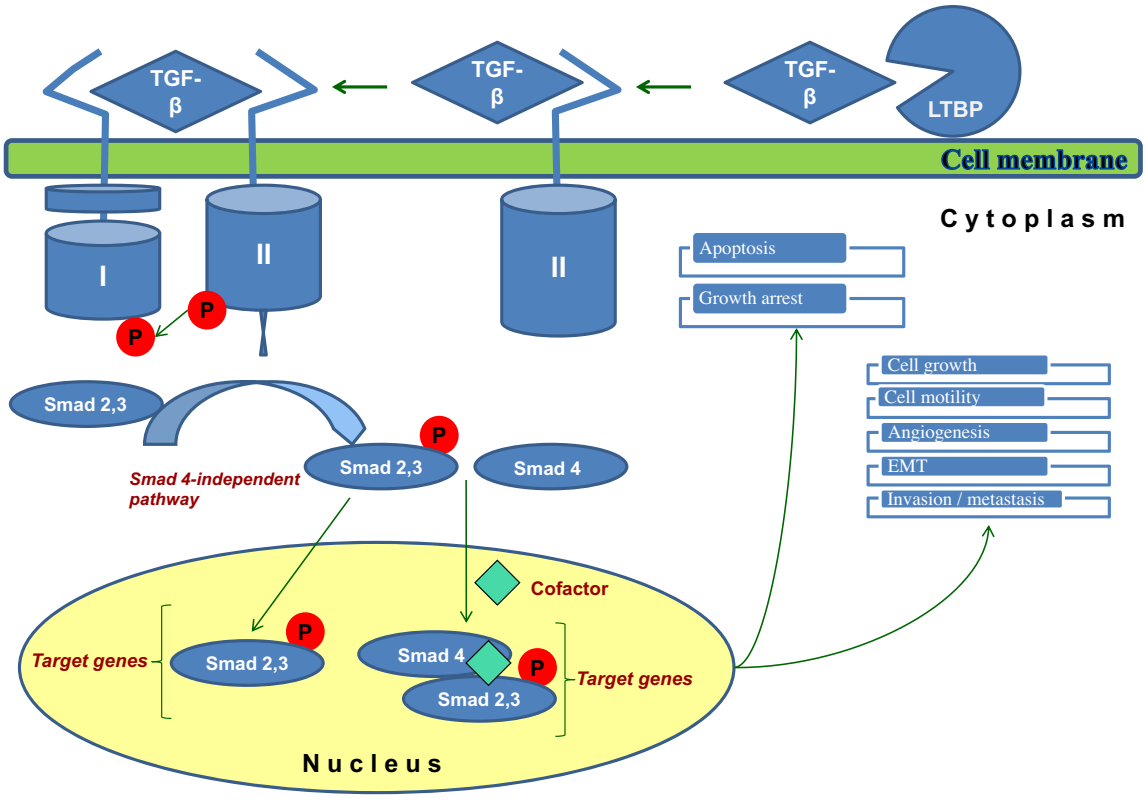

Fig. 1. Schematic diagram of TGF-β/Smad signalling pathway. Detachment of TGF-β from LTBP allows binding to TGF-β Receptor II, which in turn attracts and potentiates TGF-β Receptor I. Phosphorylation at the C-terminal serines of Smad 2 and Smad 3 by activated TGF-β Receptor I triggers complex formation with Smad 4, which accumulates in the nucleus. Phosphorylated Smad 2,3 may also enter the nucleus in a Smad 4-independent fashion. Once in the nucleus, the Smad complex binds to a cofactor and regulates expression of target genes engaged in both tumor promotion and tumor repression. Blockade of this signalling pathway at different levels provides an attractive strategy for the development of anticancer therapies. LTBP, latent TGF-β binding protein; EMT, epithelial to mesenchymal transition.

carcinoma cells responded to TGF-β by proliferation, whereas moderate to well-differentiated primary site colon carcinomas were growth inhibited by TGF-β [13,14]. Intense staining for TGF-β1 correlates significantly with disease progression to metastasis and is independent of nodal status and the degree of differentiation of the primary tumor. Patients with high TGF-β protein levels in their primary site CRC are more likely to experience recurrence of their disease than were patients whose tumors exhibited low levels of TGF-β [14]. Preoperative TGF-β level is a predictive factor for liver metastasis after curative resection, suggesting that high TGF-β-producing CRC cells may have a biological potential for liver metastasis. The postoperative level of the cytokine is highly predictive of liver metastasis after curative resection. Elevation of the postoperative levels suggests that preclinical liver metastasis might have occurred at surgery. A single point measurement of the plasma cytokine levels at 2 weeks after surgical removal of the primary tumor may be useful for predicting clinical recurrence due to liver metastasis. The value of a prospective trial of liver-targeted adjuvant therapy for patients with increased postoperative levels of the cytokine, to prevent the development of liver metastasis, might be essential [15]. In another study it was shown that there was a correlation between Duke's stage and plasma concentration of active TGF-β and that after curative resection the active TGF-β levels from patients recovering from curative resection are similar to those of healthy individuals. These results suggest that active TGF-β can be used as a tumor marker for CRC [16].

In the past years, it has become clear that TGF-β plays a role in the regulation of neurotransmission, which extends beyond its well-established role as neurotrophic and neuroprotective factors [17]. Recently, it was shown that there is a close correlation of TGF-β with the nervous system by using recombinant human TGF-β (rhTGF-β) [18]. It was proved that addition of rhTGF-β in human colon adenocarcinoma cells decreases nitric oxide (NO) secretion, an effect which also seen after the application of the neurotransmitter acetylcholine (Ach). These results suggest that TGF-β could increase colon tumor progression through a new mechanism of neuromodulation.

TGF-β has also been reported to play an important role in carcinogenesis through immune suppression. TGF-β has been found to affect multiple components of the immune system. TGF-β inhibits the function of natural killer cells, CD8+ cytotoxic T lymphocytes, B-cell proliferation and the secretion of IgA [19]. This property of TGF-β, however, is accomplished without compromising immune responses to pathogens. Furthermore, TGF-β induces infiltration and polarization of infiltrating immune cells at the tumor–host interface [20]. TGF-β expression strongly correlates with the level of expression of immune CD10+ cells at the tumor invasion front, which represents an independent prognostic biomarker in stages I–III CRC [21].

4. TGF-β receptor II mutations in colon carcinogenesis

The overall incidence of TGF-βRII mutations is close to 30% in CRC and is the most common mechanism identified so far, that results in TGF-β signalling alteration [22]. TGF-βRII frame shift mutations are found in approximately 80% of MSI + colon cancers [23]. Mutation of TGF-βRII in MSI + colon cancer may inactivate tumor suppressor activity of TGF-β. Moreover, restoration of TGF-βRII expression resulted in suppression of the tumorigenicity, thus demonstrating that *TGF-βRII* gene is in fact a tumor suppressor gene [24]. Transfection of SW48 colon cancer cells with TGF-βRII causes growth inhibition and a reduction in malignant properties. Thus, RII is a tumor suppressor protein that is required for TGF-β induced growth inhibition [25]. However, it has been shown that TGF-βRII activity can be bypassed and thus TGF-βRII mutations in RER + cancers may be just "bystander" events [26]. Additionally, TGF-βRII mutation is a late event in MSI adenomas and correlates tightly with progression of these adenomas to cancer, and these mutations have no role in the early events of carcinogenesis [27,28]. So, what is the true significance of TGF-βRII mutations in colon cancer? Is it a coincidence? Lately, it was shown with the use of defined cell line system that both genomic instability and clonal selection of TGF-β resistant cells contribute to the high frequency of TGF-βRII mutations in MSI colon cancer [29].

TGF-βRII mutations grant a growth advantage for RER + cancers of both the upper and lower gastrointestinal tract, even though this finding does not apply to other cancers such as endometrial tumors [30]. Moreover, TGF-βRII mutations are more prevalent in proximal colon than in distal rectosigmoid cancer [31]. This finding suggests that either the gene encoding TGF-βRII has no role in the carcinogenesis of the rectosigmoid region, or that another mutational abnormality downstream of the TGF-β signalling cascade acquires responsibility for tumorigenesis in the rectosigmoid locus.

With a new in vivo model system, it was demonstrated that loss of TGF-βRII contributes to colon cancer formation and metastasis by cooperation with a mutant Kras through a beta-catenin-independent pathway [32]. Furthermore, it was shown that this effect is autonomous and is unrelated to impaired TGF-β signalling regulation of T or stromal cells.

5. TGF-β receptor I and colon cancer

Given the fact that both TGF-β RI and RII are equally essential for TGF-β signal transduction, it can be predicted that mutations in either gene might yield equivalent functional results. Recently, a TGF-βRI polymorphic allele has been described that has a three alanine deletion from a nine alanine stretch, in the N-terminus. Pasche et al. confirmed that TGF-βRI (6A) homozygosity is associated with cancer. A predisposition to cancer, predominantly of the colon, was observed both in TGF-βRI (6A) homozygotes and in TGF-βRI (6A) heterozygotes. Hence, TGF-βRI (6A) acts as a tumor susceptibility allele that may contribute to the development of cancer, especially colon cancer, by means of reduced TGF-β mediated growth inhibition [33]. Overall, TGF-βRI (6A) carriers have a 26% increased risk for cancer, while TGF-βRI (6A) homozygotes have twice that risk. Kaklamani et al. in a meta-analysis from seven case-control studies concluded that carries from the US

are at increased risk for CRC but not carries from the Southern Europe [34]. Same findings were reported from Skoglund et al. in a meta-analysis: no evidence that homozygosity, heterozygosity or carrier status for the TGF-βR1∗6A allele confers an increased risk of CRC [35]. Therefore, the question still remains, is TGF-βRI (6A) associated with increased risk of cancer? The answer comes from Zhang et al., who state in their meta-analysis including 13,113 individuals that TGF-βR1∗6A is associated with increased cancer risk [36].

6. The role of Smads in CRC

Signal impairment of TGF-β pathway can also take place by way of Smad mutations. *Smad 4* was originally described as tumor suppressor gene deleted in pancreatic cancer (DPC 4), and is located at chromosome 18q21.1 [37]. *Smad 4* gene mutation plays a significant role in colon carcinogenesis. Loss of Smad activation and/or expression occurs in approximately 10% of CRCs. This subset has a poor prognosis because of its association with advanced disease and the presence of lymph node metastases at diagnosis [38]. Loss of Smad 4 function occurs at later stages of malignancy, playing a role in the acquisition of advanced phenotypes. The frequency of mutational events of *Smad 4* gene increases with the progression of carcinogenesis, being 0% in adenomas, 10% in intramucosal carcinomas, 7% in invasive carcinomas without distant metastasis, 35% in primary invasive carcinomas with distant metastasis, and 31% in carcinomas metastasized to the liver and distant lymph nodes, or disseminated [39]. Smad 4 mutations have also been shown to be associated with the occurrence of juvenile polyposis, a syndrome that predisposes to hamartomatous polyps and CRC [40,41]. In addition, some other studies have reported frequent somatic mutations of DPC4 in human colorectal tumors, suggesting an important role of Smad 4 in colorectal carcinogenesis [42,43]. Furthermore, PcDNA3.1-DPC4 plasmid transfection results in steady expression of Smad 4, suggesting that DPC4 inhibits cell growth, not only by TGF-β signal transduction pathway, but also by other cascades [44]. Some investigators have revealed that overexpression of wild-type DPC4 may re-construct a new pathway of negative regulation of cell proliferation other than TGF-β signal transduction cascade [45]. In Smad 4 null (MC38 and SW620) cell lines, TGF-β induced invasion, migration, tumorigenicity and potentiality for metastasis, while Incubation with LYLY2109761 (a potent TGF-β-receptor kinase inhibitor) reversed these effects, suggesting that loss of Smad 4 might underlie the functional shift of TGF-β from a tumor suppressor to a tumor promoter [46]. An adaptor protein, ELF (β spectrin), from stem cells is committed to gut lineage. ELF activates and modulates Smad 4 activation to confer cell polarity, to maintain cell architecture, and to inhibit epithelial-to-mesenchymal transition. There is an indication that by modulating Smad 4, ELF has a key role in TGF-β signalling in the suppression of nonmetastatic colon cancer [47].

As aforementioned, an activated TGF-β receptor phosphorylates Smad 2 and Smad 3, which then complex with Smad 4 and translocate to the nucleus. It has been shown that Smad 4 is not required for nuclear translocation of Smad 2 and Smad 3, but is needed for activation of at least certain transcriptional responses [48]. Previous data show that mutations in Smad 2 are specifically associated with sporadic colorectal carcinoma and suggest that Smad 2 is a candidate tumor suppressor [49]. Regardless of the mutational mechanism, mutated Smad 2 protein allows an escaped TGF-β pathway mechanism to take place, and deprive cells from the antiproliferative effect of TGF-β, leading to cancer. Smad 4 mutations take place in advanced stage cancers (lymph node involvement and metastasis) whereas Smad 2 mutation occurs in early stages of cancer [50]. Smad 3 is another potent tumor suppressor for colonic epithelium. Animal studies demonstrated that inbred Smad 3 homozygous mutants develop colon adenocarcinoma in multiple stages [51,52]. Interestingly enough, to date, Smad 3 mutations have not been detected in human CRC. A logical explanation for this might be the fact that human tumor studies are insufficiently sensitive to detect a broad spectrum of inactivating mutations.

7. Regimens targeted against TGF-β signalling pathway

Signalling pathway alterations are responsible for CRC development and progression. TGF-β enhances angiogenesis, suppress immune surveillance, promote epithelial to mesenchymal transition (EMT), and directly influence the expression of metastasis genes, properties which makes it a pro-metastatic cytokine [53]. Therefore, an attractive therapeutic strategy could be to interrupt the tumor promoter properties of the TGF-β signalling, without altering its physiologic tumor suppressor actions exhibited in early stages of tumorigenesis.

Various classes of TGF-β antagonists are under development, such as TGF-β neutralizing antibodies, soluble TGF-βR:Fc fusion proteins, antisense TGF-β oligonucleotides, and inhibitors of TGF-β receptors (see Table 1). The antibody 2G7 neutralizes TGF-β1 -β2, and -β3, and the MDA-231 human breast cancer cell line. Intraperitoneal injections of 2G7 starting 1 d after intraperitoneal inoculation of tumor cells suppressed intraabdominal tumor formation and lung metastases [54]. These effects act primarily through natural killer (NK) cell activity, as 2G7 does not stimulate NK cell-mediated cytotoxicity in beige NK-deficient nude mice. 1D11 is another pan-TGF-β antibody which when administered to immunocompetent mice bearing subcutaneous GL261 tumors resulted in complete remission, but failed to arrest growth in immune deficient mice. Treatment with 1D11, however, to intracranially implanted gliomas in immunocompetent mice showed no reduction of the tumor, but reduced invasion to normal adjacent tissue [55]. GC1008 is a pan-neutralizing IgG4 human antibody directed against all three isoforms of TGF-β. Having been encouraged by preclinical data, Genzyme Inc studies GC1008 in a Phase I/II dose-escalation study in patients with advanced metastatic melanoma or renal cell carcinoma (Genzyme, Press release).

Soluble sTGF-βRII:Fc is a chimeric protein composed of the extracellular domain of the TGF-βRII and the Fc portion

Table 1
Regimens against TGF-β signalling pathway.

Agents	Drug class	Source	Clinical phase	Refs.
TGF-β neutralizing antibodies				
2G7	Pan TGF-β	Genentech	Phase III	[54]
1D11	Pan TGF-β	Genzyme/CAT	Preclinical	[55]
GC1008	Pan TGF-β	Genzyme/CAT	Phase I/II	Genzyme
Soluble TGF-βoligonucleotides				
sTβRII:Fc	TGF-βRs	Biogen	Preclinical	[56–58]
Betaglycan	TGF-βRIII		Preclinical	[59–61]
Antisense TGF-β oligonucleotides				
AP 12009	mRNA TGF-β2	Antisense Pharma	Phase III	[62]
AP 11014	mRNA TGF-β1	Antisense Pharma	Preclinical	[63]
AP 15012	Oligonucleotide	Antisense Pharma	Early stage	Antisense Pharma
Inhibitors of TGF-β receptors				
LY 364947	TGFβRI,RII kinase	Eli Lilly Research	Preclinical	[64]
LY 2109761	TGFβRI,RII kinase	Eli Lilly Research	Preclinical	[65,66]
LY 573636	TGFβRI kinase	Eli Lilly Research	Phase II	[67]

of the murine IgG1 heavy chain (Fc:TβRII). This ligand trap interferes with TGF-β receptors and in vivo blocks TGF-β induced fibrosis [56]. Systemic administration of Fc:TβRII on models of breast cancer metastases increased apoptosis in primary tumors expressing MMTV-PyV mT, while reducing tumor cell motility, intravasation, and lung metastases [57]. Administration of the kinase inhibitor for 12 weeks in mice showed no obvious toxicity, a result of paramount importance. Another study showed that sTGF-βRII has the potential to be a potent suppressor of PANC-1-derived tumor growth and metastasis [58]. Betaglycan a type III class of TGF-β receptors is a transmembrane glycoprotein with large extracellular regions and small cytoplasmic regions [59]. Apart from Fc:TβRII, sTGF-βRIII has been used in preclinical trials, as a therapeutic mean against cancer, demonstrating its inhibitory effects. Systemic continuous or bolus sTGF-βRIII administration can inhibit human prostate cancer DU145 xenograft growth and angiogenesis, probably due to the attenuation of TGFbeta-induced MMP-9 expression [60]. Moreover, use of sTGF-βRIII inhibits tumor growth formed by human colon carcinoma HCT116 as well as tumor growth and metastatic potential of breast carcinoma MDA-MB-435 cells in nude mice [61]. This action seems to be partly due to inhibition on angiogenesis.

Using antisense technology, targeting mRNA sequence specific degradation, Antisense Pharma has developed two antisense oligonucleotides (AP-12009, AP-11014), specific for TGF-β2 and TGF-β1 mRNA, respectively [62,63]. AP-12009 has been reported to be a promising therapy approach for malignant gliomas, as in Phase I/II trials showed prolonged survival over standard therapy, and even complete long lasting remission in two patients [62]. Currently, AP-12009 is under Phase III trial for high grade gliomas, Phase II trial for pancreatic and malignant melanoma, Phase I trial for CRC and in preclinical stage for lung prostate and renal carcinomas (Antisense Pharma reports). AP-11014, on the other hand, is under preclinical investigation for the treatment of non-small cell lung carcinoma, colorectal and prostate cancers. Last, a third antisense drug, AP-15012, also developed by Antisense Pharma, is under investigation for malignant melanomas.

Other small molecules which inhibit TGF-β signalling cascade at the kinase domain receptor I level have been developed with promising results. Two small molecules namely, LY-364947 and LY-2109761 are able to block both kinase receptors I and II, thus having the advantage of being metabolically stable and being attractive molecules for in vivo studies [64,65]. In a recent study, the dual kinase inhibitor LY-2109761 was shown to be able to inhibit TGF-β signalling pathway in a canonical and non-canonical way in CT26 colon adenocarcinoma cells having K-Ras mutation [66]. In addition, LY-2109761 attenuates cell migration, invasion and tumorigenicity of CT26 cells, decreases liver metastases and prolongs survival in a metastatic model. The only TGF-β receptor I kinase inhibitor used so far in a clinical trial setting is LY-573636, developed by Eli Lilly Research. This drug is used in a Phase II trial and administered as an intravenous infusion on Day 1 of a 28-Day cycle as a second-line treatment in patients with unresectable or metastatic melanoma [67].

It has become clear that new agents targeted against specific steps in the TGF-β signalling cascade have been developed in the last decade. Many of these have been entered in clinical trials with promising therapeutic results. Bearing in mind that TGF-β pathway has a dual role in homeostasis as both a tumor suppressor and a tumor promoter, caution must be given as to when, how and how much of anti-TGF therapy might be beneficial. Finally, effects of TGF-β signalling which drive tumor expansion need to be identified and provide researchers with new weapons against cancer.

8. Conclusions

There has been an increasing interest, lately, concerning the impact of TGF-β signalling pathway on colorectal carcinogenesis. It has been recognized that altered function of this pathway plays a crucial role on initiation, propagation and advance of CRC. In the era of signal transduction-based anticancer treatment, research efforts in this area might be helpful for future therapeutic strategies with regimens targeted against TGF-β signalling components.

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