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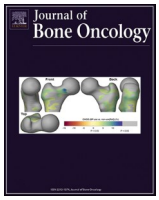

Targeting transforming growth factor beta signaling in metastatic osteosarcoma

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HIGHLIGHTS

- The transforming growth factor beta (TGF-β) signaling functions as a metastasis promoter by promoting tumor growth, inducing epithelial-mesenchymal transition (EMT), blocking antitumor immune responses, increasing tumor-associated fibrosis, and enhancing angiogenesis.
- TGF-β antagonists have been shown to have effects on osteosarcoma *in vitro* and *in vivo*.
- Several phase 1/2/3 clinical trials have shown TGF-β antagonists are safe and well tolerated.
- Clinical trials evaluating the effect of TGF-β antagonists on osteosarcoma are promising.

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ABSTRACT

Osteosarcoma is a rare type of bone cancer, and half of the cases affect children and adolescents younger than 20 years of age. Despite intensive efforts to improve both chemotherapeutics and surgical management, the clinical outcome for metastatic osteosarcoma remains poor. Transforming growth factor β (TGF-β) is one of the most abundant growth factors in bones. The TGF-β signaling pathway has complex and contradictory roles in the pathogenesis of human cancers. TGF-β is primarily a tumor suppressor that inhibits proliferation and induces apoptosis of premalignant epithelial cells. In the later stages of cancer progression, however, TGF-β functions as a metastasis promoter by promoting tumor growth, inducing epithelial-mesenchymal transition (EMT), blocking antitumor immune responses, increasing tumor-associated fibrosis, and enhancing angiogenesis. In contrast with the dual effects of TGF-β on carcinoma (epithelial origin) progression, TGF-β seems to mainly have a pro-tumoral effect on sarcomas including osteosarcoma (mesenchymal origin). Many drugs that target TGF-β signaling have been developed: neutralizing antibodies that prevent TGF-β binding to receptor complexes; ligand trap employing recombinant Fc-fusion proteins containing the soluble ectodomain of either type II (TβRII) or the type III receptor ((TβRIII), preventing TGF-β from binding to its receptors; antisense nucleotides that reduce TGF-β expression at the transcriptional/translational level; small molecule inhibitors of serine/threonine kinases of the type I receptor (TβRI) preventing downstream signaling; and vaccines that contain cell lines transfected with TβRII antisense genes, or target furin convertase, resulting in reduced TGF-β signaling.

TGF-β antagonists have been shown to have effects on osteosarcoma *in vitro* and *in vivo*. One of the small molecule TβRI inhibitors, Vactosertib, is currently undergoing a phase 1/2 clinical trial to evaluate its effect on osteosarcoma. Several phase 1/2/3 clinical trials have shown TGF-β antagonists are safe and well tolerated. For instance, Luspatercept, a TGF-β ligand trap, has been approved by the FDA for the treatment of anemia associated with myeloid dysplastic syndrome (MDS) with ring sideroblasts/mutated SF3B1 with acceptable safety. Clinical trials evaluating the long-term safety of Luspatercept are in process.

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1. Introduction

Sarcomas are a rare and heterogeneous group of malignant tumors of mesenchymal origin. Approximately 80 % of sarcomas originate from soft tissue, and the rest originate from bone. Osteosarcomas are characterized by the production of osteoid tissue or immature bone by malignant cells. Approximately 750 to 900 new cases are diagnosed each year in the United States, of which 400 arise in children and adolescents younger than 20 years of age [1–3]. Most osteosarcomas are high-grade tumors, located around anatomic regions of high growth rate. Despite intensive efforts to improve both chemotherapeutics and surgical management, osteosarcoma patients have a 40 % mortality rate. Specifically, the clinical outcome for metastatic osteosarcoma remains poor; less than 30 % of patients who exhibit metastases will survive five years after the initial diagnosis [1,4,5]. Treating metastatic osteosarcoma thus remains a challenge. The transforming growth factor (TGF-β) is one of the most abundant growth factors in the bone matrix. TGF-β functions as a metastasis promoter by promoting tumor growth, inducing epithelial-mesenchymal transition (EMT), blocking antitumor immune responses, increasing tumor-associated fibrosis, and enhancing angiogenesis [6–10]. Many studies have generated considerable enthusiasm for targeting the TGF-β signaling pathway as a novel therapy in osteosarcoma. In this review, we present the mechanism of targeting the TGF-β signaling pathway and the status of drug development of TGF-β signaling antagonists in osteosarcoma. See (Table 1).

2. Transforming growth factor beta signaling pathway

In the early 1980 s, TGF-β was biochemically isolated from tumor cells and named after its ability to transform normal rat kidney fibroblasts [11,12]. TGF-β is produced by many parenchymal cell types and is also produced or released by infiltrating cells such as lymphocytes, monocytes/macrophages, and platelets. TGF-β is found in all tissues, but is particularly abundant in bone, lung, kidney, and placental tissue [13,14].

TGF-β is a prototypic member of a transforming growth factor superfamily that includes three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3), activins, growth and differentiation factors, bone morphogenetic proteins, inhibins, nodal, and anti-mullerian hormone [13,15].

TGF- β is stored in the extracellular matrix (ECM) as a large latent complex of TGF-β, the latency associated peptide (LAP), and latent TGF-β-binding protein (LTBP) [13,16,17]. Two key mechanisms that mediate the activation of latent TGF-β have been uncovered: LAP portion of latent TGF-β was cleaved by ECM proteases such as matrix metalloproteases (MMPs) in cooperation with the tolloid-like family of proteases, and the integrin receptor complex, via its intracellular association with the actin cytoskeleton can exert a force that distorts the folded structure of LAP, enforcing a mechanical release of the mature ligand from the well-designed cage generated by the TGF-β prodomain [18–21] (Fig. 1). Mature TGF-β comprises highly conserved dimeric proteins with a molecular weight of approximately 25 kDa. TGFβ1 is the most abundant isoform in most cells and tissues.

The TGF-β signal is transduced by a pair of transmembrane serine/threonine kinase receptors, the TGF-β type II receptor (TβRII), and the TGF-β type I receptor (TβRI), expressed in all cell types. TβRI is also known as activin receptor-like kinase 5 (ALK-5) [22,23]. In endothelial cells, the TGF-β1/2/3 ligands can also engage with another type I receptor known as ALK-1 [24]. The TGF-β type III receptors (TβRIII) including endoglin and beta-glycan are transmembrane proteoglycans with no intrinsic signaling capacity which bind to all three TGF-β isoforms with high affinity and, a preference for TGF-β2. It is thought that the binding of TGF-β to the TβRIII increases the local concentration of ligands and enhances presentation of ligands to TβRII [25].

TβRI and TβRII both contain an extracellular ligand binding domain, a transmembrane serine-threonine domain, and a cytoplasmic serine-threonine kinase domain. Binding of biologically active TGF-β to TβRII homodimers results in the recruitment of two TβRI molecules into the heterotetrametric complexes. The TβRII kinase phosphorylates serine residues in the juxta membrane subdomain of TβRI, characterized by a short glycine and serine-rich motif (GS), and thus, activates the TβRI kinase [26–28] (Fig. 2).

In response to receptor activation, two cytosolic proteins, receptor associated SMADs (R-SMADs), SMAD2 and SMAD3, become transiently associated with and phosphorylated by the TβRI kinase at specific Ser residues on the Mad homology 2 (MH2) domain, allowing them to form heteromeric complexes with a third homologue, SMAD4 [29,30]. SMADs contain the Mad homology 1 (MH1) domain at their N-terminus and the MH2 domain at their C terminus, and a linker region [31,32]. These SMAD2/3/4 complexes are translocated from the cytoplasm to the nucleus and bind to DNA, which results in the transcriptional regulation of wide range target genes involved in cell differentiation, proliferation, apoptosis, migration, and extracellular matrix production such as p15, p21, parathyroid hormone–related peptide (PTHrP), and hundreds of other genes [33–35]. The endocytic protein Smad anchor for receptor activation (SARA) promotes the activation of R-SMADs as it is responsible for the recruitment of SMAD2 and SMAD3 to the TGF-β receptor. TGF-β signaling may be controlled by several inhibitory mechanisms. Among them, SMAD7 competes with R-SMADs to bind to activated TβRI and recruits E3-ubiquitin ligases to degrade the receptor. Additionally, SMAD7 may recruit protein phosphatases to dephosphorylate the receptor complex [36] (Fig. 3).

In addition to the canonical pathway, TGF-β can activate Smad-independent or non-canonical pathways. TβRI phosphorylates the adaptor protein Shc on tyrosine, which initiates a docking site for downstream signaling mediators, Grb2 and Sos, then activates the Ras GTPase, leading to the sequential activation of c-Raf, MEK (MAP kinase/ERK kinase) and ERK1/2 kinases [37–40]; TGF-β activates JNK and p38 via activation of MAP kinase kinases (MKKs)[41,42] and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling via the direct interaction of p85, the regulatory subunit of PI3K with the TGF-β receptor complex [43,44]; TGF-β promotes the phosphorylation of Janus kinase 2 (JAK2), which leads to the phosphorylation and activation of Signal transducer and activator of transcription 3 (STAT3) [45,46]; TGF-β activates Rho- and Rho-like GTPases, important regulators of cytoskeletal organization and cell motility and induces EMT [47,48].

TGF-β signaling outcomes also depend on the TGF-β pathway crosstalks with other signaling pathways such as FGF, Hippo, Indian Hedgehog/Parathyroid hormone-related protein (Ihh/PTHrP), Notch, PTH, and Wnt signaling pathways [13,49–61]. The function of TGF-β receptors and SMADs is modulated by other signaling effectors. Additionally, many SMAD-interacting transcription factors are also regulated by other signaling pathways, adding to the complex and integrated nature of the regulation of different cellular responses. For instance, Wnt signaling inhibits glycogen synthase kinase and stabilizes Smad proteins. In response to stimulation by Wnt, the canonical Wnt pathway and the Smad pathway can synergize and activate transcription of target genes. Smad3 facilitates β-catenin nuclear translocation and coordinates with the complex of β-catenin and T-cell factor or lymphoid enhancer–binding factor 1 at regulatory promoter sequences of target genes to regulate gene expression [49,60,62–66]. Similar scenarios promote the crosstalk of Smad signaling with Notch and Hedgehog signaling in the control of target gene expression [13,61,67–71].

Therefore, TGF-β plays key roles in many biological functions including embryonic development, angiogenesis, wound healing, hematopoiesis and immune function, as well as disease states, including cancer, chronic inflammation with fibrosis, and immune disorders [26,72].

3. Transforming growth factor beta signaling in bones

Bone is made up of an extracellular matrix surrounding osteoclasts, osteoblasts, osteocytes, and bone marrow stromal cells. The

Table 1
Examples of antagonists of TGF-β signaling used in current clinical trials as of 09/2023.

Agent	Drug target	Cancer Type	NCT Number
Neutralizing antibodies			
Fresolimumab (GC-1008)	pan-TGF-β neutralizing antibody	Mesothelioma	NCT01472731
		Non-small cell lung carcinoma	NCT01401062
		Renal cell carcinoma	NCT01112293
		Melanoma	NCT02581787
		Breast cancer	NCT01291784
			NCT00356460
			NCT00923169
JYB1907	monoclonal antibody targeting GARP-TGF-β1	Advanced solid tumor	NCT05821595
NIS793	human anti-TGF-β IgG2 monoclonal antibody	Advanced malignancy	NCT04390763
		Pancreatic adenocarcinoma	NCT04952753
		Colorectal cancer	NCT04935359
		MDS	NCT04810611
			NCT05417386
			NCT04097821
			NCT05546411
			NCT02947165
Y101D	recombinant Anti-PD-L1 and TGF-β Bispecific Antibody	Advanced solid tumor	NCT05028556
Ligand Trap/Soluble TβRII, TβRIII			
AK130	Fc-mutant anti-TIGIT antibody fused with TβRII protein	Advanced malignant tumor	NCT05653284
AVID200	fusing TβRII ectodomains to IgG Fc regions, TGFβ trap against TGFβ1 and β3	Myelofibrosis	NCT03895112
BCA 101	anti-Epidermal growth factor receptor (EGFR) IgG1 antibody linked to an extracellular domain of TβRII	EGFR driven advanced solid tumor	NCT04429542
BinTrafusp (M7824)	fusion protein with PDL1 antibody and the extracellular domain of TβRII	Advanced solid tumor	NCT04349280
		HPV Positive cancer	NCT05005429
		Head and Neck cancer	NCT05061823
		Biliary tract cancer	NCT04396886
		Esophageal cancer	NCT04246489
		Ovarian cancer	NCT04878250
		Breast Cancer	NCT04874311
		Mesothelioma	NCT05012098
		Brain metastasis	NCT03620201
		Small cell lung cancer	NCT04396535
		Prostate cancer	NCT04551950
			NCT04457778
			NCT05445882
			NCT03833661
			NCT04708470
			NCT03524170
			NCT04708067
			NCT04789668
			NCT05145569
			NCT04481256
			NCT04417660
			NCT04247282
			NCT04574583
			NCT04220775
			NCT03840915
			NCT04066491
			NCT03436563
			NCT04235777
			NCT04835896
			NCT03631706
			NCT04491955
			NCT02723955
			NCT02517398
BJ-005	anti-PD-L1 IgG1 monoclonal antibody fused with the extracellular domain of human TβRII	Advanced solid tumor Lymphoma	NCT05115292
ES014	fusing extracellular domain of TβRII to antibody targeting CD39	Advanced solid tumor	NCT05717348
HCW9218	protein complex comprising dimeric extracellular domains of the human TβRII and human interleukin (IL)-15	Advanced solid tumor	NCT05304936
			NCT05322408
Luspatercept	protein containing the extracellular domain of human TβRII fused to the human IgG1 Fc domain	Myelodysplastic syndrome	NCT04064060
			NCT04477850
			NCT05520749
			NCT03900715
			NCT03194542
			NCT05925504
			NCT05732961
			NCT05181592
			NCT06006949
			NCT02631070
			NCT02268383
			NCT05384691
			NCT04539236

(*continued on next page*)

Table 1 (*continued*)

Agent	Drug target	Cancer Type	NCT Number
			NCT01749514
			NCT04717414
			NCT05949684
			NCT03682536
			NCT05924100
			NCT05181735
			NCT05005182
			NCT05568225
PM8001	fusing extracellular domain of TβRII to anti PD-L1 IgG1	Advanced solid tumor	NCT05537051
SHR1701	fusing extracellular domain of TβRII to anti PD-L1 IgG1	Advanced solid tumor	NCT04407741
		Breast cancer	NCT05106023
		Colorectal Cancer	NCT04580498
		Gastric cancer	NCT05177497
		Hodgkin's lymphoma	NCT05896046
		Melanoma	NCT04856774
		Non-small cell lung carcinoma	NCT04324814
		Head and Neck cancer	NCT03710265
		Pancreatic cancer	NCT05020925
			NCT03774979
			NCT04937972
			NCT04679038
			NCT05300269
			NCT05179239
			NCT05671822
			NCT04950322
			NCT05503888
			NCT05132413
			NCT05048134
			NCT04856787
			NCT04355858
TQB2858	anti-PD-L1 monoclonal antibody fused with the extracellular domain of human TβRII	Advanced cancer.	NCT04805060
		Soft-tissue sarcoma	NCT05262101
		Cervical cancer	NCT05068921
		Nasopharyngeal cancer	NCT05154630
		Endometrial cancer	NCT05198531
			NCT05121363
			NCT05193604
TQB2868	anti-PD-1 monoclonal antibody fused with the extracellular domain of human TβRII	Advanced solid tumor	NCT05198505
			NCT05998941
QLS31901	fusing extracellular domain of TGF-βRII to anti-PD-L1 heavy chain	Advanced malignant tumor	NCT04954456
		Cervical cancer	
TST005	extracellular domain of the TβRII fused to anti-PD-L1 IgG1 antibody with ablated Fc immune effector function	HPV related cancer	NCT04958434
		Advanced or metastatic cancer	
Antisense nucleotides			
STP705	drug substance composed of two siRNA oligonucleotides, targeting TGF-β1 and Cyclooxygenase-2 mRNA, respectively	Basal cell carcinoma	NCT04669808
		Squamous cell carcinoma	NCT05421013
TASO-001	TGF-β2 targeting anti-sense oligonucleotide	Metastatic solid tumor	NCT04862767
Trabedersen AP12009	TGF-β2-specific antisense oligodeoxynucleotide	Glioblastoma	NCT00844064
OT-101		Refractory astrocytoma	NCT00431561
		Melanoma	
		Pancreatic adenocarcinoma	
		Colorectal adenocarcinoma	
		Non-small cell lung cancer	
		Mesothelioma	
Small molecule inhibitor of the receptor serine/threonine kinases			
Galunisertib	TβRI/ALK5 inhibitor	Prostate adenocarcinoma	NCT05700656
(Ly2157299)		Rectal Adenocarcinoma	NCT02734160
		Carcinosarcoma of uterus or	NCT02304419
		ovary	NCT02906397
		Pancreatic cancer	NCT02423343
		Hepatocellular carcinoma	NCT02008318
		MDS	NCT02672475
			NCT02452008
			NCT02240433
			NCT03206177
			NCT02688712
			NCT01722825
			NCT02178358
			NCT01373164
			NCT01582269
			NCT01220271
			NCT01246986
			NCT01682187
			NCT04031872

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Table 1 (*continued*)

Agent	Drug target	Cancer Type	NCT Number
GFH018	TβRI/ALK5 inhibitor	Advanced solid tumor	NCT05051241
		NSCLC	NCT04914286
			NCT05386888
Ly3200882	TβRI/ALK5 inhibitor	Advanced solid tumor	NCT04031872
		Colorectal adenocarcinoma	NCT02937272
TU2218	ALK5/ vascular endothelial growth factor receptor dual inhibitor	Advanced solid tumor	NCT05784688
			NCT05204862
Vactosertib (TEW-7197, EW-7197)	TβRI/ALK5 inhibitor	Colorectal caner	NCT05436990
		Urothelial cancer	NCT03143985
		Melanoma	NCT04103645
		Pancreatic cancer	NCT04064190
		Hematologic malignancy	NCT04515979
		Multiple Myeloma	NCT04893252
		MPN	NCT05588648
		MDS	NCT04258072
			NCT03724851
			NCT05400122
			NCT04656002
			NCT03732274
			NCT03844750
			NCT03698825
			NCT02160106
			NCT03074006
YL-13027	TβRI/ALK5 inhibitor	Advanced solid tumor	NCT05457517
			NCT05228600
			NCT03869632
Vaccine			
Belagenpumatucel-L (Lucanix™)	TGF-β antisense gene modified allogeneic tumor cell vaccine	Non-Small Cell Lung Cancer	NCT00676507
			NCT01058785
Gemogenovatucel-T (Vigil)	autologous tumor cell vaccine comprising the immunostimulatory Granulocyte-macrophage colony-stimulating factor gene, and proprotein convertase furin which knock down downstream targets (TGF-β1 and TGF-β2)	Ewings Sarcoma	NCT02574533
		Non-Small Cell Lung Cancer	NCT02725489
		Liver Cancer	NCT02511132
		Ovarian cancer	NCT02346747
			NCT01309230
			NCT01551745
			NCT01867086
			NCT01061840
TGF-β-B-15 Peptide	TGF-β derived epitope vaccine	Pancreatic adenocarcinoma	NCT05721846
Genetically Modified T cell			
CAR-EGFR-TβR-KO T cell	knocking out TβRII through CRISPR/Cas9	Advanced solid tumor	NCT04976218
CART-PSMA-TGFβRDN cells	Prostate-specific membrane antigen (PSMA)-specific/ TGFβ-dominant negative receptor (DNR) Chimeric antigen receptor (CAR) modified autologous T cells	Prostate cancer	NCT03089203
CB-NK-TGF-betaR2-/ NR3C1- cells	Engineered natural killer (NK) cells containing deleted TβRII and NR3C1	Glioblastoma	NCT04991870
DNR-CTL	TGF-β -resistant LMP-specific cytotoxic T lymphocytes (CTL)	Lymphoma	NCT00368082
DNR.NPC-specific T cells	TGF- β resistant cytotoxic T-lymphocytes	Nasopharyngeal carcinoma	NCT02065362
MSLN-CAR-T cell	co-infecting T cells with two lentiviral vectors. One vector expresses CD19-CAR and tEGFR molecular safety switch, and the other vector expresses mesothelin (MSLN)- CAR and DN-TβRII.	Relapsed/refractory MSLN-positive solid tumors	NCT05783089
TGF-β resistant HER2/EBV-CTLs	Her2 chimeric receptor and TGF-β DNR expressing Epstein-Barr virus specific lymphocytes	Advanced Her2 positive malignancy	NCT00889954
TGF-β resistant infiltrating lymphocytes	TGF-β DNRII-transduced autologous tumor infiltrating lymphocytes	Metastatic melanoma	NCT01955460

extracellular matrix contains both an organic component, formed by type I collagen, proteoglycans and glycoproteins, and inorganic ions (calcium and phosphate) organized in hydroxyapatite crystals. Osteoblasts, which are derived from mesenchymal cells, deposit new bone matrix in the resorbed area and facilitate mineralization. Osteoclasts, which are derived from hematopoietic cells, resorb old bone matrix. TGF-β is one of the most abundant growth factors in the bone matrix. TGF-β regulates a number of important bone processes and uniquely coordinates the activity of bone cells to maintain normal bone homeostasis [73].

TGF-β regulates the differentiation and function of both osteoblasts and osteoclasts [74]. Bone-forming osteoblasts secrete TGF-β, which remains embedded in a latent form in a mineralized bone matrix [75,76]. In response to bone resorption by osteoclasts, TGF-β is activated and released, recruits perivascular mesenchymal cells to the surface of resorption lacunae and differentiates them into osteoblasts. Runt-related transcription factor 2 (Runx2) is a master regulator of osteoblast differentiation by regulating the expression of several genes including type I collagen, alkaline phosphatase, osteopontin, osteonectin, and osteocalcin [77]. TGF-β increases Runx2 expression during the early differentiation, accelerating the proliferation of osteoblasts [78]. In later stages of differentiation, TGF-β inhibits terminal osteoblast differentiation and bone matrix synthesis by repressing Runx2 through a Smad3-dependent mechanism [76,79]. TGF-β also inhibits osteocyte apoptosis, partially via Smad3-dependent and vitamin D receptor-dependent mechanisms [80].

TGF-β plays a role in the differentiation of osteoclasts. In vitro studies demonstrated TGF-β affects bone resorption in a dose-dependent manner [81,82]. Low concentrations of TGF-β stimulate the migration of osteoclast precursors to the bone resorption site, and their differentiation into mature osteoclasts. TGF-β promotes the recruitment of osteoclast precursors (bone marrow macrophages) to the bone site and

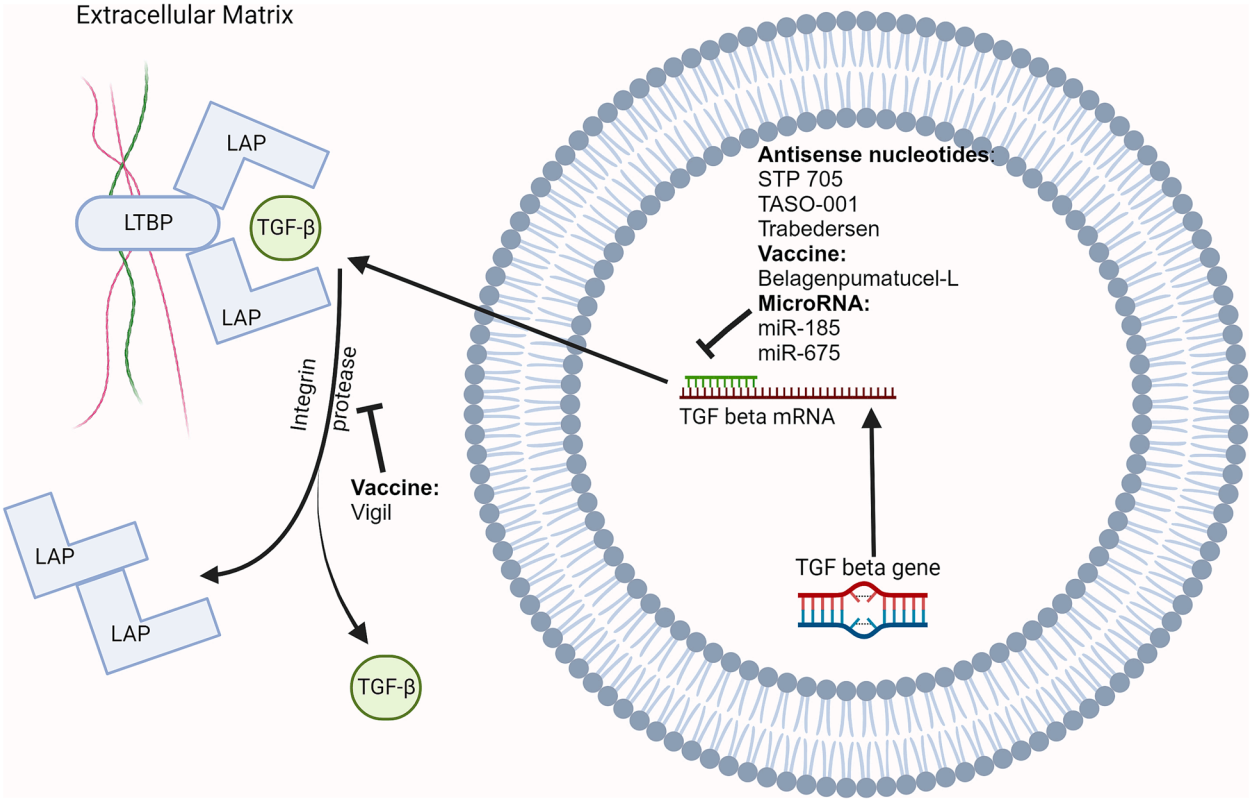

Fig. 1. A. Activation of a large latent complex which comprises of TGF-β, the latency associated peptide (LAP), and latent TGF-β-binding protein (LTBP). B. Antisense nucleotides and MicroRNA reduce TGF-β expression at the transcriptional/translational level.

stimulates their proliferation and differentiation [75,83]. During active bone resorption, osteoclasts secrete cathepsins, which proteolytically activate and release TGF-β from the latent complex. TGF-β activates SMAD2/3 which binds directly to Tumor necrosis factor receptor associated factor 6 (TRAF6)-TGF-β activated kinase 1 binding protein 1 (TAB1)-TGF-β activated kinase 1 (TAK1) to form a complex that can promote Receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast differentiation. Contrastingly, high dose TGF-β inhibits the migration of osteoclast precursors and their differentiation through the modulation of RANKL and Osteoprotegerin expression by osteoblasts [81,82,84]. In vivo experiments indicate that TGF–β favor bone resorption and destruction [81,85–88].

4. Transforming growth factor beta signaling and osteosarcoma

The TGF-β signaling pathway has complex and contradictory roles in the pathogenesis of human cancers. In epithelial cells, TGF-β is primarily a tumor suppressor that inhibits proliferation or induces apoptosis of premalignant epithelial cells [22,89]. In the later stages of cancer progression, however, TGF-β functions as a metastasis promoter by promoting tumor growth, stimulating EMT, promoting migration and invasiveness, blocking antitumor immune responses, increasing tumor-associated fibrosis, and enhancing angiogenesis [8,9,90]. TGF-β signaling affects osteosarcoma via its prominent role in EMT by influencing key transcription factors, including Snail, zinc-finger E-box-binding, and basic helix–loop–helix transcription factors. TGF-β increases fibroblast proliferation, stimulates the synthesis and deposition of connective tissue, and inhibits connective tissue breakdown [91–93]. Release of TGF-β from the bone matrix by osteoclastic bone resorption induces tumor production of osteolytic factors such as interleukin 11, connective tissue growth factor, MMP-1, CXCR4, and PTHrP [94,95]. Several *in vitro* experiments have demonstrated that TGFβ stimulated osteosarcoma cell migration and invasion [87,96,97]. TGF-β signaling plays a critical role in the regulation of immune cell function in normal and tumor-associated lymphocytes, in particular, the activation of T-regulatory cells. Lamora et al reported TGF-β levels are higher in serum samples from patients with osteosarcoma compared with healthy volunteers and TGF-β /Smad3 signaling pathway is activated in clinical samples [87]. Xu et al reported patients with metastatic tumors presented a significantly higher TGF-β level than those without metastasis [98]. These results indicated that serum TGF-β may play roles in the progression of osteosarcoma. In addition, Zhou et al reported higher expression of TGF-β is more likely to induce chemotherapy resistance among patients with osteosarcoma and lead to poor prognosis [99].

5. Antagonists of TGF-β signaling

Many drugs that target the TGF-β signaling pathway have been developed. Preclinical and clinical studies indicate the utility of these agents in fibrosis and oncology. There are many ways to inhibit TGF-β superfamily signaling: (1) Neutralizing antibodies that prevent TGF-β binding to receptor complexes. There are TGF-β1 neutralizing antibodies (metelimumab, LY2382770, CAT-192), the TGF-β2 neutralizing antibody (Lerdelimumab), and the pan-TGF-β neutralizing antibody (Fresolimumab/GC-1008); (2) Ligand traps which employ recombinant Fc-fusion proteins containing the soluble ectodomain of either TβRII (Luspatercept, AVID200) or TβRIII (glycosaminoglycan modified beta-glycan), preventing TGF-β from binding to its receptors [100]. Bintrafusp is a bifunctional anti-Programmed death-ligand 1 (PD-L1) / TβRII trap fusion protein; (3) Antisense nucleotides reduce TGF-β expression at the transcriptional/translational levels. Trabedersen/AP 12,009 and TASO-001 are TGF-β2-specific antisense oligodeoxynucleotide; (4) Small molecule inhibitors of the receptor (TβRI/ALK5, TβRII) serine/threonine kinases prevent downstream signaling [101,102]. In contrast to tyrosine kinase inhibitors, Ly2157299/Galunisertib, GFH018, Ly3200882, Vactosertib, and YL-13027 inhibit TβRI/ALK5

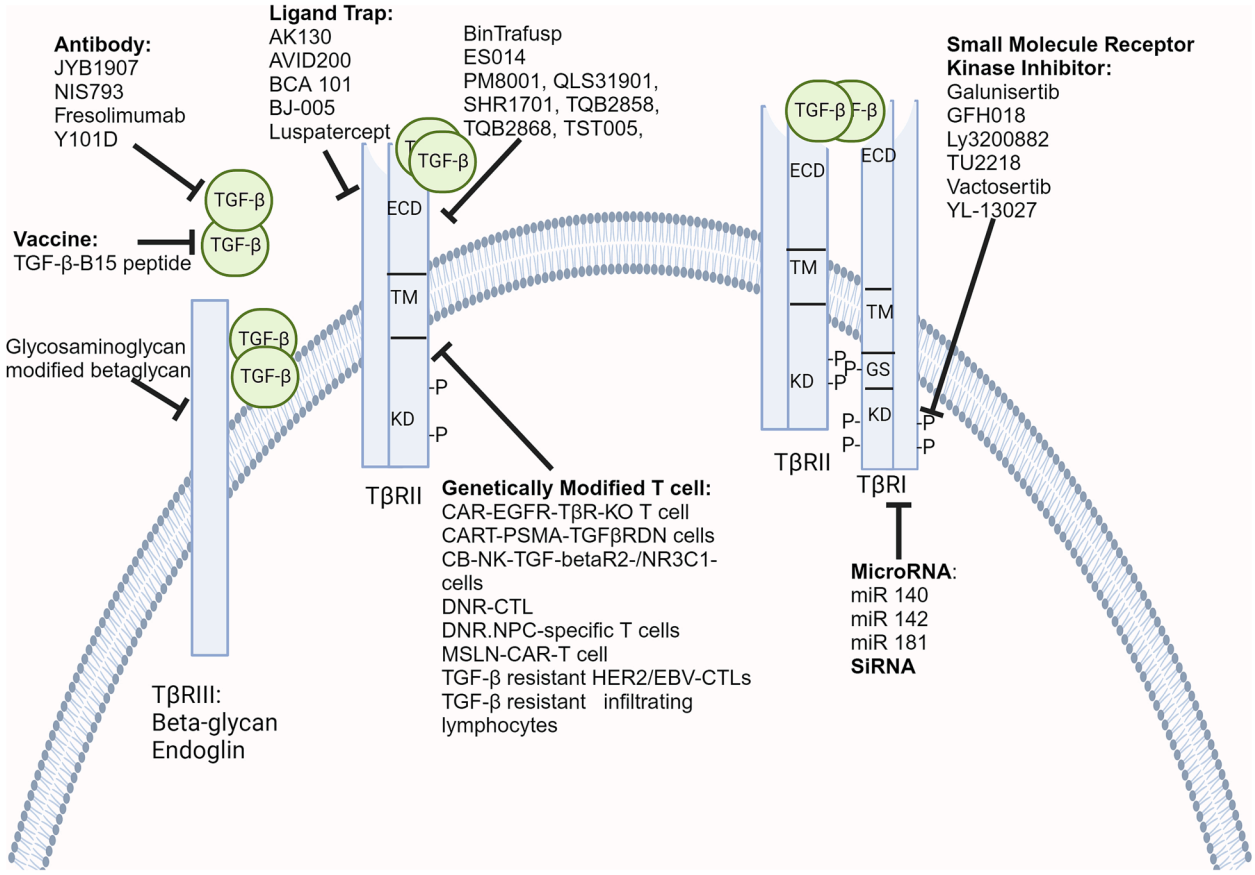

Fig. 2. A. TβRI and TβRII contain an extracellular ligand binding domain (ECD), a transmembrane serine-threonine domain (TM), and a cytoplasmic serine-threonine kinase domain (KD). TGF-β activates TβRII, and TβRII kinase phosphorylates serine residues in the juxta membrane subdomain (GS motif) of TβRI. B. Antagonists: TGF-β neutralizing antibody; Ligand trap containing the soluble ectodomain of either TβRII or modified TβRIII; Small molecule inhibitors of TβRI serine-threonine kinase; MicroRNA (miR) or Small interfering RNA (SiRNA) targeting TβRI; Genetically modified T cell targeting TβRII.

serine/threonine kinase activity and have been developed through preclinical to clinical trials; (5) Vaccines: Belagenpumatucel-L contains cell lines transfected with TGF-β2 antisense gene. Vigil is a vaccine targeting furin convertase, resulting in reduced TGF-β1 and TGF-β2 activation; (6) "peptide aptamers": Trx-SARA aptamer is made of a rigid scaffold Trx (the Escherichia coli thioredoxin A protein) linked to the SMAD-binding domain of SARA. Trx-SARA binds specifically to SMAD2 and SMAD3 and inhibits TGF-β induced gene expression. [103]; (7) MicroRNAs, which are small non-coding RNA molecule nucleotides. Many miRNAs targeting TGFβ signaling components have been identified. For example, miR-185 and miR-675 target TGF- β1 and the let-7 family, miR140, 142, and 181, target TβR1 [102]; (8) Small interfering RNA (siRNA) targeting TGFβ receptors. Chen et al used TβRI and TβRII siRNAs packaged into 7C1 nanoparticles to suppress the target receptor. (9) "SMAD7 overexpression": SMAD7 competes with R-SMADs for binding to activated TβRI and recruits E3-ubiquitin ligases to degrade the receptor protein phosphatases to dephosphorylate the receptor complex [104]. (Figs. 1, 2, &3).

6. Effects of TGF-β antagonists on osteosarcoma cell lines *in vitro*

Several small molecular inhibitors have been studied on osteosarcoma cell lines. SB-431542, a specific small molecule inhibitor of ALK-4, ALK-5, and ALK-7, inhibited the phosphorylation of both Smad2/3 and Smad1/5 induced by TGFβ in MG63 human osteosarcoma as well as TGFβ -induced Id-1 expression and PAI-1. SB-431542, inhibited cell proliferation of MG63 human osteosarcoma cells induced by TGF-β [105] and dedifferentiation and clonogenicity of osteosarcoma cells [106]. LY2109761, a small molecule ALK-5 inhibitor, was able to induce apoptosis and inhibit the growth and invasion of osteosarcoma MG-63 cells *in vitro* [107]. RepSox, a small molecule inhibitor of TβRI/ALK5, effectively inhibited the proliferation of Osteosarcoma (OS) cells by inducing S-phase arrest and apoptosis. RepSox inhibited cell migration and invasion *in vitro* and reduced the protein levels of molecules associated with the EMT phenotype, including E-cadherin, N-cadherin, Vimentin, MMP-2, and MMP-9. Concurrently, the JNK and Smad3 signaling pathways were inhibited [9,108]. Vactosertib inhibited the growth of mouse osteosarcoma (mOS) and human osteosarcoma cells (hOS) tumor cells and down-regulated three molecules associated with OS tumor progression and metastasis: Ephrin-2 (EFNB2), IL-11, and Prostate transmembrane protein androgen induced1 (PMEPA1). Vactosertib inhibited the expression of genes of PMEPA1, LTBP1, IL-11 and JUNB; these genes are involved in tumor progressions and metastasis in cancer. Furthermore, JUNB has also been reported to bind to the promoter of c-myc and regulate its expressions [109].

Grilli et al., reported the expression of miR-34a and down-modulation of TGF-β signaling emerge as pivotal events to drive CD99-mediated reversal of malignancy and activation of differentiation in Osteosarcoma cells [110]. Fu et al., reported miR-181c overexpression prominently repressed osteosarcoma cell proliferation, invasion, and migration abilities via modulating EMT and TGF-β signaling pathway. SMAD7 functioned as an important target for miR-181c in osteosarcoma cells [111].

7. Effects of TGF-β antagonists on osteosarcoma *in vivo*

Several animal studies have shown the effects of TGF-β antagonists on osteosarcoma and investigated the mechanisms of the effects.

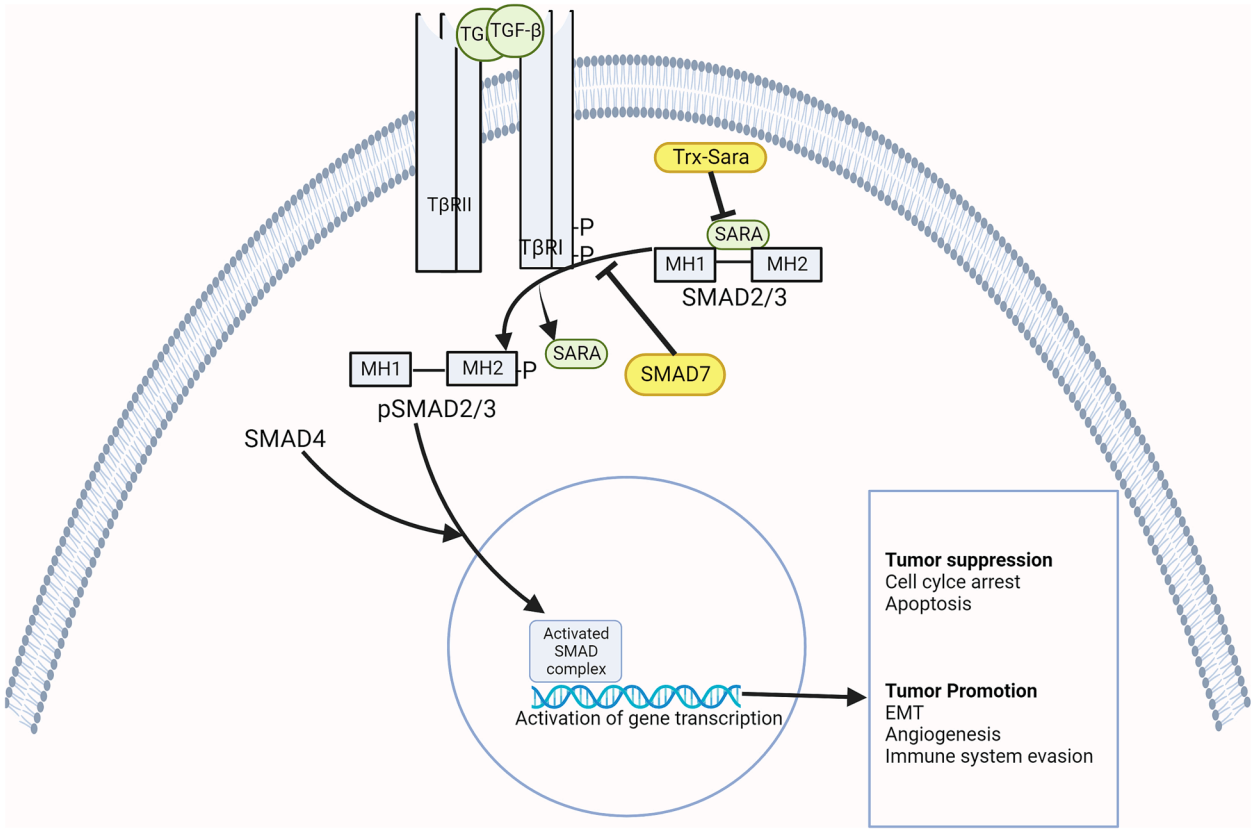

Fig. 3. A. TβRI kinase phosphorylates SMAD2 and SMAD3 at Mad homology 2 (MH2). SARA promotes the activation of SMAD2/3. The SMAD2/3/4 complexes are translocated from the cytoplasm to the nucleus and result in the transcriptional regulation of wide range target genes. B. Antagonists: Trx-SARA binds specifically to SMAD2 and SMAD3 and inhibits TGF-β induced gene expression; SMAD7 competes with R-SMADs for binding to activated TβRI.

Lamora1 et al., demonstrated Smad7 overexpression slowed the growth of the primary tumor, inhibited lung metastases, and increased mice survival. In addition, Smad7-osteosarcoma bone tumors expressed lower levels of osteolytic factors such as RANKL, suggesting that Smad7 overexpression affects the "vicious cycle" established between tumor cells and bone cells by its ability to decrease osteoclast activity. In their study, the treatment of mice with SD208, a small molecule ALK-5 inhibitor, inhibited the development of lung metastasis in Rj:NMR1-nude mice injected with hOS SAOS intramuscularly close to the tibia [76].

In NSG mice injected with SAOS2 cells subcutaneously, Vactosertib reduced the tumor volume, lung metastasis and increased survival rate. The p-Smad2 as well as c-Myc mRNA expression were significantly inhibited in Vactosertib-treated tumors *in vivo* [109]. In BALB/c mice injected subcutaneously with the mOS cells K7M2, Vactosertib treatment significantly inhibited K7M2 tumor growth. Vactosertib enhanced CD4 T-cells, CD8 T-cells, and NK cell infiltration while suppressing Treg, tumor associated M2-like macrophages, and PD-1-expressing T cells in the K7M2 tumor microenvironment. These results suggest that tumor-infiltrating T lymphocytes were activated upon the inhibition of TGF-β1 [109].

In addition, *in vivo* findings using a xenograft model also revealed that RepSox markedly inhibited the growth of tumors. Furthermore, upregulation of miR-181c dramatically suppressed osteosarcoma tumorigenesis *in vivo* [108].

The mice that received dendritic cells exposed to cryotreated tumor lysates with the monoclonal antibody 1D11 (mouse IgG1 neutralizes all three isoforms of TGF-β) showed increased numbers of CD8(+) T lymphocytes, reduced regulatory T lymphocytes in the metastatic lesion, and inhibited metastatic growth [112].

8. Clinical role of targeting transforming growth factor beta signaling in metastatic osteosarcoma

Currently the TGF-β antagonists are used in many clinical trials for colon adenocarcinoma, pancreatic adenocarcinoma, prostate adenocarcinoma, glioblastoma, advanced solid tumors, and MDS. In contrast with the dual effects of TGF-β on epithelial carcinoma progression, TGF-β seems to mainly have a pro-tumoral effect on sarcoma including osteosarcoma. MP-VAC-209 is a Phase I/II, open label, single arm, multicenter study to assess safety and efficacy of Vactosertib in adolescents and adults with recurrent, refractory, or progressive osteosarcoma (ClinicalTrials.gov, NCT05588648). However, a very small phase 1b study reported anti-PD-L1/TGF-beta antibody TQB2858 did not demonstrate evidence of efficacy as 0/5 osteosarcomas had any objective response [113].

Several phase 1/2/3 clinical trials have shown TGF-β antagonists are safe and tolerated well. Luspatercept, a recombinant fusion protein that contains a modified form of the extracellular domain of human TβRII and links to the human IgG1 Fc domain, increases differentiation and proliferation of erythroid precursors. Luspatercept is first-in-class approved for anemia associated with MDS with ring sideroblasts/mutated SF3B1 based on the phase III trial (MED-ALIST; ClinicalTrials.gov identifier: NCT02631070). The most frequently reported adverse events during the trial were fatigue (27 %), diarrhea (22 %), asthenia (20 %), nausea (20 %), dizziness (20 %), and back pain (19 %) [114,115]. AVID200 was constructed by fusing TGFβR ectodomains to a human IgG Fc region. TGF-β2 plays a key role in normal cardiac function and is thus an undesirable drug target [116,117]. Interestingly, AVID200 is believed not to hit TGF-β2, which may be crucial to minimize cardiac toxicities.

Galunisertib (LY2157299), a small molecule TβRI/ALK5 inhibitor,

has shown some clinical benefits in several types of cancers. The most frequently occurring treatment-emergent adverse events overall (>20 % of patients) were fatigue, anemia, peripheral edema, and abdominal pain. The most common grade 3/4 treatment-related adverse events were neutropenia (2.7 %), fatigue, anemia, increased bilirubin, hypoalbuminemia, and embolism (1.3 % each) [118,119]. Overall, the comprehensive cardiovascular monitoring for Galunisertib did not detect medically relevant cardiac toxicity [120]. Vactosertib, also a TβRI/ALK5 inhibitor, was safe and well tolerated (ClinicalTrials.gov, NCT03143985). The most common treatment-related adverse events were fatigue, abdominal pain, AST elevation, and less frequent pulmonary embolism [121].

Fresolimumab (GC1008) is a human anti- TGF-β monoclonal antibody that neutralizes all isoforms of TGF-β. Treatment with Fresolimumab was well tolerated (ClinicalTrials.gov NCT03064074). Observed adverse events included gingival bleeding, epistaxis, headache, and fatigue. These adverse events were ≤ grade 2. The development of reversible cutaneous keratoacanthomas/squamous-cell carcinomas and hyperkeratosis were the major adverse events observed [122].

Belagenpumatucel-L (Lucanix) is a vaccine comprised of TβRII-antisense gene-modified, irradiated, allogeneic NSCLC cell lines. Belagenpumatucel-L was well tolerated with no serious safety concerns [123,124]. Gemogenovatucel-T (Vigil/FANG/Gradalis) is an autologous tumor cell vaccine manufactured from harvested tumor tissue, which specifically reduces expression of furin and downstream TGF-β1 and TGF-β2. Gemogenovatucel-T resulted in no grade 3 or 4 toxic effects [125].

The safety and efficacy of the fusion protein composed of the extracellular domain of the TβRII (a TGF-β "trap") fused to monoclonal antibody blocking programmed death-ligand 1 (PD-L1) were evaluated in patients. A phase 2 study (ClinicalTrials.gov, NCT03833661) of Bintrafusp alfa reported the most common events were pruritus, rash, and fatigue. Treatment-related adverse events of grade 3 or higher occurred in 26.4 % of patients; the most common grade ≥ 3 treatment-related adverse events included anemia, pruritus, increased alanine aminotransferase, and increased aspartate aminotransferase. One treatment-related death occurred due to hepatic failure [126–128]. A phase 1 trial of SHR-1701 (ClinicalTrials.gov, NCT03710265), showed an acceptable safety profile with Grade ≥ 3 treatment-related adverse events occurring in 22 % of patients, mainly including increased gamma-glutamyltransferase(4 %), increased aspartate aminotransferase (3 %), anemia (3 %), hyponatremia (3 %), and rash (2 %) [129].

9. Discussion

One reasonable concern of targeting TGF- β signaling is the increased risk of secondary primary tumors because TGF-β antagonists could block the tumor suppressing effect in the early stage of epithelial cancer. So far, all clinical trials of TGF-β antagonists are targeting advanced/metastatic cancer. One of the challenges of clinical trials for advanced osteosarcoma would be recruitment because of the rarity of metastatic or recurrent/refractory osteosarcoma. International muti cancer center collaboration may be necessary.

So far, the effects of antagonists of TGF-β on cancer from phase I/II studies have been moderate [118,119,123–125,130,131]. Overexpression of TGF-β inhibits the function of cytotoxic T cells and natural killer cells, inhibits maturation and antigen presentation by dendritic cells, increases the production of T_{reg} cells, and thus leads to immune suppression. Hence, a combination of a TGF-β inhibitor and a PD-1/PD-L1 inhibitor could potentially improve immune response and augment deeper effects. The safety and efficacy of the fusion protein composed of the extracellular domain of the TβRII (a TGF-β "trap") fused to monoclonal antibody blocking PD-L1 such as Bintrafusp alfa and SHR-1701 have been evaluated in patients with carcinoma. Clinical trials using this fusion protein in osteosarcoma could help improve the outcome. The bisphosphonates and RANKL inhibitor indirectly prevent TGF-β release from the bone by preventing bone resorption, and thus bisphosphonates combined with TGF-β inhibitors can have greater therapeutic effectiveness than either agent alone. Similarly, cytotoxic chemotherapies combined with TGF-β inhibitors could improve efficacy.

Studies to investigate which patient population would benefit more from blocking TGF-β signaling would also be important. The most direct biomarkers for patient selection for anti-TGF-β antagonists are circulating TGF-β in blood and p-SMAD2 levels in peripheral mononuclear cells (PBMC). A pSMAD2 assay was established to measure the reduction of pSMAD2 in PBMCs during the FHD trial. A plasma TGF-β1 ELISA was developed and subsequently used for pharmacodynamic assessments. Other plasma proteins such as c-MYC, PTHrP, von Willebrand factor, and interleukin-10 correlated with TGF-β1 levels can potentially act as biomarkers [132,133].

^{89}Zr-fresolimumab small-animal PET was shown to be preclinically feasible for imaging and quantification of Fresolimumab tumor uptake and organ distribution. This technique can be used to guide further clinical development of Fresolimumab and could identify patients most likely to benefit [134].

With ubiquitous distribution and cross talk of TGF- β signaling, there are concerns of disruption of physiological function with TGF-β signaling. A global knockout of the TGF-β1 gene during development in mice results in severe multiorgan inflammation and early death [135], while mice with a global TGF-β2 knock out die perinatally with multiple cardiac, craniofacial, cleft palate, non-cranial skeletal, eyes, inner ears, and urogenital developmental defects [117]. Mice with a global TGFβ3 deletion die within 20 h of birth due to abnormal lung development and the presence of cleft palate [136]. In some cancers, instead of systemic delivery, local delivery of these antagonists might be a good option to minimize systemic toxicity. One of the examples is Trabedersen, a TGF-β2-specific antisense oligodeoxynucleotide. Trabedersen was injected into glioblastoma intratumorally. About 25 % of patients did experience drug-related serious adverse events such as meningitis, hyponatremia, and brain edema [131].

A study to evaluate the long-term safety in participants who have participated in other Luspatercept (ACE-536) clinical trials is ongoing (ClinicalTrials.gov ID NCT04064060). The long-term adverse effects of other TGF-β antagonists need to be evaluated.

10. Conclusion and outlook

TGF-β antagonists have been shown to have effects on osteosarcoma *in vitro* and *in vivo*. The clinical trial to evaluate the effect of TGF-β antagonists on advanced osteosarcoma seems promising. In addition, the combination of a TGF-β inhibitor and a PD-1/PD-L1 inhibitor, a bisphosphonate, a RANKL inhibitor, or cytotoxic chemotherapies could improve efficacy. Many phase 1/2 clinical trials have shown TGF-β antagonists are safe and well tolerated. Clinical trials evaluating long-term safety are in process.

Author contributions

Rongrong Ge contributed to drafting the manuscript. Gavin M Huang contributed to review & editing. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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