

Consequences of Mutations and Abnormal Expression of SMAD4 in Tumors and T Cells

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Rongxue Wan¹⁻³
Jianguo Feng^{2,4}
Liling Tang¹

¹Key Laboratory of Biorheological Science and Technology, Ministry of Education, College of Bioengineering, Chongqing University, Chongqing, 400044, People's Republic of China; ²National Key Discipline of Human Anatomy, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong Province, People's Republic of China; ³Department of Human Anatomy, School of Basic Medical Sciences, Guangdong Medical University, Zhanjiang, Guangdong Province, People's Republic of China; ⁴Department of Anesthesiology, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan Province, People's Republic of China

Abstract: SMAD4 is a typical tumor suppressor in the TGF- β signaling pathway. In human cancers, SMAD4 is frequently mutated and inactivated. In recent years, the consequences of mutations and inactivation of SMAD4 are gradually becoming clearer. Most of the mutations have negative consequences and reduce the chances of survival of their carriers. Loss of SMAD4 functions due to mutations or abnormal expression can suppress the inhibition of tumor growth and support the tumor progression. Functions of SMAD4 and its variants in T cells are being studied extensively, to better understand the SMAD4 functions in T cells. In this review, we mainly discuss the recently reported consequences of mutations and abnormal expression of SMAD4 in tumors, and the effects of loss, deficiency or mutation of SMAD4 and its T cells, to show the use of SMAD4 mutations in cancer diagnosis and therapeutic strategies.

Keywords: SMAD4, mutation, abnormal expression, T cell, TGF- β

Background

The nomenclature of “Smad” was first termed in 1996, a contraction of the names of prototypic members Sma and Mad.¹ These are intracellular transcription factors that regulate the TGF- β signal transduction. The eight members of the Smad family of regulatory proteins are classified into three groups, according to their functions: receptor-activated Smads (R-Smads), co-mediator Smad (Co-Smad) and inhibited-Smads (I-Smads). Upon activation of TGF- β signaling pathway, TGF- β type I/II receptors (T β R-I/T β R-II) complex phosphorylates SMAD2 and SMAD3, which are R-Smads,² while the other R-Smads, viz., SMAD1/5/8, participate in the BMP/Smads pathway, after being phosphorylated by the BMP type I/II receptors (BMPRI/BMPRII) complex.³ SMAD4, the co-Smad, also named as deleted in pancreatic cancer 4 (DPC4), is a tumor suppressor in many types of tumor cells.^{3,4} SMAD4 can complex with all the activated R-Smads, and transfer signals into the nucleus, to regulate the transcription of the target genes, controlling cell differentiation, proliferation and apoptosis.⁵ The I-Smads, including SMAD6 and SMAD7, inhibit the phosphorylation of SMAD4, competing to complex with R-Smads or disturbing the binding of Smad complex binding to DNA, resulting in negatively regulating TGF- β /Smads signaling pathways.⁶⁻⁸

In the canonical TGF- β /Smad signaling pathway, Smads regulate the communication between the nucleus and the cell microenvironment by shuttling between the cytosol and nucleus.⁹ Briefly, the signal is triggered by the binding of TGF- β and T β RII, activating the T β RII, which further activates the T β RI. This phosphorylates

Correspondence: Jianguo Feng
1023-1063 Shatai South Road, Baiyun District, Southern Medical University, Guangzhou, 510515, People's Republic of China
Tel +86-1820-835-7473
Email ffengjianguo@163.com

Liling Tang
83 Shabei Street, Shapingba District, Chongqing University, Chongqing, 400044, People's Republic of China
Tel +86-1399-605-1730
Email tangliling@cqu.edu.cn

R-Smads, forming the phosphorylated-R-Smads/SMAD4 complexes, which are then transported into the nucleus, and interact with other transcription factors to regulate the expression of target genes. Transduction of this kind of signaling can be inhibited by I-Smads, to regulate the extent of activation of TGF- β /Smad signaling.

The mechanisms of initiation and development vary among different tumors. Abnormal expression and accumulation of mutations in tumor-related genes are the major contributors to oncogenesis.^{10,11} SMAD4 is frequently mutated during cancerization of cells.^{12–16} Using high throughput sequencing technology, increasing number of SMAD4 mutations are being reported in the cancer cells and immune cells. These mutations can be new targets for cancer therapy. In this review, we discuss the structure and functions of SMAD4 and the consequences of abnormal expression and mutations of SMAD4 in tumor cells and T cells. This knowledge may provide new insights into the diagnoses and therapeutic strategies for SMAD4-associated tumors.

Mutations That Affect the Functions of SMAD4 Protein

Structure of SMAD4

Human *SMAD4* gene is located on the chromosome location 18q21.1, and is composed of 11 exons, spanning the full-length (1659 bp) transcript of SMAD4.¹⁷ SMAD4 protein is composed of three domains: Mad Homology1 (MH1) domain at the N-terminus, Mad Homology2 (MH2) domain at the C-terminus, and a linker region between the MH1 and MH2 domains.^{17,18} As shown in Figure 1, the conserved MH1 domain is encoded by most parts of the exon 1 and 2, and contains sub-regions such as a nuclear localization signal (NLS) in the exon 1, essential for the role of SMAD4 in transcription, a DNA binding motif in the junction of exon 1

and exon 2, necessary for the binding of SMAD4 to the Smad-binding elements (SBE) in the promoters of the target genes, and a functional, leucine-rich nuclear export signal (NES) in the junction of exon 2 and 3, important for the role of SMAD4 in TGF- β /Smads signaling pathway.^{19–22}

The MH2 domain of SMAD4, necessary for forming the R-Smad/co-Smad complex, is encoded by most of the exon 8 and exon 11 and entire of the exon9 to exon10 (Figure 1).²³ The MH2 domain of R-Smads, but not of SMAD4, can be phosphorylated by the T β R-I/T β R-II complex. In addition, the MH1 and MH2 domains are antagonistic; as in the R-Smads, the MH2 domain of SMAD4 is negatively regulated by the MH1 and this regulation could be removed when MH2 domain was phosphorylated.^{24,25} The L3 loop, a motif in MH2 domain, could be recognized by Ski that has been identified affecting cell growth and muscle differentiation,^{26,27} disturbing the formation of SMAD4/R-Smads complex, and negatively regulate the TGF- β , BMP and activin signaling pathways.²⁸

Between the MH1 and MH2 domains is the proline-rich linker region. In the full-length SMAD4, the linker region is encoded by a part of exon 2 and exon 8, and the entire of exon 3 to exon 7 (Figure 1). The linker region carries a nuclear export signal, crucial for the subcellular localization of SMAD4.^{29,30} A proline-rich Smad-activation domain (SAD), which is necessary for the role of SMAD4 in transcription, is also in the linker region.³¹ Crystal structure analysis showed that because of the proline-rich sequence, the SAD elements can activate SMAD4 for transcription by interacting with other transcription factors.³² Even though the mutated SMAD4 without SAD can complex with R-Smads or other transcription factors, these complexes are not sufficient to activate transcription.³³ Because the lengths of

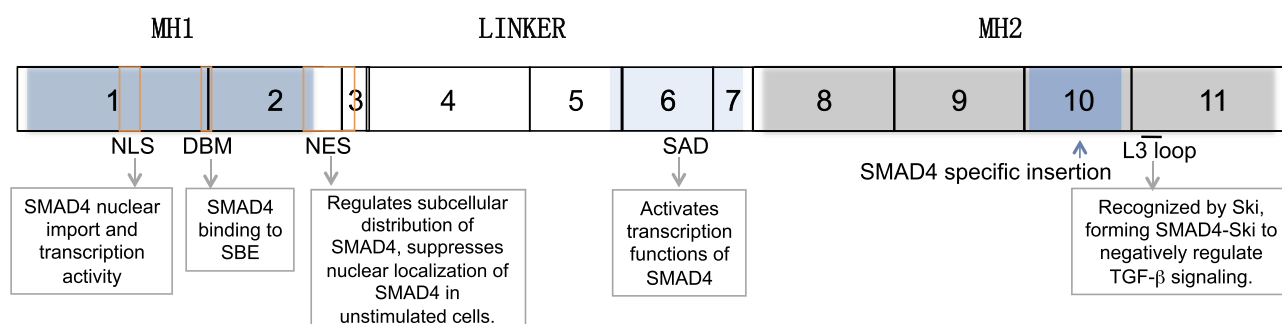


Figure 1 The schematic diagram to show the structure of SMAD4. SMAD4 is composed of three different domains, MH1, linker region and MH2 domain. Nuclear localization signal (NLS), DNA binding motif (DBM) and nuclear export signal (NES) are in MH1 domain. And linker region has a Smad-activation domain (SAD). In MH2 domain, there is a region that is called SMAD4 specific insertion and MH2 mutation often occur in this region. L3 loop of MH2 domain is important for forming complex with R-Smads.

the spliced variants or isoforms vary, the linker region is not conserved, unlike the MH1 or MH2 domains. Frequency of mutations in the linker region is high, and include silencing, missense, insertion-deletion, and frame-shift mutations.¹⁸

Mutations of SMAD4 Occurring in Tumors

Proliferation of cancer cells is inhibited by the TGF- β signaling, which requires the participation of a functional SMAD4.^{34,35} However, SMAD4, a tumor suppressor gene, is frequently mutated or silenced during tumor initiation and development. About 50% of the pancreatic cancers have mutated SMAD4, and 15% of colorectal cancers carry SMAD4 mutations in the homozygous or hemizygous state.^{36,37} Mutations in SMAD4 inactivate the TGF- β signaling in gastric tumors.³⁸ Loss of SMAD4 leads to formation and promotion of metastases in the head and neck cancer, and the pancreatic cancer.³⁹ In mice, SMAD4 and PTEN synergistically inhibit the forestomach squamous cell carcinoma, and mutations in them enhance the cell growth and carcinogenesis.^{40,41} In addition, the MH2 domain is more frequently mutated than the MH1 domain or the linker region. It is reported that the MH2 domain is a mutation hotspot region in SMAD4.⁴² In Juvenile Polyposis, 40–60% patients were diagnosed with germline mutation of SMAD4, and about 85% of these mutations are seen in the MH2 domain, and just 15% of them occur in the MH1 and linker domains.^{43,44}

In colorectal cancers, primary cancer lesions harbor a variety of mutations, including nonsense, frame-shift, missense, insertion, and deletion, while the most frequent ones are the missense mutations,⁴⁵ which always have severe consequences. The somatic mutation of SMAD4 analysis showed that as high as 78.8% missense mutations were in MH2 domain, and mainly within the R-Smad binding region.⁴⁵ Three other reported missense mutations in the MH2 domain may affect the binding of C-terminal region of SMAD4 with SMAD2 and SMAD3. A large number of missense mutations in the MH1 and MH2 domains of SMAD4 alter the stability of the protein, and particularly the K45N mutation in the MH1 domain disrupts the SMAD4 localized to the nucleus.^{45,46} Some mutations, such as missense mutation of L172M and T197I in the linker region, do not affect the functions of SMAD4 protein.⁴⁵

Mutations Negatively Regulate the Functions of SMAD4

SMAD4 is involved in the TGF- β /Smad signaling pathway, mainly in inhibiting cellular growth. Mutations in SMAD4 result in decreased activation of TGF- β /Smad signaling pathway. SMAD4 shuttles between the cytoplasm and the nucleus, using the NLS and NES present in the MH1 and MH2 domains.²² Mutations in the NLS disrupt this nucleocytoplasmic shuttling, reducing the accumulation of SMAD4 in the nucleus, eventually inactivating the transcriptional function of SMAD4.²² In acute myelogenous leukemia, a heterozygous missense mutation in the MH1 domain prevented its DNA-binding, and a frame shift mutation in the MH2 domain inhibited the nuclear translocation (Table 1).⁴⁷ Mutated SMAD4 with homozygous deletion of a 38 amino acid at the MH2 domain, failed to form the complexes with SMAD2 or SMAD3, and could not be recruited to DNA binding sites by other transcriptional factors.⁴⁸

In some cases, mutations in the individual amino acids can have serious consequences. An overall 38% point mutations in the SMAD4, detected among the patients with juvenile polyposis syndrome, occur mainly in the exon 8 and 9.⁴⁹ In the MH2 domain, I500, which is conserved in many species, is close to the K519, a site of protein ubiquitination. A report showed that a single codon mutation in I500 can cause Myhre syndrome.⁵⁰ K507 in the L3 loop is a target site for ubiquitination, and participates in the recognition of phosphorylated R-Smads.⁵¹ When K507 is mutated, the complexes of SMAD4/R-Smads become non-functional and the transcriptional activity of SMAD4 decreases.⁵¹ Another set of two point mutations in the MH2 domain of SMAD4 from colorectal cancer cell lines also result in SMAD4 failing to form complexes with activated SMAD2, and a weaker response of SMAD4 to the TGF- β stimulation (Table 1).³⁶ R361C mutation in the C-terminus was detected in the patients of juvenile polyposis and HHT, negatively regulating the SMAD4 protein function by preventing its oligomerization.^{52,53} Similar spectra of somatic mutations were found in the pancreatic cancers and colorectal cancers.^{53–55} Carriers of SMAD4 mutations appear to have a higher risk of carcinogenesis. In colorectal cancers, a higher frequency of mutations in SMAD4 represents relatively advanced stages of cancer, probably distant metastasis.⁵⁴

Table 1 Representative Mutations of SMAD4 in Different Cancers and the Impacts on the Functions of SMAD4

Disease	Mutated Domain	Mutation Type	Results	References
Colorectal Cancer	MH2	Deletion, Insertion, Transition	Involve in distant metastasis	[54]
	MH2	Missense (D ³⁵¹ → H, D ⁵³⁷ → Y)	Disrupt the interaction with SMAD2	[36]
	MH1	Transition (L43 to R135)	Reduce the ability to bind DNA	[96]
Myhre Syndrome	MH2	Missense (p.R496C)	Impair the ubiquitination of SMAD4; Affect the stability of SMAD4/p-R-Smads complexes	[97]
	MH2	Missense (I500)	Increase the stabilization of SMAD4; Perturb the binding specificity or affinity of SMAD4	[98, 99]
Breast Cancer	MH2	c.1350G>A (p.Q450Q) c.1214T>C (p.F362F)	Disrupt the exonic splicing enhancers (ESE)	[100]
Acute Myelogenous Leukemia	MH1	Missense (P102L)	Inhibit DNA-binding ability	[47]
	MH2	One frame shift (D(483 ± 552))	Block nuclear translocation	[47]
Cervical Cancer	MH2	Transition G/A	—	[101]
Liver Cancer	MH1	R100T	No disruption on SMAD4 functions	[102]

Note: “—” means no reports showing the consequence of the mutations.

Chung and Mortel⁵⁶ reported a case, in which the patients with combined juvenile polyposis syndrome and hereditary hemorrhagic telangiectasia (JPS/HHT) diagnosed through MRI and endoscopic correlation, and recommended screening the patients using combined JPS/HHT for mutations in *SMAD4* gene mutations. Due to mutation or deletion, SMAD4 was inactivated and posed a risk to the patients. In the pancreatic cancer, inactivation of SMAD4 reduces the chances of survival of the patients, and result in their poorer prognosis.⁵⁷ Mutated SMAD4 is rapidly degraded through SCF^{β-TrCP1} E3 ligase-mediated protein ubiquitination.⁵⁸ Wild type SMAD4 can also be ubiquitinated, leading to its inactivation. Recently, αB-crystallin, a small heat-shock protein, was found to be important for nuclear localization of SMAD4, and it was able to interact with E3-ubiquitin ligase to prevent the ubiquitination of SMAD4.⁵⁹ However, not all mutations are harmful to SMAD4. The single codon mutation of I500 has two distinct consequences: causing Myhre syndrome and stabilizing SMAD4 (Table 1).⁵⁰ These findings suggest that the mutations of SMAD4 are important in diagnosing cancer and devising effective therapies.

Abnormal Expression of SMAD4 Benefits to Tumors

SMAD4, the only co-Smad, is important in signal transduction through the classical TGF-β/Smad pathway, and interacts with other transcription factors, including R-Smads, FAST-1, and TIF1-β, to regulate the proliferation, growth, and differentiation of cells.^{31,60–62} Delta-FosB was shown to positively regulate the mRNA level of SMAD4 in the goat mammary epithelial cells.⁶³ In the rod photoreceptors of mature retina, neural retina leucine (Nrl) is reported to control the expression of SMAD4.⁶⁴ Methylation of the promoter of SMAD4 affects its expression as well.

Loss of SMAD4 expression was reported in many invasive cancers, such as ampulla of pancreatic cancer, colorectal cancer, vater carcinomas, breast carcinoma, resulting in failure to inhibit cancer cell growth.^{54,65–67} In different stages, expression patterns of different genes vary in the tumors. In pancreatic cancer, the expression of SMAD4/DPC4 was suppressed by miR483-3p and down-regulation of SMAD4 expression was reported to have a role in the carcinogenesis of adrenocortical carcinoma.⁶⁸ Expression of SMAD4 was also reported to

be lost in gastrointestinal cancer due to the genomic deletion at chromosome 18q, resulting in the cytologic grade and cellularity higher, also with destructive invasion, and lower overall survival.⁶⁹ Park et al⁷⁰ recently reported that the loss of expressions of SMAD4, p53, and E-cadherin, together promote the development and metastasis of gastric adenocarcinoma. It is reported that loss of SMAD4 enhances aggressive tumor behavior through up-regulating PGK1 in pancreatic ductal adenocarcinoma (PDAC) and Yu et al showed that the loss of SMAD4 expression predicts a poor overall survival in PDAC.^{71,72} It is becoming clear that switching of tumor-suppressive activity of TGF- β to tumor-promoting interactions is the result of loss of SMAD4, and triggered by the Smad4-independent TGF- β signaling.⁷³ Loss of SMAD4 promotes the BMP-induced epithelial-mesenchymal transition (EMT) through the ROCK pathway in colorectal cancer.⁷⁴ Loss of SMAD4 decreases the expression of Brca/Fanc, resulting in the accumulation of DNA damage leading to tumor formation.^{73,75}

Although SMAD4 expression is lost in many tumors, the SMAD2/3 expression remain unaltered,⁷⁶ suggesting that there may be some molecules that, at least partially, compensate for the loss of SMAD4, allowing the TGF- β /Smad signaling pathway to continue. Ahmed et al demonstrated that, when the human SMAD4 is mutated in breast cancer cells, resulting in its loss of function, the SMAD4 of *Schistosoma mansoni* could replace it, and respond to the TGF- β signaling pathway.⁷⁷ Mutations in the MH2 domain reduce the affinity of binding between SMAD4 and DNA, and weaken the transcriptional responses, and this is compensated by SMAD3.^{78,79} However, the mechanism of such a compensation for inactive SMAD4 due to mutations or abnormal expression remains unclear.

Functions of SMAD4 and Its Mutants in T Cells

SMAD4 Regulates T Cell Differentiation, Proliferation and Growth in TGF- β -Dependent or -Independent Manner

TGF- β plays crucial roles in modulating the immune response and inflammation. Effector Th1 cells were associated with autoimmune diseases. IL-10, a cytokine produced by Th1, which was resulting from TGF- β mediated SMAD4 binding to the promoter of IL-10, was reported restrained autoimmune inflammation.⁸⁰ In the human T cell line HuT78, treatment with TGF- β induces the

expression of TTP, which is regulated by Smad proteins.⁸¹ Th17 cells derived from the naïve T cells, can be induced by IL-6 and IL-21. In the context of IL-21, SMAD4 could suppress the differentiation of Th17 cells, by directly binding to the *Rorc* promoter region along with SKI.⁸² In presence of TGF- β , this suppression is eliminated, because of the degradation of SKI.⁸³ SMAD4 is also reported important for regulating the proliferation of activated T cells in TGF- β -independent manner, which mediated by the transcription factor Myc.⁸⁴ Although SMAD4 deficiency does not affect the survival of T cells significantly, the proliferation, growth, and the TGF- β -induced differentiation of SMAD4-deficient T cells were reported to be impaired.⁸⁵ The cytotoxic function of CD8⁺ T cells is important for the immune response. Generation of memory-precursor of CD8⁺ T cells was controlled by SMAD4, and its deficiency weakens the response of memory CD8⁺ T cells, as their cytotoxic function is partially impaired.⁸⁶ During chronic viral infection, the CD8⁺ and CD4⁺ T cells are suppressed. The differentiation and accumulation of CD4⁺ T cells were required SMAD4.⁸⁷ Kim et al explored the role of SMAD4 in T cells from the nonobese diabetic mice with an autoimmune disease, and found that SMAD4 plays a protective role in the development of autoimmune Sjogren's syndrome.^{85,88} These findings suggest that the development of autoimmune diabetes benefits from SMAD4 deletion in T cells.

Loss or Deficiency of SMAD4 in T Cells Promoting the Incidence of Diseases

Although TGF- β /SMAD4 signaling was important for T cell function, SMAD4 loss or deficient in T cells was reported associated with incidence and progress of diseases, especially autoimmune diseases. The increased levels and functions of type 9 T-helper (Th9) cells involved in inflammatory disease, accompanied with increased Th2-cell activity.⁸⁹ Further study indicated that SMAD4 was involved in Th2 differentiated into Th9 through TGF- β /SMAD3/SMAD4 and interferon-regulatory factor4 (IRF-4) signaling pathway and loss expression of SMAD4 lead to Th2-related cytokines, such as IL-4 and IL-13, and serum IgA over-production, which was associated with the pathogenesis of human IgA nephropathy.^{89,90}

Sjögren's syndrome (SS), an autoimmune disease, which usually studied in nonobese diabetic mice for pathology, targets the exocrine glands including salivary

glands and lacrimal glands where mainly CD4⁺ T cells infiltrated in affected individuals.^{91,92} It is reported that deletion of T-cell-specific SMAD4 increased the activation of effector T cells leading to up-regulation of IL-17 in nonobese diabetic (NOD) mice.⁸⁸ Donghee Kim et al reported that loss of SMAD4 increased the number of activated/memory CD4⁺ T cell and elevated proliferation potential of effector T cells, and dysregulated T cell activation, which promoted the progress of autoimmune diabetes and the incidence of the disease.⁸⁵ These results indicated that SMAD4 in T cells plays critical role in immune response affecting the development of SS in NOD mice.

Mutations of SMAD4 in T Cells Promote the Tumor Development Showing Potential Targets for T Cell-Related Therapy

Although SMAD4 deletion or deficiency is well studied in regulating T cells differentiation, proliferation, and growth,

it is reported to be mutated in T cells as well, which usually promote tumor development.^{88–90} Two mutations of SMAD4, SMAD4 (P102L) and SMAD4Δ (483–552), have been reported in acute myelogenous leukemia.⁴⁷ SMAD4 (P102L) lost its ability to bind to the Smad binding element (SBE), while SMAD4Δ (483–552) blocks the translocation of SMAD4 into the nucleus, leading to loss of the transcription function of SMAD4. Thus, both of these interrupt the TGF-β signaling triggered growth-inhibition process in leukemogenesis.⁴⁷ The loss of SMAD4-dependent signaling in T cells leads to spontaneous epithelial cancers throughout the gastrointestinal tract in mice.⁹³ SMAD4^{-/-} T cells produce abundant Th2-type cytokines including IL-5, IL-6, and IL-13, which are the known mediators of stromal expansion of plasma cells.⁹³

With the development of immunotherapy, SMAD4 or its mutants are considered as potential targets for T cell-related therapy. SMAD4 mutations and other infiltrating immune markers, including CD15, CD117, and CD206, are thought to predict the cancer recurrence and survival of patients with pancreatic ductal adenocarcinoma (PDAC) patients

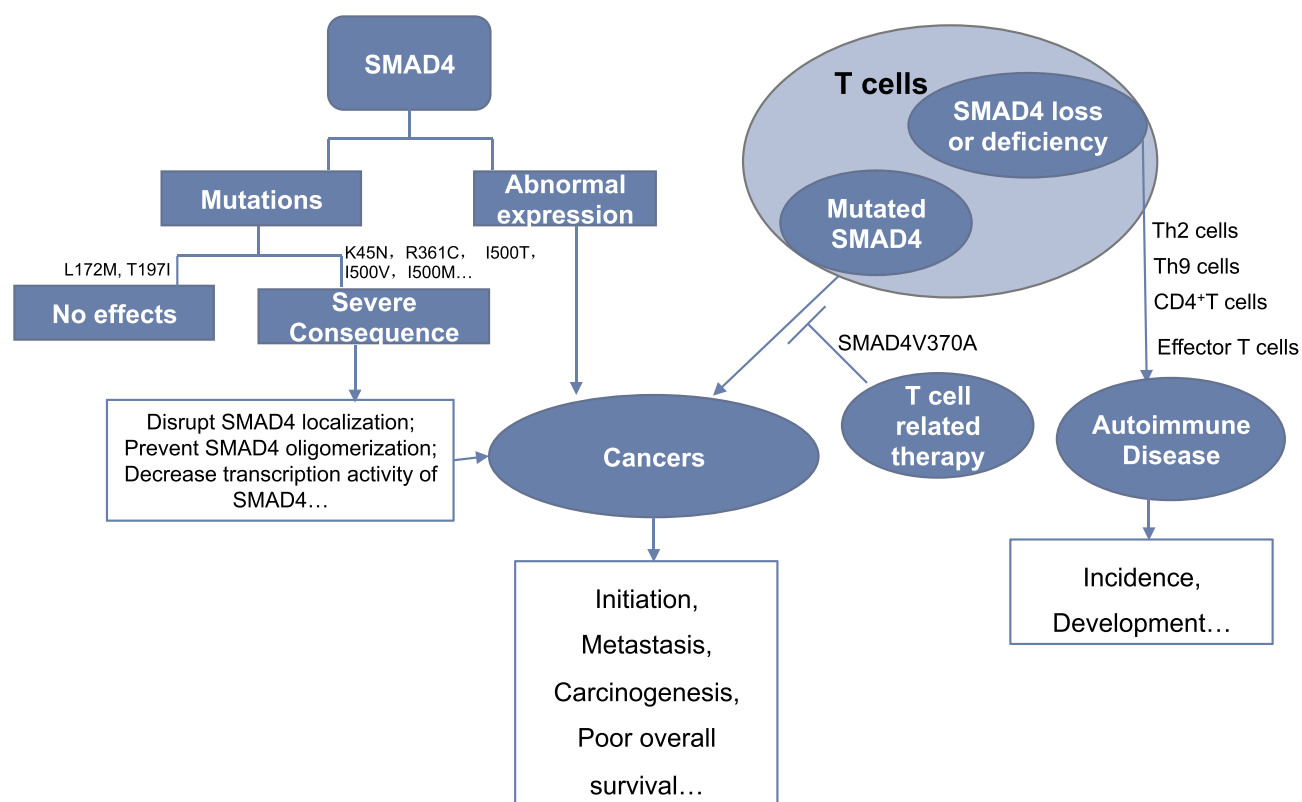


Figure 2 SMAD4 was reported mutated or abnormally expressed in tumors and T cells. These mutation and abnormal expression mainly resulted in severe consequence, which facilitates tumor initiation and growth, metastasis, carcinogenesis, and prognosis poor overall survival. T cells with mutated SMAD4 were reported promote tumor development. Recently, T cell-related therapy targeted on mutated Smad4 showed positive outcomes in tumor therapy. Loss or deficiency of SMAD4 in T cells was reported associated with the incidence and development of autoimmune diseases.

after surgery.⁹⁴ Mennonna et al⁹⁵ reported SMAD4V370A mutation in the colorectal cancer samples, using high-throughput DNA sequencing. When they used peptides containing SMAD4V370A residue to stimulate T cells, the CD8⁺T cells were induced, which could specifically recognize the CRC cells expressing SMAD4V370A.⁹⁵ These new epitopes of SMAD4 can be new targets for T cell-related cancer therapy.

T cell-specific SMAD4 was involved in T cell differentiation, proliferation and growth. The loss or deficiency of SMAD4 resulted in dysfunction of T cells and even the immune diseases; however, the mechanisms of SMAD4 loss or deficiency are still unclear and need more exploration. Interestingly, some mutations of SMAD4 could be targets for immunotherapy. Therefore, it is worthy to explore the functions or consequences of mutated SMAD4 to find more targets related to T cell-specific SMAD4.

Conclusion

SMAD4, a tumor suppressor gene is involved in the TGF- β /Smads signal pathway to inhibit tumor cell growth and is frequently mutated in human tumors, leading to loss of function and put the patients at risk (Figure 2). Most of the mutations occur in the MH1 and MH2 domains, resulting in inactivation of SMAD4 and promoting the growth and distant metastasis of tumors. The consequences of mutations are complex and attractive, and the studies focused on this may provide new insights for new tumor therapeutic. The abnormal expression of SMAD4 also has negative consequences for patients, and new studies on the regulation of SMAD4 expression are needed. SMAD4 in T cells regulates cell survival, proliferation, differentiation, and growth. However, loss or deficiency of SMAD4 in T cells often leads to immune diseases and mutations of SMAD4 in T cells promote tumor development. Interestingly, some mutations could activate the immune system, showing their potential as targets for T cell-related therapies.^{94,95}

Future studies need to explore the functions of mutated SMAD4, and new epitopes based on SMAD4 mutants in cancers and T cells. This work is expected to provide new insights into cancer diagnoses, anti-cancer drug design, and novel strategies for cancer therapeutics.

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Disclosure

The authors declare that they have no competing interests.

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