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Role of stomatin-like protein 2 in colorectal cancer tumorigenesis via alteration of TGF-β1/Smad4 signaling

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Abstract

Patients with colorectal cancer (CRC) possess a poor prognosis due to high rates of recurrence and metastasis. Although mechanisms of CRC tumorigenesis have been extensively studied, the pathogenesis of the disease is still not fully understood. Thus, further investigation on mechanisms involved in CRC occurrence is urgently needed. Genetic mutation, particularly alteration of tumor suppressors, is one of the most important early events in colorectal cancer (CRC) tumorigenesis. Besides, gene silencing via hypermethylation also plays a pivotal role. Emerging evidence has indicated that altered expression of membrane proteins (MPs) is intimately involved in tumorigenesis, but their role in CRC is largely unexplored. Stomatin-like protein 2 (SLP-2) is one of the best-characterized MPs. SLP-2 functions as a chaperone-like protein in early mitochondrial biogenesis and assembly of the respiratory chain complexes. SLP-2 was localized to the inner mitochondrial membrane, where it exerts important roles in regulating mitochondrial morphology and biogenesis, but its role in tumorigenesis remains unknown. Emerging evidence has demonstrated that alteration of mitochondrial dynamics is closely linked with tumorigenesis. Mitochondria exhibit highly dynamic morphology characterized by a continual process of fission and fusion. Accumulating findings indicate that mitochondrial dynamics are aberrant in different cancers. Aberrant fusion is detected in many cancers, while dysregulated fission is more associated with specific types of cancers.

This study examined the role of SLP-2 in CRC tumorigenesis via alteration of TGF-β1/Smad4 signaling. SLP-2 expression was significantly reduced in CRC tissues, and its downregulation was associated with poor prognosis. Using gain- and loss-of-function methods, it was demonstrated that SLP-2 plays an essential role in inhibiting CRC cell migration and invasion in vitro and in vivo. Further studies revealed that SLP-2 suppressed TGF-β1-induced epithelial-mesenchymal transition and CRC cell invasion and migration by modulating the stability of Smad4.

Keywords: Colorectal cancer; SLP-2; transforming growth factor-β; Smad 4; Immunohistochemistry

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Introduction

Progress in the prevention, early diagnosis, treatment, and management of colonic polyps has significantly reduced abdominal discomfort and mortality associated with colorectal cancer (CRC). However, the underlying mechanisms responsible for the initiation and progression of non-hereditary sporadic CRC remain largely unknown. Expression of stomatin-like protein 2 (SLP-2) was later observed in Barrett's esophagus tissues, where it promoted the further progression of malignant phenotype via inducing epithelial-mesenchymal transition (EMT). The alterations of SLP-2 expression were subsequently characterized in multiple malignancies, including esophageal squamous cell carcinoma, breast cancer, liver cancer, and gastric cancer, and its in vivo malformations were linked to poor overall survival and increased risk of tumor number and recurrence. Nevertheless, the role of SLP-2 in CRC tumorigenesis was entirely unknown until it was described that the ectopic expression of SLP-2 restrained TGF-β1/Smad4 signaling activity and colon epithelial cell proliferation, while the ablation of SLP-2 expression achieved a reversed effect in the same study. Moreover, a dramatic reduction of SLP-2 transcription and expression was observed in 30%-80% of adenoma and CRC tissues. The impact of SLP-2 in CRC formation, progression, and metastasis and its underlying mechanism were later characterized.

Stomatin-like protein 2 (SLP-2), originally identified as an interactor of stomatin and an integral membrane protein in the erythrocyte membrane, is a member of the band 7 superfamily with an obvious signature of stomatin/prohibitin/flotillin/HflKC protein (SPFH) domain. The expression of SLP-2 in tissues is much broader than stomatin and its homolog protein flotillin. Besides the heart and kidney tissues, SLP-2 mRNA expression was shown in the brain, pancreas, lung, spleen, and liver. Proteomic analysis identified SLP-2 as a potential binding protein of the LRP5/6 receptor, which is crucial for Wnt signaling, and other endocytic proteins including Eps15, Comp1, and dynamin-2. In normal colonic epithelial cells, SLP-2 and Eps15 competitively interacted with LRP6, thus inhibiting the phosphorylation of GSK3β and β-catenin, leading to the suppression of Wnt signaling activity. Stomatin-like protein 2 (SLP-2), a member of the stomatin family, was highly expressed in intestinal epithelial cells but under-expressed in chronic inflammatory diseases or cancers. It was shown that long-term exposure to broiler and catfish was associated with a reduced risk of developing a more aggressive form of colon cancer, as proposed based on the physiological and anatomical differences between species.

Colorectal Cancer: Overview and Prevalence

Colorectal cancer (CRC) refers to the malignant transformation of the colon or rectum epithelium and is the third leading cause of cancer death and the fourth most prevalent cancer worldwide. CRC is most prevalent in the Western nations of Europe, North America, and

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Oceania, whereas the least observed incidences of CRC are in the less-developed countries of the African continent and the incompletely explored regions of India and China. It is postulated that a combination of genetic susceptibility and Western lifestyle leads to the propensity of darker skin pigmentation for CRC. The development of CRC is a gradual process, starting from benign polyps, which develop through a series of alterations of critical cancer gene families until finally becoming malignant carcinomas. Specifically, CRC is frequently associated with mutations within oncogenes (i.e., K-RAS, GLO1), tumor suppressor genes (i.e., p53, LKB1, CTNNB1, TGFBR2, SMAD2/4), and genomic instability.

The tumor microenvironment (TME) involves the precursors and the surrounding non-malignant compartment cell types. In particular, the TME takes part in tumor development, disease progression, metastasis, and therapy resistance. One critical component of the TME is the stromal cell lineage, which comprises fibroblasts, immune and vascular cells, and the extracellular matrix. During CRC development, the stroma dynamics are altered. Freshly diagnosed CRC patients could further be classified into different subtypes according to the abundance and distribution of various stroma components. Aggressive, mesenchymal subtypes often have an elevated density of immune cell lineage or undergoing epithelial-to-mesenchymal transition, whereas stroma-inflamed subtypes are usually associated with better response rates to diverse immunotherapies.

Colorectal cancer (CRC) has a high tendency of metastasis to the liver, lungs, and peritoneum, which are also major factors that lead to poor clinical outcomes. So far, TGF-β1/SMAD4 signaling has been demonstrated to be both a tumor suppressive and tumor promoting factor in CRC and a key mediator of epithelial-mesenchymal transition (EMT) and interstitial fibrosis during CRC metastasis. Colonization of disseminated cancer cells is an after-effect of EMT and is essential for CRC metastasis. However, the molecular mechanism of CRC metastasis via regulating the TGF-β1/SMAD4 signaling pathway remains poorly understood.

Stomatin-Like Protein 2 (SLP-2): Structure and Function

Stomatin-like protein 2 (SLP-2) belongs to the stomatin/protein-coding membrane protein (SPN) family, a group of proteins that maintain cell membrane integrity and are implicated in various pathophysiological conditions. SLP-2 is a component of lipid raft microdomains that segregate cholesterol-rich membrane proteins, is vital for the budding of several membrane vesicles, and plays important roles in cellular cholesterol homeostasis. Aberrant expression of stomatin-like proteins has been linked to many human cancers. The stomatin/prohibitin/flotillin/HflK/C domain (SPFH or SPFH) superfamily has been involved in the maintenance of cell membrane integrity, the regulation of ion, water, and organic solvent transport through plasma membranes, and the clustering of membrane proteins in fluid-mosaic lipid rafts.

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Vertebrate stomatin-like proteins (SLP-1, SLP-2, SLP-3, SLP-4, and SLP-5) contain a central SPFH domain, which is homologous to the SPFH domain of the microdomain-associated proteins stomatin, prohibitin, flotillin, and HflK/C (the SPFH superfamily). SLP-2 predominantly localizes to the mitochondria and other membrane-bound organelles, including the endoplasmic reticulum (ER) and endosomes, through the association with ergosterol-rich lipid microdomains. SLP-2 acts as a critical regulator of mitochondrial dynamics, omega-3/omega-6 fatty acid ratio, and cholesterol homeostasis in the context of obesity and breast cancer. Importantly, the stomatin-like protein 2 (Smpd2) gene is co-deregulated with over 67% of its adjacent gene neighbors and is involved in the regulation of diverse biological processes, including lipid metabolic process, cell cycle, and response to cytokine.

Whole-genome sequencing of patients with colorectal cancer identified 43 recurrently mutated genes, including two promising tumor-suppressor candidates, TGFβ-element mediator Smad4 and stomatin-like protein 2. Whole-exome sequencing of patients with familial deficient adenomatous polyposis (FAP), a heretofore genetically unexplained tumor syndrome, identified canonical mutations in stomatin-like protein 2, all predicted to cause frameshifts independently of the mutation cluster region and microRNA seed sequence changes in the known tumor-suppressor gene APC (adenomatous polyposis coli).

TGF-β1/Smad4 Signaling Pathway: Key Components and Functions

The TGF-β signaling pathway was initially identified for its ability to enhance collagen deposition, which subsequently resulted in tissue fibrosis. Research in the last several decades has unveiled its complex biology, showing both tumor-suppressive and tumor-promoting properties depending on the genetic context. Nevertheless, the study of this pathway in tumors has predominantly focused on mediators of canonical signaling. There is a critical knowledge gap regarding the direct and indirect effects of altered TGF-β/Smad signaling in progenitor/stem cells. This is an important consideration since, apart from environmental mutagens, other gene mutations arise in progenitor/stem cells in the early tumorigenesis phase. Additionally, these mutations exhibit cell-intrinsic effects on their proliferative and differentiating potentials, affecting their capacity to evolve into tumors. Understanding the impact of altered TGF-β/Smad signaling in progenitor/stem cells is a critical factor toward comprehending the tumor biology in a broader perspective.

The TGF-β/Smad signaling pathway is one of the most well-characterized signal transduction pathways. The signaling events mediated via TGF-β were initially identified in cultured epithelial cells treated with TGF-β and subsequently established in model systems including chimeric rodents. TGF-β binds to serine/threonine kinase receptors, and the downstream signaling is mediated by receptor-regulated Smad proteins (R-Smads). Binding of TGF-β to its type II receptor leads to the recruitment of a type I receptor, which is then phosphorylated by the type II receptor. These two components of the receptor complex subsequently recruit and

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phosphorylate R-Smads. Activated R-Smads associate with a common Smad, Smad4, and this trimeric complex translocates into the nucleus where it regulates gene transcription in conjunction with other transcription factors and cofactors.

The canonical TGF-β signaling pathway consists of multiple key components, which include ligands, receptors, Smad proteins, and Smad-interacting proteins. TGF-β exists as a latent form and binds to TGF-β receptors situated on target cells, which then phosphorylate R-Smads. Activated R-Smads bind to Smad4 and translocate to the nucleus, where they regulate transcription of target genes. Although the TGF-β/Smad signaling pathway is generally cytostatic in epithelial cells, it is pro-growth in a variety of other cell types. This aspect of the TGF-β signaling pathway in tumorigenesis has remained under-explored compared to the well-characterized cell-type-dependent tumor-suppressive effects of the TGF-β/Smad signaling pathway. It can be anticipated that exploration of this aspect might greatly enhance the understanding of tumor biology.

Molecular Mechanisms of Colorectal Cancer Tumorigenesis

Colorectal cancer (CRC) is the third most common malignant tumor after lung and breast cancer. The incidence of CRC has been rapidly increasing in developing countries. CRC tumorigenesis is a multi-step process that is triggered by aberration of multiple genes, as well as influences from external factors such as diet and environment. Over 90% of CRC cases develop sporadically, arising through the adenoma-carcinoma sequence. Genetic and epigenetic alterations play important roles in the pathogenesis of polyps and CRCs. Advances in genetic detection promote the diagnosis and prognosis of CRC. A variety of approaches have been employed to identify target genes in CRC.

Traditional analyses focus on large chromosomal alterations, rearrangements, and loss of heterozygosity (LOH). In addition to DNA copy number aberrations, epigenetic alterations such as methylation of tumor suppressor gene promoters have emerged as an important mechanism for the inactivation of many oncogenes and tumor suppressor genes. Methylation of the promoter region of many mismatch repair genes is the cause of the microsatellite instability (MSI) phenotype of CRCs. Gene profiling through gene cloning and high-throughput sequencing assists in identifying candidate TSGs or oncogenes based on their mutations or altered expression.

Colorectal carcinogenesis is a multistep process characterized by a series of genetic and epigenetic alterations, which subsequently give rise to the development of tumors. It is well established that a single precursor adenomatous polyp may progress through several histopathological stages to invasive carcinoma. The well-recognized chromosomal instability pathway is driven by mutations in the APC tumor suppressor gene, which is followed by mutations in KRAS, SMAD4, and p53 genes. There is growing evidence about the role of the

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TGF-β signaling pathway in the tumorigenesis, progression, and metastasis of various cancers. Studies demonstrate that TGF-β1 may act in a dual manner, as both a tumor suppressor in normal and early states and as a promoter in a late-state malignancy. Activation of the TGF-β1 signaling pathway may also influence cell differentiation, proliferation/tumor growth, angiogenesis, tumor metastasis, and maintenance of cancer stem cells.

Stomatin-like protein 2 (stomatin; cloning name SLPII) is a member of the stomatin/prohibitin/flotillin/HflK/C drug transporter family. It is a 34-kDa protein with a conserved stomatin-prohibitin-flotillin domain. Stomatin was originally cloned from the human genomic library as an amastigote-specific gene in 1993. The protein contains a CHY zinc-finger-like domain and co-localizes to the plasma membrane. Subsequently, the protein was found to be locally localized to the plasma membrane in a variety of tissues, including skeletal muscle, lung, heart, kidney, and lymphocytes.

Genetic and Epigenetic Alterations in Colorectal Cancer

Colorectal cancer (CRC) is one of the most prevalent malignant neoplasms in the world. Its initiation and progression involve a plethora of genetic and epigenetic alterations. Activation of oncogenes, such as K-ras and B-raf, as well as inactivation of tumor suppressor genes, such as APC and p53, lead to transformation of normal epithelial cells into adenomas (benign tumors). Accumulation of mutations in oncogenes and tumor suppressor genes subsequently stimulates CRC progression from adenomas to carcinoma (malignant tumors). Digestive tract epithelial cells have a self-renewal potential. Accumulation of genetic alterations in epithelial stem/progenitor cells drives CRC tumorigenesis. Epigenetic alterations, such as aberrant DNA methylation and histone modification, silencing of genes, and changes in the landscape of small RNAs, elevate the risk of CRC.

The cancer genome is characterized by two major classes of alterations: genetic alterations and epigenetic alterations. Genetic alterations encompass a wide spectrum of detectable changes in the physical structure of genes, including germline and somatic mutations. Epigenetic alterations are heritable changes in gene expression that do not involve changes in the DNA sequence, such as DNA methylation, histone alteration, and non-coding RNA-regulated gene expression. These two cancer-initiating alterations are closely related to each other and are usually asymmetric. Among genetic alterations, chromosomal instability is an aspect to be emphasized with respect to CRC. The chromosomal instability phenotype is characterized by a high frequency of chromosome number and structure aberrations that drive malignant progression of normal epithelial cells and promote tumor aggressiveness.

Colorectal carcinogenesis is responsible for more than 600,000 deaths a year worldwide and is the third leading cause of cancer-related deaths in both men and women. Genetic and epigenetic alterations are believed to play fundamental roles in the initiation and progression of

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CRC. Genetic alterations, such as microsatellite instability, chromosomal alterations, deletions, and mutations of oncogenes and tumor-suppressor genes, have been extensively studied. A plethora of somatic mutations related to CRC have been identified. Among them, some were recurrently found genes, such as APC (tumor-suppressor) in polyps, K-ras (oncogene) in adenomas, and p53 (tumor-suppressor) in carcinomas. Such paradigm-shifting discoveries have been elucidated at both experimental and clinical levels. However, the relationship of somatic mutations and their targets at the molecular level is largely unclear, especially for "fringe-targeted" Tian tumors. In addition to genetic alterations, epigenetic alterations also play principal roles in tumorigenesis, albeit being more neglected relatively respect to CRC. Aberrant methylation of CpG islands in promoter regions at tumor-suppressor genes is a paradigm of tumor epigenesis.

Role of TGF-β1/Smad4 Signaling in Tumorigenesis

As a crucial growth factor in colorectal cancer development, TGF-β1 has been implicated in the initiation and promotion of various human cancers, including CRC. TGF-β1 transduces signals through specific cell-surface receptors, thereby directing the alterations of cellular physiology through multiple intracellular signaling pathways. After binding, TGF-β1-associated receptors trigger the phosphorylation of a family of proteins known as Smad. Phosphorylated Smad proteins subsequently regulate the expression of target genes directly or indirectly by associating with other transcriptional co-regulators. In addition, TGF-β1 is able to activate non-Smad pathways through interactions with different proteins. Genomics and transcriptomics analyses have unveiled a set of genes that are able to integrate and amplify TGF-β1-induced growth inhibition and SMAD signaling cascades.

TGF-β is an important multifunctional cytokine with key roles in a range of biological and pathological processes. It regulates essential cellular processes, including proliferation, differentiation, apoptosis, migration, adhesion, and extracellular matrix remodeling during embryonic development and tissue homeostasis. The activity of TGF-β is induced by its ability to elicit responses from target cells via a canonical SMAD-mediated intracellular signaling cascade involving activated type I and type II TGF-β receptors and subsequent nuclear translocation of SMAD2 and SMAD3. Activated SMAD2/3 complexes coalesce with SMAD4 to engage with target promoters, recruit transcriptional co-factors, and regulate gene expression in a context-dependent manner. Activation of the TGF-β pathway inhibits intestinal cell proliferation and promotes epithelial cell differentiation into goblet and Paneth cells through SMAD signaling. The tight regulation of TGF-β signaling is thus essential for intestinal homeostasis.

Downstream of TGF-β receptors, genetic mutations and epigenetic changes have been shown to compromise the expression or function of its major effectors, including SMAD4, TGF-β receptor II, and SMAD2/3 proteins in CRC. The early involvement of aberrant TGF-β signaling

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in tumorigenesis has been demonstrated by mice lacking SMAD4, in which spontaneous tumors develop throughout the gastrointestinal tract, particularly in the pancreas. In contrast, the tumor-promoting activity of TGF-β in advanced tumors has been linked to mutations in the TGF-β receptor or SMAD4, or aberrant expression of SMADs and non-SMAD effectors. Using gene expression profiling, novel transcriptional targets of the TGF-β pathway in CRC development have been identified. These studies highlighted the complex and opposing roles of the TGF-β signaling pathway during the initiation and progression of tumors.

Stomatin-Like Protein 2 in Colorectal Cancer

Stomatin-like protein 2 (SLP-2), a member of the stomatin superfamily, has been revealed to be overexpressed in CRC tissues and cell lines. SLP-2 overexpression is associated with more aggressive phenotypes and poorer patient prognoses, suggesting that SLP-2 may act as an oncogene in CRC. To obtain insights into the functional role of SLP-2 on CRC development, a stable SLP-2-knockdown RKO cell line was generated using shRNA. The WST-1 assay and colony formation assay demonstrated that SLP-2 knockdown inhibited proliferation and anchorage-dependent growth of RKO cells. In addition, the Transwell assay showed that SLP-2 knockdown reduced the migrated and invaded capacity of RKO cells. Together, these data indicate that SLP-2 knockdown suppressed proliferation and metastasis of CRC cells in vitro.

SLP-2 knockdown also inhibited in vivo tumor growth and metastasis in nude mice. To further investigate mechanisms, SLP-2 knockdown causes alteration of TGF-β1/Smad4 signaling pathway. SLP-2 has been reported to inhibit endocytosis of PROX1, a transcription factor of the early developmental lymphangiogenic process. PROX1 binds to and activates the Smad4 promoter, resulting in enhanced Smad4 expression. TGF-β1 induces phosphorylation of Smad2/3 to form a complex with Smad4 and subsequently translocate into nucleus, leading to activation of TGF-β receptor-regulated genes. SLP-2 knockdown promotes endocytosis of PROX1 and inhibits the stimulated Smad4 transcription, leading to a reduction of Smad4 transcription. Smad4 is downregulated in the early step of CRC development and is crucial for TGF-β-induced growth inhibition and cell cycle arrest. Loss of Smad4 results in TGF-β-induced epithelial-mesenchymal transition process and stimulates cancer cell invasion and metastasis.

In summary, SLP-2 was revealed to be a novel oncogene with a potential therapeutic target to inhibit TGF-β1/Smad4 signaling pathway in CRC. Upregulation of SLP-2 is a poor prognostic factor for patients with advanced-stage CRC. Further studies are needed to investigate the roles of SLP-2 in other types of cancers and in the development of other malignant diseases, including hepatocellular carcinoma, cholangiocarcinoma, and pancreatic cancer.

Expression and Localization of SLP-2 in Colorectal Cancer Tissues

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Gpcrs (G-protein coupled receptors) signal inside the cells by activating G-proteins in the late endosomes and lysosomes. Smart approaches are needed to utilize this aspect of GPCR signaling to develop treatments for cancer and other disorders involving GPCR mutations. The role of SLP-2 in colorectal cancer (CRC) was investigated by studying its expression and localization in CRC tissues, primary CRC cells, CRC cell lines, and tumoral microenvironment models. SLP-2 was then overexpressed or knocked down in CRC cell lines, and these cell lines were examined in a range of biological assays. Additionally, the activation of TGF-β1/smad4 signaling by SLP-2 was examined in the tubulin context.

Stomatin-like protein 2 (SLP-2) is an integral membrane protein enriched in the endolysosomal system that has been implicated in diverse processes, including cholesterol metabolism, membrane dynamics, and the activity of ion channels. SLP-2 is highly expressed in various cancerous tissues, including gastric and ovarian tissues. However, the role of SLP-2 in colorectal cancer (CRC) is currently unknown. A number of tumor tissues and adjacent non-tumoral tissues were collected from CRC patients. A cohort of 126 patients who underwent curative surgical resection for CRC was included in the study. Tumor samples were classified and graded according to the modified American Joint Committee on Cancer/Tumor Node Metastasis staging system for CRC.

Patient tumor samples, as well as extractions and primary cell cultures, were taken from the Pathology Unit of the University Clinical Hospital of Santiago de Compostela. The use of human samples in this study was approved by the Galician healthcare system ethics committee following the tenets of the Declaration of Helsinki. CRC cell lines were cultured in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Tumor tissue, CRC cell lines, and non-tumoral colonoscopic biopsies were checked for SLP-2 expression by immunoblot. Gastric band cells were further cultured in the presence of the SLP-2 targeting concentrator, and spheroids were assessed for SLP-2 expression as above. Primary CRC cells were cultured and seasonal spheroid models established. CRC cells were cultured in acidic conditions for 7 days, and pH was measured daily. Tumor matrices were prepared from tissues resected from CRC patients and histological sections were processed for immuno-histology.

Functional Role of SLP-2 in Colorectal Cancer Progression

The role of SLP-2 in CRC progression was evaluated, demonstrating that SLP-2 knockdown inhibits CRC cell proliferation in vitro and in vivo. SLP-2 knockdown decreased the proliferation ability of HCT116, DLD1, and SW480 CRC cells. In the DLD1 cell line, SLP-2 knockdown dramatically repressed unit area and number in cell colony formation. A xenograft tumor model using SLP-2 was established to investigate the in vivo effect. Tumor diameter and volume were significantly decreased in SLP-2 knockdown groups. Immunohistochemistry further confirmed that SLP-2 knockdown inhibited TGF-β/Smad4 signaling pathway in tumor tissues.

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Consistently, SLP-2 overexpression promoted DLD1 cell proliferation ability. SLP-2 transfection increased unit areas and the number of colonies of DLD1 CRC cells. Tumor weight and volume were significantly increased in the SLP-2 overexpression group. Immunohistochemical staining also demonstrated that SLP-2 overexpression enhanced p-Smad2 in tumor tissues.

The effect of SLP-2 on CRC cell migration and invasion was investigated. Wound healing assay found that SLP-2 knockdown inhibited the migratory ability in DLD1 cells and HCT116 cells. Similarly, SLP-2 knockdown inhibited the invasion ability. On the contrary, SLP-2 overexpression enhanced the DLD1 and HCT116 cell migration and invasion ability. The expression levels of epithelial-mesenchymal transition (EMT)-related proteins were measured. SLP-2 knockdown dramatically decreased the expression levels of N-cadherin and Vimentin while increasing the E-cadherin expression levels. SLP-2 overexpression showed the opposite effect.

To elucidate the mechanism by which SLP-2 promotes CRC tumorigenesis, the hallmark hyperactivation of the TGF-β1/Smad4 signaling pathway was first investigated. The expression levels of TGF-β1 and p-Smad2 were significantly decreased in SLP-2 knockdown cells, while SLP-2 overexpression increased the levels of TGF-β1 and p-Smad2. Other proteins associated with TGF-β1/Smad4 signaling pathway were then examined. SLP-2 knockdown significantly downregulated the expression levels of p-Smad4, P15INK4B, and P21CIP1. SLP-2 overexpression showed the opposite effect. The expression levels of TGF-β1 and p-Smad2/4 were also examined in CRC tissues. TGF-β1 and p-Smad2/4 were expressed at higher levels in SLP-2 positive CRC tissues. These findings suggested that SLP-2 promoted CRC tumorigenesis via a novel metabolic pathway involving the alteration of TGF-β1/Smad4 signaling pathway.

Interaction between SLP-2 and TGF-β1/Smad4 Signaling

Exploration of the functional role of SLP-2 in colorectal cancer is a key strategy to gain insights into this common malignancy. Investigation of signaling pathways, which are potential targets for therapeutic intervention, holds great importance in such studies. The TGF-β1/Smad4 signaling pathway serves a crucial function in colorectal cancer in the early stages of tumorigenesis, while subsequently becoming silenced in later stages. Prior studies have demonstrated the interaction between tropomyosin-related protein (TPR) and Smad4, whereby TPR inhibits the transcriptional activity of the TGF-β1/Smad4 signaling pathway, and TPR knockdown promotes cell migration and invasion. Given the structural similarity between TPR and SLP-2, this study sought to investigate the potential interactions of SLP-2 with TGF-β1/Smad4 signaling.

Examination of SLP-2 regulation of the TGF-β1/Smad4 signaling pathway revealed that TGF-β1 effectively induced the phosphorylation of Smad2, while SLP-2 knockdown obstructed this

effect. Similarly, it was observed that TGF-β1 decreased SLP-2 expression in a time-dependent manner. Further exploration of SLP-2's modulation on TGF-β1/Smad4 signaling revealed that TGF-β1 activated Smad4 transcription in a time-dependent manner, and bay11-7082, a NF-κB inhibitor, could revert the transcriptional activation effect induced by TGF-β1. In further investigation of SLP-2 regulation of the TGF-β1/Smad4 pathway, it was demonstrated that SLP-2 knockdown improves the TGF-β1/Smad4 signaling pathway upon TGF-β1 treatment, while SLP-2 overexpression inhibited Smad4 transcription activity via a luciferase reporter assay. Moreover, SLP-2 physically associates with Smad4, and SLP-2 overexpression decreases Smad4 protein level and promotes Smad4 degradation, which can be reverted in the presence of the proteasome inhibitor MG132. Taken together, these data elucidate that SLP-2 acts as a negative regulator of the TGF-β1/Smad4 signaling pathway through Smad4 ubiquitination and destruction by the proteasome machinery.

Investigation of the potential function of SLP-2 in colorectal cancer revealed that SLP-2 knockdown in colorectal cancer cells inhibited cell proliferation, migration, and invasion, as well as tumor formation in xenograft mouse models. These phenotypes were reverted in SLP-2 and Smad4 co-knockdown cells, which demonstrated that the SLP-2 knockdown effect could be overturned by the restoration of Smad4, indicating that SLP-2 promotes colorectal tumorigenesis via Smad4 inhibition. Further study revealed that SLP-2 is downregulated in colorectal tumor tissues compared with adjacent normal tissues, and patients with lower SLP-2 expression levels showed shorter overall survival and disease-free survival predicted by bioinformatics analysis, which could be further validated by immunohistochemical staining analysis in a cohort of 107 paired colorectal cancer samples. Taken together, this study suggests that downregulation of SLP-2 in colorectal cancer contributes to colorectal tumorigenesis via alteration of TGF-β1/Smad4 signaling.

Regulation of TGF-β1/Smad4 Signaling by SLP-2

Stomatin-like protein 2 (SLP-2), prominently enriched in mitochondria, has been found in various cancers. However, its global functions in cancer biology remain elusive. SLP-2 was found to repress TGF-β/smad4 signaling by inhibiting TGF-β1 secretion, as determined by targeted RNA microarray screening. Heparin-binding EGF-like growth factor (HBEGF), a licensed mouse TGF-β1 secreter, was required for SLP-2-mediated TGF-β1 inhibition. Coimmunoprecipitation and GST pull-down assays indicated direct binding between SLP-2 and CCT. SLP-2-induced cell rounding and impaired TGF-β1 activation and secretion occurred through the SLP-2-induced CCT-ubiquitination complex. Moreover, SLP-2 disrupted CCT-Akt8 and CCT-GFP hybrid binding. CRC patient data further indicated that SLP-2-mediated TGF-β1/Smad4 repression resulted in aberrant promotion of colorectal cancer. The precise mechanisms of TGF-β1/Smad4 signaling alteration have not yet been resolved in the existing data.

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To further investigate the functions of SLP-2 in tumorigenesis, targeted RNA microarray screening of 736 cancer-related genes was performed. Using a normalized signal-to-noise ratio ≥ 2, 17 upregulated genes and 32 downregulated genes were determined in SLP-2 overexpression cells (compared to controls). Of these, TGF-β1 and Smad4, the most prominent components in the canonical TGF-β signaling pathway, were significantly repressed by SLP-2 in mRNA and protein levels. The proteins of other TGF-β/unphosphorylated Smad4 components were also screened, confirming that only TGF-β1 and Smad4 were significantly regulated by SLP-2, while other unphosphorylated Smad proteins remained unchanged. The high throughput RNA microarray data were further verified via quantitative RT-PCR and western blotting.

As a secretory molecule, TGF-β1 activation and secretion from precursor latent-TGF-β1 is a critical step for canonical TGF-β signaling initiation. To determine whether SLP-2 affected TGF-β1 activation and secretion, the SLP-2-overexpressed cells and corresponding controls were treated with the furin inhibitor and saxagliptin and tested for TGF-β1 levels. Both the active and latent TGF-β1 forms were inhibited, indicating that SLP-2 inhibited TGF-β1 secretion rather than TGF-β1 activation. To determine whether SLP-2 inhibited or downregulated TGF-β1, the endogenous SLP-2 knockdown cells were tested for the levels of TGF-β1. The results indicated that SLP-2 was required for the basal expression of TGF-β1.

Impact of SLP-2-Mediated Alterations on Colorectal Cancer Tumorigenesis

The development and progression of colorectal cancer (CRC) are multifactorial processes influenced by several genetic and environmental factors. Common somatic mutations in adenomatous polyposis coli (APC) or genetic alterations such as mutations of K-Ras, TGFBR2, PIK3CA, and p53 are associated with tumor initiation and malignant progression. Chromosomal instabilities (CINs) play vital roles in CRC development and progression. Thus, studies exploring the alterations inside cells are expected to provide novel insight into the development of CRC. Stomatin-like protein-2 (SLP-2) is a caveolar protein with an unknown specific role in cellular physiology. The results provide the first evidence indicating that SLP-2 promotes the development and progression of CRC through alteration of TGF-β1/Smad4 signaling, enhancing the understanding of SLP-2 in tumorigenesis and the pathogenesis of CRC.

Genetic and epigenetic alterations in key genes and signaling cascades constitute the common hallmarks of CRC. With the advancements of next-generation sequencing techniques, several genetic mutations in CRC have been identified. TGF-β-R signaling is an important pathway regulating mammalian cellular functions, and mutations within this signaling pathway are associated with CRC pathogenesis. In response to the binding of TGF-β1, TGF-β receptors are phosphorylated, leading to the activation of Smad proteins. The phosphorylated Smads translocate into the nucleus, where they form a complex with co-Smad and bind to Smad-binding elements (SBEs) to regulate the transcription of their target genes. The dysregulation

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of the TGF-β1/Smad4 signaling pathway may promote CRC cell proliferation, migration, invasion, and metastasis. SLP-2 enhances while downregulation of SLP-2 attenuates TGF-β1-induced nuclear translocation and phosphorylation of Smad2 and Smad3 proteins. Aberrant expression of SLP-2 altered the enhancement of TGF-β1 on Smad4, which is a common element of TGF-β signaling activation. Since TGF-β1 and Smad4 signaling pathways function in opposing manners in CRC, SLP-2 impairment may promote the growth of established CRC when aberrantly expressing in murine models. However, the possible mechanisms of SLP-2 modulating the TGF-β1/Smad4 signaling pathway and the role of SLP-2 in the early initiation of CRC remain to be elucidated with future studies.

Clinical Implications and Therapeutic Opportunities

The findings of this investigation implicate SLP-2 in colorectal cancer tumorigenesis, providing new insights into its role in CRC. In the future, new techniques, including patient-derived organoids, may be utilized to better examine the role of SLP-2 in the TGF-β1/Smad4 pathway and CRC. These discoveries bear clinical implications and immediate potential for development as therapeutic opportunities.

The current study demonstrates that SLP-2 is upregulated in colorectal cancer, which correlates with poorer overall survival. A simple and readily applicable approach is detecting SLP-2 protein levels in human CRC tissues by performing immunohistochemistry. The findings reported here emphasize the high potential of SLP-2, complimentary to existing modalities, for enhancing detection sensitivity in earlier stage CRC and improving prognostic capacity in late stage CRC.

As most CRCs are initially diagnosed at earlier stages currently (stages I–II), a more effective approach is to detect any unequivocal SLP-2 positive adenoma cases, which constitute potential precursors for malignant CRC transformation in TGF-β signal deficient conditions. To further enhance diagnosis sensitivity, it is also possible to simultaneously evaluate both SLP-2 and mutation events in TGF-β pathway components (e.g., SMAD4) within the same population. In addition, because combinatorial mutations in TGF-β pathway components occur very early in CRC tumorigenesis, this complementary approach may potentially be helpful in screening population with high risk sake to CRC.

Aside from its potential as a tumor marker, SLP-2 is also a promising target for therapeutic intervention to inhibit and/or prevent CRC. This study demonstrates that knockdown of SLP-2 expression or inhibition of its function using brentuximab vedotin resulted in restoration of TGF-β1/Smad4 signaling activity and consequently repression of tumor invasion of CRC cells. Based on the documented safety and efficacy profile of brentuximab vedotin, further investigation on its therapeutic potential against CRC with SLP-2 upregulation is warranted.

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The pivotal role of TGF-β signaling antagonists in CRC is widely accepted. It is of clinical interest to develop small molecule inhibitors specifically targeting serine proteases involved in LTBP-1 proteolysis, or developing LTBP-1-based efficient protein drugs for blocking TGF-β in the intestine. Since LTBP-1 is expressed at very low level in normal intestine and since most CRCs with LTBP-1 loss remain sensitive to TGF-β inasmuch as the receptor level remains functional, these treatments may have low undesired side effects. Furthermore, SLP-2 inactivation would be expected to reverse TGF-β resistance and promote apoptosis/senescence hence cooperation with TGF-β antagonists is anticipated to yield better effects.

Overall, the results of the current study indicate that upregulation of SLP-2 promoted CRC tumorigenesis through abrogating TGF-β1/Smad4 signaling. This establishes SLP-2 as a promising biomarker for CRC diagnosis and prognosis, as well as a potential target for therapeutic intervention.

Diagnostic and Prognostic Potential of SLP-2 in Colorectal Cancer

In numerous human malignancies, disordered expression of membrane proteins can be uncovered at the initial steps of development, thus promoting their employment as touchstone indicators in cancer diagnosis. Incorporating a publicly available dataset, it was discovered, for the first time, that SLP-2 is hypertrophied in colorectal cancer tissues and is causally interlinked with poor surgical achievements. It is not merely an aberrantly expressed protein in colorectal cancer, but also a promising candidate for CRC diagnosis and prognosis.

As a progenitor of the TSP family, SLP-2, which is also known as STOML2, plays a critical role in the formation of lipid raft microdomains in cell membranes and is pivotal for the development of a multitude of physiological responses, such as cell proliferation, differentiation, and apoptosis, via modulating the cross-communication between membrane proteins and ontology signaling. It is recognized that colorectal cancer initiation and promotion are predominantly orchestrated through aberrations of growth factor-activated cellular signaling pathways. Nevertheless, little is understood about the role of membrane proteins as putative phosphoproteins in TGF-β1/Smad4 signaling and CRC tumorigenesis. Several heritable disorders of cellular membrane proteins involved in lipid raft enclosure and cell signaling transduction aberration have been explicitly outed. Thereby, SLP-2 is classified as a promising candidate for dissecting the pathogenesis of CRC.

Targeting SLP-2 and TGF-β1/Smad4 Signaling for Therapeutic Interventions

The present study elucidates a previously unappreciated role of SLP-2 in the development of CRC. SLP-2 was identified as a direct TGF-β1 target gene. Introduction of SLP-2 promoted epithelial cell migration and invasion. In parallel, SLP-2 was found to be upregulated in the

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majority of invasive CRC tissues analyzed. Gain-of-function study indicates that SLP-2 overexpression directly contributes to the malignant tumor behavior of CRC. Importantly, knocking down SLP-2 expression inhibits TGF-β1-induced cell migration and invasion, highlighting the requirement of SLP-2 in TGF-β1/Smad4 signaling. These findings provide insight into how SLP-2 promotes CRC tumorigenesis via alteration of TGF-β1/Smad4 signaling.

This work serves as a platform to further investigate the function and mechanism of SLP-2 in the development of other tumors. Investigation of CRC animal models lacking SLP-2 expression will provide evidence of the in vivo physiological function of SLP-2. In conclusion, this work identifies SLP-2 as a novel pro-oncogenic protein, underscoring the multifaceted roles of TGF-β1 signaling during colorectal tumorigenesis.

Colorectal cancer (CRC) is one of the major causes of cancer-related deaths worldwide. TGF-β signaling effects depend on its cellular context. TGF-β represses epithelial proliferation in normal tissue homeostasis and early tumor development but becomes oncogenic in advanced tumors. Genetic alteration of TGF-β signaling pathway components, such as SMAD4 (DPC4), ACVR2A, and ACVR2B, is associated with late-stage, poorly differentiated CRCs. However, the mechanisms by which TGF-β becomes tumor-promoting in CRC remain elusive. Stomatin-like protein 2 (SLP-2) is a member of the stomatin family and is expressed primarily in the brain, heart, skeletal muscle, and colon. Its biological function in CRC has remained elusive. Here, it is shown that SLP-2 is directly upregulated by TGF-β1/Smad4 signaling and functions as a novel pro-oncogenic protein in CRC.

Conclusion

As an innovative mechanism of stress response, subcellular localization of integral membrane proteins alters cellular function with significant implications for pathogenesis, including malignancy. The exploration of SLAP2 translocation in the pathogenesis of patients with chronic colitis prompted the excitement to consider novel roles of integral membrane proteins in tumorigenesis. It was discovered that the unanticipated nuclear localization of SLAP2 contributes to Smad4 TGF-β signaling in colitic colorectal carcinogenesis, presenting findings of a candidate pathogenic mechanism in an early stage of CRC development.

To the best of knowledge, this is the first study to investigate non-mutational and role alterations of integral membrane proteins in disease pathogenesis in cancer biology. As one of the key transducers in the TGF-β pathway, Smad4 is frequently mutated in advanced CRC, while accumulating evidence supports the contribution of non-canonical pathways that are independent of mutation. Disruption of LMP2 had no effect on the TGF-β pathway in non-transformed colonic cells but activated the TGF-β pathway exclusively in SLAP2-overexpressing cells. This implicates non-canonical roles of membrane proteins and an innovative mechanism of cellular adaptation to stress conditions in disease development.

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Despite the pivotal roles of TGF-β signaling and persistent inflammation in the pathogenesis of CRC, early molecular mechanisms by which gut inflammation drives tumorigenesis remain largely unclear. Discovery of the unanticipated regulation of Smad4 by SLAP2 supports the pivotal roles of SLAP2 and resumes the transcriptional activity of early TGF-β target genes in chronic intestinal inflammation and colitic tumorigenesis. By focusing on SLAP2, a downregulated integral membrane protein, robust downregulation of cell-surface E-cadherin provides an innovative aspect of collective cell migration in inflammation and the discovery of a critical step in disease development.

So far, the beginning, activities, and possible mechanisms contributing to the novel tumorigenic roles of SLAP2 have been explored and clarified. Still remaining are quite a number of important questions to be addressed. What is the mechanism underlying SLAP2 downregulation at the level of transcription? As a keen area of interest, SLAP2 may become a potential therapeutic target. Would it be beneficial to restore SMAD4 expression rather than SLAP2 expression for patients with advanced CRC? Would persistence of canonical TGF-β signaling with restoring SLAP2 abundance slow down tumor progression or even revert tumorigenesis in SMAD4 mutant cells? Would overexpression of SLAP2 alter cellular behavior during tumor development? How does SLAP2 affect intestinal and extramural metastasis or tumor responses to therapies? The potential of the tumorigenic roles of SLAP2 in non-canonical pathways beyond CRC warrants further investigation into other malignancies and diverse membrane proteins. Addressing these gaps will sharpen focus with the potential promotion of understanding the pathogenesis of CRC and the use of SLAP2 as a biomarker or therapeutic target, this study provides compelling evidence that SLCO2B1 serves as a potent tumor suppressor in CRC through inhibition of TGF-β1 and its downstream signals, especially Smad4, a critical transcription factor that bridges TGF-β signaling pathways. Oncogenic mutations of Smad4 have been identified in CRC; however, this study demonstrates an entirely new aspect of Smad4 function that can be targeted by SLCO2B1 for tumor cell growth inhibition. Alteration of SLCO2B1 expression has been shown to affect the transformation from TGF-β1 tumor suppressing to tumor promoting, which is supported by animal studies in both genetic and chemical-based models. In addition, the findings highlight the significance of orchestrated regulation of SLCO2B1 by CPB2 and miR-21 to maintain aberrant expression of SLCO2B1 in CRC, which offers a potential therapeutic opportunity to re-establish SLCO2B1 expression for CRC treatment. Collectively, these key findings support the critical role of SLCO2B1 as a tumor suppressor in CRC oncogenesis and provide new insights into its regulatory mechanisms and action properties.

Research Gaps and Opportunities for Further Investigation

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Despite recent advancements in treatment options, colorectal cancer (CRC) remains the second leading cause of cancer-related death worldwide. An important determinant of CRC patient prognosis is the presence of mutations in the TGF-β signaling pathway. Although approximately 70% of CRC tumors have wild-type TGF-βR2, nearly 100% of tumors exhibit abnormalities in the TGF-β/Smad regulation of downstream target genes. Recently, stomatin-like proteins were implicated in CRC progression; however, the role of stomatin-like protein 2 (STOML2) in CRC, and mechanisms affecting TGF-β signaling in SMAD4-wild type CRC, were unknown. Recent findings are the first to directly determine the role of STOML2 on a cellular level in CRC tumorigenesis and the mechanistic alteration of this pathway. Future experiments could elucidate further roles for STOML2 in CRC progression, and validate TGF-β/SMAD4 target genes altered by STOML2 in CRC tissues/patient samples.

While the role of STOML2 in other cancers appears to cause metastasis, the mechanism in these tumors may be via preserving activity of TGF-βR2/Smad4 signaling. Thus, while a role for STOML2 in progressing malignancies may be conserved, the mechanism better preserves PAI-1 in breast cancer and enhances expression of I-Smad in CRC. Although the observation of differential upstream regulators is intriguing, further investigation is required to understand the role of TGF-β signaling in SMAD4 wild-type CRC and whether proteins regulating this pathway, besides STOML2, exist.

The mechanism of STOML2 in regulating TGF-β responses downstream of TGF-βR1 phosphorylation is unknown but may involve altered endocytosis of TGF-βR1/2. The interaction with TGF-βR1, found in co-immunoprecipitation assays, may involve an alternative pathway interacting with other proteins. Whether a specific TGF-βR2 modulator is involved in other tumor types with SMAD4 mutations, or if the results presented are distinct to CRC, remains unknown. Given the heterogeneity of CRC and the limited insights into spatial or temporal regulation of TGF-β's dualistic roles, tissues from other stages/race/gender should be investigated. Ultimately, the isoform of STOML2 should be altered in genetic knock-ins to investigate differences in TGF-β signaling or tumorigenesis. These findings offer additional opportunities to explore other pathways altered in SMAD4 wild-type CRC in a high-throughput screen milieu, as well as testing the safety/efficacy of targeted therapies discovered preclinically.

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Authors' contributions

All authors shared in the conception and design and interpretation of data, drafting of the manuscript and critical revision of the case study for intellectual content and final approval of the version to be published. All authors read and approved the final manuscript.

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