Correlation Between SMADs and Colorectal Cancer Expression, Prognosis, and Immune Infiltrates

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Abstract

Background: In current years, the incidence and mortality of colorectal cancer (CRC) are increasing, and the 5-year survival rate of advanced metastatic CRC is poor. The Small mothers against decapentaplegic (SMAD) superfamily is an intracellular signal transduction protein associated with the development and prognosis of a variety of tumors. At present, no study has systematically analyzed the relationship between SMADs and CRC.

Methods: Here, R3.6.3 was used to analyze the expression of SMADs in pan-cancer and CRC. Protein expression of SMADs were analyzed by HPA. GEPIA was used to evaluate the correlation between SMADs and tumor stage in CRC. The effect of R language and GEPIA on prognosis was analyzed. Mutation rates of SMADs in CRC were determined by c-BioPortal and potentially related genes were predicted using GeneMANIA. R analysis was used for correlation with immune cell infiltration in CRC.

Results: Both SMAD1 and SMAD2 were found to be weak expression in CRC and correlated with immune invasion level. SMAD1 was correlated with patient prognosis, and SMAD2 was correlated with tumor stage. SMAD3, SMAD4 and SMAD7 were all low expressed in CRC and associated with a variety of immune cells. SMAD3 and SMAD4 proteins were also low expressed, and SMAD4 had the highest mutation rate. SMAD5 and SMAD6 were overexpressed in CRC, and SMAD6 was also associated with patient OS and CD8+ T cells, macrophages and neutrophils.

Conclusions: Our results reveal innovative and strong evidence that SMADs can be used as biomarkers for the treatment and prognosis of CRC.

Introduction

Colorectal cancer (CRC) is widely known as one of the most pervasive malignancies due to its own third morbidity (10.0%) and second mortality (9.4%) in all cancers worldwide, and its morbidity and mortality are on the rise year by year^[1]. The 5-year survival rate for advanced metastatic colorectal cancer is less than 20%^[2]. The main treatment methods for CRC are surgery, radiotherapy and chemotherapy, which are good for early colorectal cancer, but poor for advanced and metastatic colorectal cancer^[3]. There is no good treatment for advanced metastatic colorectal cancer. In order to eliminate the high incidence and mortality of CRC, further exploration of its meaningful biomarkers is urgently needed to strengthen its therapeutic efficacy.

There are eight Small mothers against decapentaplegic(SMAD)s codes in the human genome^[4]. SMAD proteins are a family of signal transduction molecules involved in the transforming growth factor β (TGF- β) ligand pathway^[5]. SMADs belong to the intracellular protein family with a total length of 500 amino acids, among which SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8 act as TGF- β receptors in mammals, of which SMAD8 is generally expressed as SMAD9. SMAD4 is a common pathway mediator, and SMAD6 and SMAD7 inhibit SMAD^[6]. The main function of SMADs is to control gene program, transcriptional

regulation and signal transduction, which can mediate TGF- β /SMAD, Notch, ERK/MAPK, Hippo, JAK/STAT, Hedgehog, BMP/SMAD and so on^[7]. SMADs have been found to be implicated to cell proliferation, migration, apoptosis and immune regulation of cancer cells^[8–10]. Smads are associated with lung, pancreas, liver ,gastrointestinal tumors and so on^[11–12]. However, there are few comprehensive studies on the expression, prognosis and immune infiltration of SMAD superfamily as a whole and colorectal cancer.

In this study, we used public databases and R language for in-depth analysis of the correlation between SMADs and the occurrence and development of CRC, as well as the prognostic analysis and immune infiltration analysis of CRC patients. To demonstrate the value of different SMADs in the occurrence, prognosis and immune infiltration of colorectal cancer.

Materials And Methods

The Human Protein Atlas(HPA)

HPA (www.proteinatlas.org/pathology) maps human proteins by analyzing the effects of clinical results on various omics, primarily based on the relationship between the genome-wide transcriptome of protein-coding genes of 17 cancer types and clinical results^[13]. In this study, we used this database to investigate the relationship between SMADs protein and CRC.

GEPIA

GEPIA(<http://gepia.cancer-pku.cn/>) is an online web address based on TCGA and GTEx consisting of thousands of tumor and healthy tissue sample data, using standard processing pipelines, providing key interactive and customizable functionality^[14]. In this study, GEPIA was used to analyze the correlation between SMADs and pathological stage of CRC , and its prognostic value was analyzed by this method.

c-BioPortal

c-BioPortal (<http://cbioportal.org>) is a free open platform for multi-dimensional cancer genome analysis, detection and visualization at the DNA level^[15]. In this study, cBioPortal was used to predict mutation rates of the SMAD gene family in CRC.

GeneMANIA

GeneMANIA (<http://www.genemania.org>) is a rich and friendly website for hypothesis of gene function, analysis of gene lists, identification of functionally similar genes, biofunctional genomics, and more^[16]. In

this study, we explored the SMADs interaction network and associated genes through GeneMANIA database.

Statistical Analysis

All statistical analyses were performed using R (V3.6.3). The differences were visualized using the ggplot2 software package. Paired *t*-test and Mann-Whitney *U*-test were used to detect differences between colorectal cancer tissues and adjacent normal tissues. R Package survminer was used for visualization of prognostic value, and survival software package was used for statistical analysis of survival data. The ssGSEA package of GSVA^[17] was used for immune infiltration analysis, shapiro-Wilk normality test and Spearman correlation coefficient calculation were used to detect the correlation of immune infiltration.

Results

Differential Expression of SMADs in Pan-Cancer and CRC

The expression differences of SMADs in pan-cancer were detected by ggplot2 software package, as shown in Fig. 1. Then, the same package was used to detect the differential expression of SMADs in 51 normal samples and 647 colorectal cancer samples (Fig. 2), and the results showed that the expression levels of SMAD1-4, SMAD7 and SMAD9 were significantly down-regulated, while the expression levels of SMAD5 and SMAD6 were significantly up-regulated. The specific situation was analyzed as follows.

Unpaired sample analysis showed that the expression of SMAD1 in CRC was significantly lower than that in adjacent colorectal normal tissues (Fig. 2a, 3.207 ± 0.539 vs. 3.654 ± 0.236 , $p < 0.001$), the expression of SMAD2 in CRC was absolutely lower than that in adjacent colorectal normal tissues (Fig. 2b, 2.604 ± 0.591 vs. 2.823 ± 0.246 , $p < 0.001$), the expression of SMAD3 in CRC was significantly lower than that in adjacent colorectal normal tissues (Fig. 2c, 4.197 ± 0.650 vs. 4.426 ± 0.384 , $p = 0.002$), the expression of SMAD4 in CRC was absolutely lower than that in adjacent colorectal normal tissues (Fig. 2d, 3.440 ± 0.644 vs. 3.908 ± 0.288 , $p < 0.001$), the expression of SMAD7 in CRC was significantly lower than that in adjacent colorectal normal tissues (Fig. 2g, 4.225 ± 0.706 vs. 4.990 ± 0.423 , $p < 0.001$), the expression of SMAD9 in CRC was absolutely lower than that in adjacent colorectal normal tissues (Fig. 2h, 2.486 ± 1.164 vs. 3.125 ± 0.625 , $p < 0.001$). The expression levels of SMAD5 (Fig. 2e) and SMAD6 (Fig. 2f) in CRC tissues were significantly higher than those in adjacent normal colorectal tissues ($p < 0.001$), and the statistical results were 4.364 ± 0.723 vs. 3.952 ± 0.446 and 2.916 ± 0.64 vs. 2.085 ± 0.394 respectively.

Protein Expression Of Smads In Crc

Protein expression of SMADs in normal intestine and CRC tissues were analyzed by HPA, as shown in Fig. 3. The results showed that the protein expressions of SMAD1 and SMAD2 were significantly

increased in CRC tissues (Fig. 3a, b), the protein expressions of SMAD3, SMAD4 and SMAD5 were significantly decreased in CRC tissues (Fig. 3c, d, e), and the protein expressions of SMAD7 and SMAD9 were not significantly different (Fig. 3f, g). SMAD6 cannot be evaluated if it is not detected in HPA.

Correlation Between Smads And Crc Tumor Stage

By evaluating the correlation between SMADs expression and tumor stage in CRC patients, the results are shown in Fig. 4. The analysis results showed that SMAD2 group had noticeable differences (Fig. 4b, $p < 0.05$), while SMAD1, SMAD3, SMAD4, SMAD 5, SMAD6, SMAD7, SMAD9 groups had no significant differences (Fig. 4a, Fig. 4c-h, all $p > 0.05$).

Prognostic Value Of Smads In Colorectal Cancer

R Package survminer and survival were used to analyze overall survival (OS), disease-specific survival (DSS) and progress free interval (PFI) indicators of survival prognosis of CRC patients by SMADs, as shown in Fig. 5. The results showed that SMAD1 was significantly correlated with DSS ($p = 0.037$) and PFI ($p = 0.02$) in CRC patients (Fig. 5a), SMAD9 was significantly correlated with OS ($p = 0.038$) and DSS ($p = 0.035$) in CRC patients (Fig. 5h), while other results showed no significant differences.

GEPIA was used to analyze OS and disease free survival (RFS) indicators of the prognostic value of SMADs for CRC patients, as shown in Fig. 6. Analysis showed that SMAD6 and SMAD9 were significantly correlated with OS in CRC patients (Fig. 6f,h), while no significant correlations were found in others.

Analysis of SMADs Gene Mutation and Interaction Expression in CRC

The frequency of SMADs changes in CRC was detected by cBioPortal. The results showed that in 881 CRC patients, the mutations of SMAD1 and SMAD6 were 1.9%, SMAD2 was 7%, SMAD3 and SMAD5 were 5%, and SMAD4 was 18%, and the mutation rate was 4% for SMAD7 and 2.8% for SMAD9. The OncoPrints contained inframe mutation, missense mutation, splice mutation, truncating mutation, structural variant, amplification, deep deletion and no alterations (Fig. 7a).

Through the GeneMANIA database, twenty genes associated to the interaction network with SMADs were analyzed, which were NFIB, NFIA, NFIX, NFIC, SKI, PNKP, ZFYVE16, PHLDB1, TIFAB, FHAD1, TIFA, STRAP, SLC4A1AP, CEP170B, FKBP1A, SLMAP, PPP1R8, MCRS1, FOXH1, CEP170 (Fig. 7b).

Correlation With Immune Infiltration

The ssGSEA Package of GSVA was used to comprehensively analyze the relationship between SMADs and immune cell infiltration, as shown in Fig. 8 and Table 1. The results showed that the expressions of SMAD1, SMAD4, SMAD7 were positively correlated with the infiltration of B cells, CD8 + T cells, dendritic

cells (DC), eosinophils macrophages, and neutrophils (Fig. 8a, d, g). SMAD2 expression was positively correlated with CD8 + T cells, macrophages, and neutrophils (Fig. 8b). SMAD3 expression was positively correlated with B cells, CD8 + T cells, eosinophils and macrophages (Fig. 8c). SMAD5 expression was positively correlated with macrophages infiltration, while SMAD5 expression was negatively correlated with DC infiltration (Fig. 8e). SMAD6 expression was positively correlated with DC infiltration, SMAD6 expression was negatively correlated with CD8 + T cells, macrophages, and neutrophils infiltration (Fig. 8f). The expression of SMAD9 was positively correlated with eosinophils infiltration, and the expression of SMAD9 was negatively correlated with neutrophils infiltration (Fig. 8h).

Table 1
Correlation between immune cell infiltration and SMADs in CRC

Gene	Spearman	Cells					
		B cells	CD8 T cells	DC	Eosinophils	Macrophages	Neutrophils
SMAD1	r	0.230	0.190	0.160	0.190	0.280	0.220
	p	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
SMAD2	r	0.049	0.210	0.037	0.037	0.290	0.130
	p	0.217	< 0.001	0.347	0.352	< 0.001	0.001
SMAD3	r	0.085	0.120	0.054	0.200	0.086	0.046
	p	0.030	0.002	0.170	< 0.001	0.029	0.246
SMAD4	r	0.130	0.230	0.130	0.084	0.340	0.220
	p	0.001	< 0.001	0.001	0.032	< 0.001	< 0.001
SMAD5	r	-0.062	0.050	-0.150	-0.041	0.110	-0.031
	p	0.116	0.205	< 0.001	0.297	0.005	0.436
SMAD6	r	0.004	-0.097	0.089	0.032	-0.310	-0.170
	p	0.910	0.014	0.024	0.417	< 0.001	< 0.001
SMAD7	r	0.260	0.170	0.160	0.260	0.130	0.078
	p	< 0.001	< 0.001	< 0.001	< 0.001	0.001	0.048
SMAD9	r	0.047	0.009	0.071	0.100	-0.033	-0.110
	p	0.234	0.816	0.072	0.010	0.396	0.007

Discussion

Studies have shown that SMADs are involved in the development, metastasis, prognosis and immune microenvironment of many tumors. The immune infiltrating cells are related to tumor microenvironment and influence tumor growth and metastasis. The high expression of SMAD1, SMAD2 and SMAD4 in gastric cancer tissues is significantly correlated with the prognosis of patients^[18]. In breast cancer, SMAD2 and SMAD3 are related to the incidence of patients^[19]. SMAD4 and SMAD7 in pancreatic cancer are significantly correlated with the progression and prognosis of patients^[20]. SMAD3 plays a bidirectional role in liver cancer, inhibiting tumor occurrence in the early stage and accelerating tumor growth in the late stage^[21]. Overexpression of DcR3 in hepatocellular carcinoma(HCC) is mediated by TGF β 3-Smads-SP1 signaling pathway to promote the differentiation and secretion of immune cells^[22]. However, studies related to SMADs and the occurrence, development, prognosis and immunity of CRC have not been fully clarified.

SMAD1 is the activation type of SMAD receptor, which is involved in modifying cell growth, differentiation, apoptosis and other processes, and plays an important role in the body's immune system. In lung cancer related studies, SMAD1 was significantly lower than the alveolar and bronchial controls^[23]. Studies related to prostate cancer have shown that down-regulated SMAD1 inhibits its proliferation, invasion and migration^[24]. These studies suggest that SMAD1 has different effects in different tumors. There are a few studies on SMAD1 and colorectal cancer. Correlation experiments showed that SMAD1 plays an oncogene role in CRC through autophagy^[25]. This study claimed that low expression of SMAD1 in colorectal cancer was related to prognosis and immune cell infiltration, but SMAD1 protein was significantly increased in colorectal cancer tissues. These results suggest that SMAD1 can be used as a biomarker for CRC diagnosis, malignancy assessment and prognosis.

SMAD2 plays an important role in a variety of physiological and pathological processes^[26]. Studies have shown that SMAD2 inhibits cell proliferation and expression of related functional genes, and is related to the transcriptional response that promotes cell apoptosis^[27]. SMAD2 plays different roles in different tumor stages by regulating a variety of biological processes^[28]. In colorectal cancer, tumor suppressor gene NIT1 is realized by activating the SMad2/3 signaling pathway^[29]. SMAD2 mutation has a prevalence rate of 3.4% in sporadic CRCs, and may be a tumor suppressor gene^[30]. In this study, SMAD2 expression in CRC was low, which was significantly different from colorectal cancer tumor stage, associated with CD8 + T cells, macrophages and neutrophils, and had a high mutation rate. The results of this study are consistent with those of other studies, suggesting that SMAD2 can be used as a marker related to CRC development, staging, and immunity.

SMAD3, as an important transcriptional regulatory signal molecule of TGF- β pathway, its transduction disorder leads to tumorigenesis and plays the dual role of oncogene and tumor suppressor gene in tumor formation^[31]. SMAD4 plays a core role in TGF- β signaling pathway transduction, making the cell cycle stay in G1 phase, leading to abnormal tumor proliferation, and is a tumor suppressor gene^[32]. In CRC, SMAD3 reduces its expression through mir-4429, and ultimately inhibits the occurrence, development and metastasis of cancer cells^[33]. SMAD3 and SMAD4 mutations are associated with tumorigenesis and can

be used as tumor suppressor genes^[34]. Meta-analysis showed that high mutation rate of SMAD4 in CRC patients was associated with poor prognosis, but not with clinical stage^[35]. This study showed that SMAD3, SMAD4 and their proteins were significantly underexpressed in colorectal cancer. But there was no significant correlation between tumor stage and prognosis. The maximum mutation rate of SMAD4 in CRC was 18%. Studies on immune infiltration have shown that SMAD3 and SMAD4 are associated with a variety of immune cells. Our results are generally consistent with previous reports, suggesting that SMAD3 and SMAD4 can act as tumor suppressor genes of CRC and influence patient immune status. However, whether SMAD4 can be used as a prognostic indicator needs further validation.

SMAD5 mediates TGF- β superfamily ligand signaling pathway to regulate cell proliferation, differentiation and apoptosis^[36]. SMAD6 can also regulate TGF- β signaling pathway, stimulate extracellular matrix, promote angiogenesis, and inhibit immunity, so it is conducive to tumor growth, diffusion and metastasis^[37]. This study found that SMAD5 and SMAD6 were significantly overexpressed in colorectal cancer. SMAD6 was significantly correlated with OS. SMAD5 has rarely been studied in tumors, only partially in gastric and esophageal cancers. In studies related to gastric cancer, SMAD5 promotes the proliferation, invasion and migration of cancer cells and inhibits the apoptosis of cancer cells^[38]. In esophageal cancer-related studies, SMAD5 acts as a microrna-145 target to promote the proliferation and metastasis of esophageal cancer cells^[39]. Overexpression of Mir-186-5p in CRC can significantly reduce SMAD6, ultimately inhibit proliferation and migration of CRC cells, and increase apoptosis of CRC cells^[40]. These results are consistent with our study of SMAD5 and SMAD6. These results demonstrated that SMAD5 and SMAD6 could be used as oncogenes of CRC, and SMAD6 could also be used as prognostic biomolecules.

SMAD7 is an inhibitor of TGF- β signaling pathway, which can antagonize TGF- β -mediated diseases, including tumors^[41]. SMAD7 plays a dual role in different tumor stages, acting as a tumor suppressor gene by inhibiting proliferation and promoting apoptosis in the early stage, and promoting epithelial-mesenchymal degeneration and increasing invasion in the late stage, which is positively correlated with the degree of malignancy^[42–43]. In CRC, SMAD7 can up-regulate mir-424 by silencing circTBL1XR1, thus promoting the proliferation, invasion and metastasis of CRC^[44]. Mir-4775 overexpressed in CRC promotes invasion, metastasis and EMT processes of cancer cells by activating SMAD7^[45]. In this study, SMAD7 expression was significantly reduced in CRC and was associated with a variety of immune cells. Our study is consistent with the current relevant experimental verification, and the current literature suggests that there is a difference in colorectal related expression between this study and SMAD7. Considering the dual role of SMAD7, CRC tissues may be in different stages, which is consistent with the actual situation. SMAD7 is both an oncogene and a tumor suppressor gene in CRC, which can be used as a marker to evaluate the state of immune microenvironment.

Although there are only eight members of the SMADs family from 1 to 8. However, some databases SMAD8 is directly named SMAD9, and some databases have both SMAD8 and SMAD9, so it is impossible to make specific analysis, so further analysis will not be conducted here.

Our study has some shortcomings. First of all, this study was mainly obtained through database analysis without relevant experimental verification. In order to better study the relationship between CRC and SMADs, experimental verification is needed to further verify the results and make the results more convincing. Secondly, due to the ambiguity between SMAD8 and SMAD9 in different databases, specific analysis is not possible.

Conclutions

In conclusion, this study used R language and several different database systems to analyze the differential expression, mutation rate, prognostic analysis and immune infiltration of SMAD family members in CRC. The results showed that SMAD1, SMAD2, SMAD3, SMAD4 and SMAD7 were significantly down-expressed in CRC, while SMAD5 and SMAD9 were significantly up-expressed in CRC. SMAD1 and SMAD2 proteins were significantly increased in CRC, SMAD3, SMAD4 and SMAD5 proteins were significantly decreased in CRC, and SMAD7 and SMAD9 protein expressions were not significantly different. Only SMAD2 was associated with tumor stage of CRC. In terms of prognostic analysis, only SMAD1 was significantly correlated with DSS and PFI, while SMAD6 was significantly correlated with OS. SMAD4 had the highest mutation rate. In immune infiltration, SMAD1, SMAD2, SMAD3, SMAD4, SMAD7 were positively correlated with a variety of immune cells. Our results provide new ideas for the selection of biomarkers related to SMADs and CRC differential expression, prognosis and immune infiltration for the diagnosis and treatment of CRC.

Abbreviations

CRC

Colorectal cancer; SMAD:Small mothers against decapentaplegic; HPA:The Human Protein Atlas; OS:overall survival; DSS:disease-specific survival; PFI:progress free interval; RFS:disease free survival; HCC:hepatocellular carcinoma

Declarations

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Authors' contributions

Ning Ding, Hongbiao Luo main design research scheme. Tao Zhang and Yanru Yao conducted data analysis. The first draft was drafted by Ning Ding and Hongbiao Luo, and revised by Yongheng He and Tianshu Peng. The authors have read and approved the final manuscript.

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Availability of data and materials

The data set used in this study is available from the correspondence and the first author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declared no conflicts of interest.

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Figures

Figure 1

Differential expression of SMADs in Pan-cancer. (a) SMAD1, (b) SMAD2, (c) SMAD3, (d) SMAD4, (e) SMAD5, (f) SMAD6, (g) SMAD7 and (h) SMAD9.

Figure 2

Differential expression of SMADs in colorectal cancer. Compared with normal tissue, (a) SMAD1, (b) SMAD2, (c) SMAD3, (d) SMAD4, (g) SMAD7, (h) SMAD9 were significantly up-regulated. (e) SMAD5 and (f) SMAD6 were significantly down-regulated.

Figure 3

SMADs protein levels in CRC patients. (a) SMAD1 and (b) SMAD2 were highly expressed. Low expression of (c) SMAD3, (d) SMAD4 and (e) SMAD5. (g) SMAD7 and (h) SMAD9 were constant.

Figure 4

Correlation between SMADs expression and tumor stage in CRC patients (GEPIA). (a) SMAD1, (c) SMAD3, (d) SMAD4, (e) SMAD5, (f) SMAD6, (g) SMAD7 and (h) SMAD9. (b) SMAD2 was significantly correlated with tumor staging ($P < 0.05$).

Figure 5

R language analysis of the prognostic value of SMADs in CRC patients. (a) SMAD1 in CRC patients was associated with DSS($p=0.037$) and PFI($p=0.02$), and (h) SMAD9 was associated with OS($p=0.038$) and DSS($p=0.035$).

Figure 6

Survival and prognosis analysis of SMADs in CRC patients (GEPIC). (f) SMAD6 and (h) SMAD9 were related to OS ($p=0.034$, $p=0.016$).

Figure 7

Mutation and expression analysis of SMADs gene in CRC(cBioportal and GeneMANIA). (a) Summary of SMADs mutations. (b)SMADs related proteins and interaction networks.

Figure 8

R language was used to analyze the correlation between the differentially expressed SMADs and immune cell infiltration. The expression of (a)SMAD1,(d)SMAD4 and (g)SMAD7 were positively correlated with the infiltration of B cells, CD8+T cells, dendritic cells, eosinophils, macrophages and neutrophils. (b)SMAD2 was positively correlated with the infiltration of CD8+T cells, macrophages and neutrophils. (c)SMAD3 was positively correlated with infiltration of B cells, CD8+T cells, eosinophils and macrophages. (e)SMAD5 was negatively correlated with dendritic cells and positively correlated with macrophages. (f)SMAD6 was positively correlated with dendritic cells and negatively correlated with CD8+T cells, macrophages and neutrophils. (h)SMAD9 was positively correlated with eosinophils and negatively correlated with neutrophils.