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Original Article

Clinicopathological characteristics of *SMAD4* gene expressions in colorectal cancer patients

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Abstract

Colorectal cancer (CRC) ranks as the third leading cause of cancer-related deaths globally. *SMAD4* gene acts as the central mediator of the signaling pathway for transforming growth factor-β (TGFβ) with a significant effect on colorectal cancer. Previous research has confirmed a relationship between the presence of the *SMAD4* gene and the survival and progression of colorectal cancer in patients. In this study, our goal was to analyze the presence of *SMAD4* in both colorectal cancer and nearby normal tissues. The expression levels of *SMAD4* were evaluated in 45 colorectal tumor tissues and 45 adjacent control tissues using the Quantitative Real-Time PCR (qRT-PCR) method. Additionally, we assessed the diagnostic effectiveness of *SMAD4* by creating a receiver operating characteristic (ROC) curve. Our findings showed that the expression of *SMAD4* was significantly reduced in colorectal cancer patients compared to the adjacent control group sample. Examination of clinicopathological characteristics of patients revealed varied correlations between *SMAD4* gene expressions and TMN stage ($p<0.0001$). These findings suggest that *SMAD4* levels could be used as possible diagnostic indicators for colorectal cancer.

Keywords: Colorectal cancer (CRC), *SMAD4,* Diagnostic biomarkers, Real-Time PCR (qRT-PCR)

1. Introduction

Colorectal cancer (CRC) is the most prevalent cancer worldwide. Several studies have shown that colorectal cancer is the result of epigenetic changes and the accumulation of genetic disorders including oncogenes products or tumor suppressors genes (such as *SMAD4*, *Kras, p53* and *APC*.(*SMAD4* plays a key role in the signaling pathway of transforming growth factor-β (TGFβ) and influences colorectal cancer. Furthermore, a decrease in *SMAD4* protein expression is observed in around 30% to 40% of cases of colorectal cancer, leading to a decreased response to chemotherapy and an increased risk of metastasis formation [1-3]. The *SMAD* family was identified as the initial target of TGF-β receptor kinases. Furthermore,

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when serine-threonine kinase activates transmembrane receptors, it leads to the activation and phosphorylation of the *SMAD* family, which plays a crucial role in cellular functions [4, 5].

Still, the molecular mechanisms of it are yet to be understood. *SMAD4* plays a critical role in intracellular signal transduction within the cell nucleus in the TGF-β pathway.[6]. *SMAD4* plays a crucial role in apoptosis, cell proliferation, and differentiation. Decreased expression of the *SMAD4* gene results in the loss of the ability of TGF-β to inhibit cell growth and apoptosis [7-9]. *SMAD4* consists of MH1 and MH2 domains located in the N- and C-terminals, respectively, separated by a proline-rich linker domain. The MH1 domain contains an intrinsic DNA linking activity, as well as the MH2 domain includes the biological impact such as interaction with regulatory proteins. Although MH1 domains can limit biological activity and transcription of MH2 domains [10]. Most of *SMAD4* gene conversion cluster occurs in the MH2 domain and often modify occurs residues in the adjacent of protein jointing mediating *SMAD4* hetero oligomerization [11]. Recent studies reported that, Mutations at the MH1 domain causes have been to increase interactions with the MH2 domain and modify DNA linking, preventing nuclear translocation and protein stability [10-12].

SMAD4 carries inactivating mutations in certain cancers, and reduced expression is a significant characteristic in the majority of human cancers [6, 13]. Moreover, prior research has confirmed a connection between the *SMAD4* gene's expression and both patient survival and the advancement of colorectal cancer [14]. Nonetheless, only a small number of research studies have demonstrated the involvement of *SMAD4* in the development of colorectal cancer. The study aimed to confirm and explore the functional role and clinical importance of *SMAD4* in colorectal cancer. It looked at how *SMAD4* is involved in the advancement and severity of colon cancer cells, compared to normal tissue, and examined its connection to clinicopathological characteristics.

2. Materials and methods

2.1. Patients and samples

This case-control study obtained tissue samples from patients who were sent to endoscopy, oncology, or surgery clinics between July 2021 and December 2023. In the end, a total of 45 patients and 45 control tissues were incorporated, with the samples obtained from biopsies or surgical resections. Additionally, healthy tissues from colorectal cancer patients were positioned adjacent to the tumor site, at a distance greater than 2 cm, and were devoid of any tumor cells. The age range for patients in the inclusion criteria is between 25 and 60 years old. A board-certified pathologist must approve the sampling of tumor tissue, ensuring that the samples are consistent with colon adenocarcinoma histologically and that patients have not undergone any colorectal cancer-related therapy prior to the biopsy. Patients undergoing treatment for colorectal cancer, those who had surgery to remove tumors, and individuals with other types of cancer were not included in the research. Additionally, their demographic, lifestyle, and histopathological data, including the clinical TNM staging, were documented.

2.2. Ethics statement

The research was conducted in accordance with the guidelines set by the Ethics Committee of Shahid Behest University of Medical Sciences, and all participants provided written consent prior to participation in the study (Code No: IR.SBMU.RETECH.REC.1402.284).

2.3. RNA extraction and cDNA synthesis

The kit from Cinnacolon in Tehran, Iran was utilized for RNA extraction, followed by RNA isolation in 40 µl of RNase-free water. Total RNA concentration and integrity were evaluated via A260/A280 measurement using a NanoDrop ND-1000 spectrophotometer from Thermo Fisher Scientific in the USA. Each sample ratio was targeted to be within the range of 1.7 to 2.1, after which the RNA suspension was stored at -80°C for future analysis and subsequently transformed into cDNA. The cDNA was generated from 2 µg of total RNA using a cDNA kit (Cinnacolon, Tehran, Iran) with Oligo (dT) and random hexamer primers in a reverse transcription reaction. Following the instructions of the manufacturer, the kit mix was processed on a PCR thermocycler gene with the following conditions: 10 minutes at 25°C, 2 hours at 37°C, 5 minutes at 85°C, and then on a PCR thermocycler Gene. The cDNA was diluted to achieve a total concentration of 5 ng/µl.

2.4. Real-Time PCR

The analysis using real-time PCR was done in duplicates with 2.0X Real Q-PCR Master Mix containing SYBR Green (Ampliqon, Odense, Denmark). Each sample's reaction includes 10 µl 2X Real Q-PCR Master Mix, 1 µl cDNA, 1 µl of each primer (10 pmol/µl), and 8 µl distilled water. Experiments were conducted on the Step One Plus Real-time PCR System (Applied Biosystems, USA) with thermal cycling conditions of 95°C for 2 min and 40 cycles of 95°C for 5 s, 60°C for 30 s. Confirmation of product specificity was done through melting curve analysis. Gene expression levels were employed for the normalization of beta-2 macroglobulin (β2 M) expression, a housekeeping gene, in each sample. The primers for *SMAD4* were strategically placed at different exon junctions to prevent inaccurate outcomes due to DNA contamination (Table 1 and Figure 1).

2.5. Statistical analysis

Efficiency values and cycle threshold (Ct) for each

Table 1. qRT-PCR primer sequences.

Genes	Primers	Sequences	Amplicon size (bp)
SMAD4	Forward Primer	TTTCCAATCATCCTGCTCCTG	194
	Reverse primer	GTCTCTCCTACCTGAACATCC	
Beta-2-microglogulin	Forward Primer	TGTCTTTCAGCAAGGACTGGT	143
	Reverse primer	TGCTTACATGTCTCGATCCCAC	

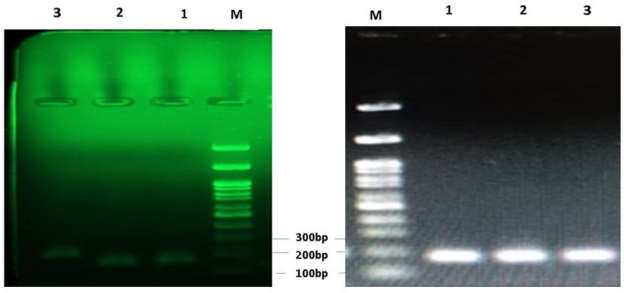

Fig. 1. PCR products from real-time PCR, agarose gel electrophoresis of PCR products.

sample were used to determine amplification efficiency with LinReg software (version: 2017.1), and the expression ratio of the *SMAD4* gene (Fold change $2^{-\Delta\Delta Ct}$) was estimated using REST 2009 software. The statistical differences in *SMAD4* gene levels between patients and control subjects were analyzed using Graph Pad Prism software version 8.0 (La Jolla, CA). Using both the Mann-Whitney test and unpaired t-test to assess the difference in *SMAD4* mRNA levels between two groups. A significance level was established at a P-value of ≤ 0.05.

3. Results

In accordance with our objectives, we examined 45 individuals with colorectal cancer (23 women and 22 men) who were between the ages of 25 and 60 years old (average ±SD = 42.6±11.79 years). Among patients, 57% of malignant tissues were located in the colon and 43% in the rectum. We noted that 5 (11.1%) CRC patients had IBD, 21 (46.7%) CRC patients had polyps, and 19 (42.2%) CRC patients had colitis. Additionally, out of these patients, 13 (28.9%) were in stage II, 14 (31.1%) were in stage III, and 18 (40%) were in stage IV based on clinical TNM staging. Our findings indicated that the expression of the SMAD4 gene was more elevated in association with a history of colitis compared to the IBD and polyp groups, with statistically significant differences (P<0.045; IBD:4.21% vs. 4.91%, polyp:5.13% vs. 4.91%) (Figure 3). Table 2 displays the chosen clinical features of the participants in the study. We quantified the levels of *SMAD4* expression in CRC tissue by using qRT-PCR. Given that we witnessed a decrease in gene expression in colorectal cancer tissues when compared to controls (p<0.0001; Figure 2). Analysis of ROC curves indicated that tissue could serve as a valuable biomarker for differentiating patients with colorectal adenocarcinoma from control individuals. Higher AUC equals greater diagnostic value. Figure 4 displays the ROC curve area for *SMAD4*. Moreover, among these patients, 13 (28.9%) were classified as stage II based on clinical TNM staging, while 14 (31.1%) were stage III and 18 (40%) were stage IV. Our findings showed that the correlation between *SMAD4* expression and TNM stage varied significantly (P<0.0001). The level of *SMAD4* expression was higher in the stage II group compared to stage III (9.73% vs. 5.93%) and stage IV (5.1% vs. 5.93%) groups (Figure 3). In this study, 22 out of 50 CRC cases (48.8%) showed LVI+, with findings indicating that the relationship between *SMAD4* expression and lymph vascular invasion was not statistically significant (p<0.6). Nevertheless, there was no corre-

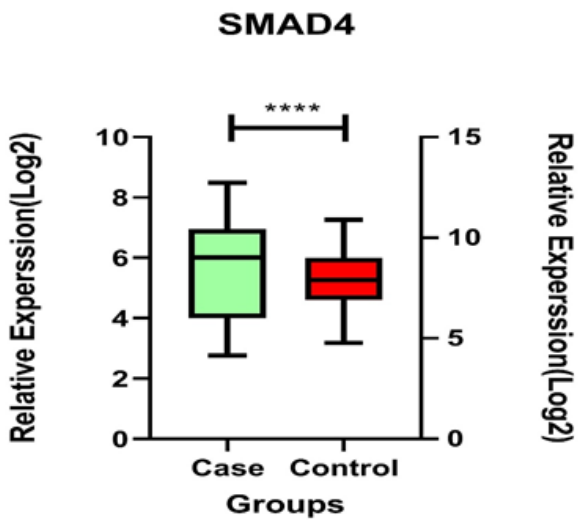

Fig. 2. The relative expression levels (−ΔCt) of *SMAD4* and B2M in colorectal cancer tissues and control noncancerous tissues.

Table 2. Patients' clinic pathologic characteristics SMAD4.

Variable	Clinic pathological parameter	Number of samples (n = 45)	Mean ± SD	p-value
Age	≥ 45	20	12.67 ± 3.02	*p*<0.783
	< 45	25	12.98 ± 4.37	
Gender	Male	22	15.65± 3.34	*p*=0.676
	Female	23	15.47 ±4.72	
TNM stage	II	13	8.52 ± 3.63	*p*<0. 0001
	III	14	11.89 ± 2.48	
	IV	18	12.32 ± 3.36	
Tumor size	< 2	11	15.84 ± 3.46	*p*=0.0001
	2-3.5	12	16.03 ± 3.42	
	3.5-5	12	16.17 ± 3.24	
	>5	10	14.09 ± 3.38	
Localization	Colon	25	15.28 ± 3.90	*p*=0.695
	Rectum	20	15.35 ± 4.29	
Lymphatic invasion	Positive	22	16.77 ± 2.26	*p*<0.667
	Negative	23	16.82 ± 3.64	

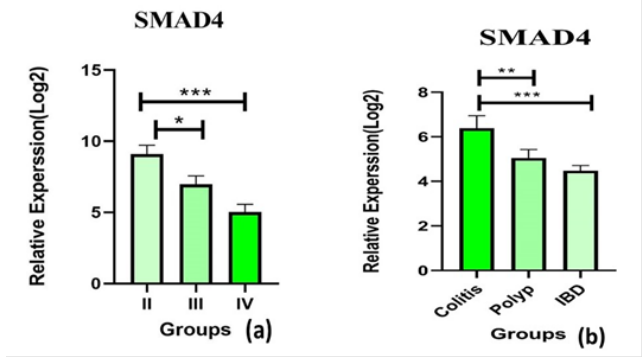

Fig. 3. The relative expression levels (−ΔCt) of *SMAD4* and relationship between clinicopathological characteristics in patients with colorectal cancer.

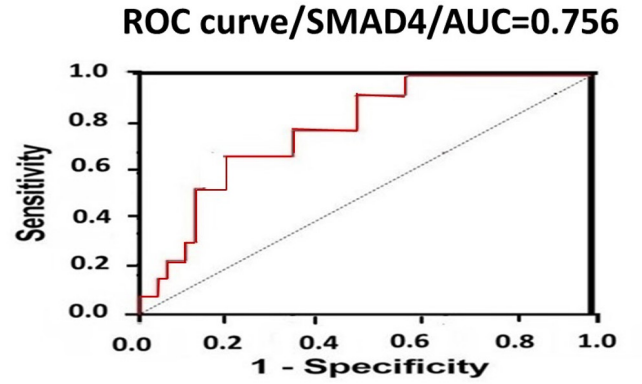

Fig. 4. ROC curve analysis of the diagnostic value of CRC-related genes to distinguish between colorectal cancer patients and healthy individuals.

lation discovered between levels of *SMAD4* and additional clinicopathological characteristics like age at diagnosis, tumor size, site, and gender.

4. Discussion

Both prognosis and biomarkers are of the utmost importance for treatment, tissue biomarkers are a specific approach in stratifying patients with CRC. The gene *SMAD4* is a tumor or suppressor that common mediator of *TGFβ* signaling [15]. The gene *SMAD4* is implicated in the regulation of differentiation, apoptosis, migration and cell proliferation. In addition, *SMAD4* loss contributes to promotion of epithelial tumors in the gastrointestinal [16]. Several studies have investigated the role of *SMAD4* in colorectal cancer patients, but the results are inconsistent [17]. In this research, it was observed that the levels of *SMAD4* expression were markedly decreased in colorectal cancer patients compared to the control group ($p<0.0001$). Analysis of 45 colorectal cancer samples and normal tissues through qRT-PCR revealed a decrease in the expression of the *SMAD4* gene in the tissue samples. Rosic J *et al*. [18], investigated the level of *SMAD4* expression in colorectal cancer patients with primary and metastatic colorectal cancer and its relationship with disease progression and therapy response, and they reported that the expression level of *SMAD4* gene was significantly lower in tumor compared to non-tumor tissues [18].

Our findings indicate that the *SMAD4* gene was more highly expressed in the colitis groups compared to the polyp and IBD groups ($P<0.045$). A recent study examined tumors at different stages of colorectal cancer to investigate how the *SMAD4* gene is inactivated by either deletion or point mutations. A connection was found between the higher occurrence of *SMAD4* gene mutations in colorectal cancer patients with metastases compared to non-metastases [19-21]. In the present investigation, the ROC curve analysis showed that the expression levels of *SMAD4* gene, they're related could be used to differentiate between colorectal cancer patients and normal tissue (Figure 4). The expression level of *SMAD4* gene might have the potential to detect CRC tissues from normal tissues, expressed by the large AUCs of ROCs (0.756 respectively). Sheng S *et al.* [22], found that miR-144 may target *SMAD4* in CRC and due to its down-regulation. The mutation and down-regulation of *SMAD4* lead to many cancers' progression. Moreover, miR-144 inhibited tumor cell invasion and metastasis by targeting *SMAD4* and the associated signaling pathway, offering a potential novel therapy for colorectal cancer (CRC) patients [22]. Our result revealed that *SMAD4* was variously associated with the TNM stage, the stage II group possessed expression of *SMAD4* gene was down-regulated compared with stage III and IV groups ($P<0.0001$). Royce SG *et al*. [23], Examined 109 colorectal cancer tumors, evaluating the *SMAD4* gene expression through quantitative real-time PCR. They discovered that the decrease in *SMAD4* protein expression was more common in rectal tumors than in tumors caused by colon cancer. [23]. Limitations of the study included a small sample size, and the analysis was done at an mRNA level; it would be more beneficial to confirm our findings at a protein level [24, 25].

5. Conclusion

In summary, the levels of *SMAD4* gene expression could potentially function as a reliable diagnostic biomarker for colorectal adenocarcinoma. More extensive research is required to establish a definite conclusion.

Conflict of Interests

None.

Authors' Contribution

All authors contributed to the study's concept and design.

Ethics approval

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study (IR.SBMU.RETECH.REC.1402.284; Link: ethics.research.ac.ir/ https://pajoohan.sbmu.ac.ir/general/cartable.action#).

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