

Basic Study

SFRP4 expression correlates with epithelial mesenchymal transition-linked genes and poor overall survival in colon cancer patients

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Author contributions: Nfonsam LE and Jandova J contributed equally to this work; Nfonsam LE, Jandova J, and Nfonsam VN designed the research; Nfonsam LE, Jandova J, Jecius HC, and Omesiete PN performed the research; Nfonsam LE and Jandova J analyzed and interpreted data; Nfonsam LE, Jandova J, Jecius HC, Omesiete PN, and Nfonsam VN wrote the paper.

Supported by the SAGES research grant (to Nfonsam VN).

Institutional review board

statement: This study does not involve human subjects. It utilizes the publicly available de-identified colon adenocarcinoma data set from The Cancer Genome Atlas.

Conflict-of-interest statement: All authors declare no conflict of interest.

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Abstract**BACKGROUND**

Colon cancer is among the most commonly diagnosed cancers in the United States with an estimated 97220 new cases expected by the end of 2018. It affects 1.2 million people around the world and is responsible for about 0.6 million deaths every year. Despite decline in overall incidence and mortality over the past 30 years, there continues to be an alarming rise in early-onset colon cancer cases (< 50 years). Patients are often diagnosed at late stages of the disease and tend to have poor survival. We previously showed that the *WNT* "gatekeeper" gene, secreted frizzled-related protein 4 (*SFRP4*), is over-expressed in early-onset colon cancer. *SFRP4* is speculated to play an essential role in cancer by inhibiting the epithelial mesenchymal transition (EMT).

AIM

To investigate the correlation between *SFRP4* expression and EMT-linked genes in colon cancer and how it affects patient survival.

METHODS

SFRP4 expression relative to that of EMT-linked genes and survival analysis were performed using the University of California Santa Cruz Cancer Browser interface.

RESULTS

SFRP4 was found to be co-expressed with the EMT-linked markers *CDH2*, *FN1*, *VIM*, *TWIST1*, *TWIST2*, *SNAI1*, *SNAI2*, *ZEB1*, *ZEB2*, *POSTN*, *MMP2*, *MMP7*, *MMP9*, and *COL1A1*. *SFRP4* expression negatively correlated with the EMT-linked suppressors *CLDN4*, *CLDN7*, *TJP3*, *MUC1*, and *CDH1*. The expression of *SFRP4* and the EMT-linked markers was higher in mesenchymal-like samples compared to epithelial-like samples which potentially implicates *SFRP4*-EMT

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Manuscript source: Unsolicited manuscript

Received: February 14, 2019

Peer-review started: February 15, 2019

First decision: March 14, 2019

Revised: April 2, 2019

Accepted: May 23, 2019

Article in press: May 23, 2019

Published online: August 15, 2019

P-Reviewer: Tarnawski AS, Kang GH

S-Editor: Wang JL

L-Editor: Filipodia

E-Editor: Zhou BX



mechanism in colon cancer. Additionally, patients overexpressing *SFRP4* presented with poor overall survival ($P = 0.0293$).

CONCLUSION

Considering the implication of *SFRP4* in early-onset colon cancer, particularly in the context of EMT, tumor metastasis, and invasion, and the effect of increased expression on colon cancer patient survival, *SFRP4* might be a potential biomarker for early-onset colon cancer that could be targeted for diagnosis and/or disease therapy.

Key words: Secreted frizzled-related protein 4; Epithelial mesenchymal transition; Colon cancer; Survival

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Core tip: We have previously shown that secreted frizzled-related protein 4 (*SFRP4*) is over-expressed in colon cancer, especially in patients younger than 50 years. As these early-onset colon cancers tend to be more aggressive and negatively affecting patients' survival, we sought to evaluate whether *SFRP4* is co-expressed with epithelial-mesenchymal transition-linked genes that play key roles in cancer progression. Our results using a large colon adenocarcinoma-The Cancer Genome Atlas cohort revealed that *SFRP4* is over-expressed in colon cancer patients together with epithelial-mesenchymal transition-linked genes. Moreover, colon cancer patients with high expression levels of *SFRP4* showed significantly poorer survival relative to colon cancer patients with lower *SFRP4* expression.

Citation: Nfonsam LE, Jandova J, Jecius HC, Omesiete PN, Nfonsam VN. *SFRP4* expression correlates with epithelial mesenchymal transition-linked genes and poor overall survival in colon cancer patients. *World J Gastrointest Oncol* 2019; 11(8): 589-598

URL: <https://www.wjgnet.com/1948-5204/full/v11/i8/589.htm>

DOI: <https://dx.doi.org/10.4251/wjgo.v11.i8.589>

INTRODUCTION

Colon cancer is an important contributor to worldwide cancer morbidity and mortality, affecting an estimated 1.2 million individuals and responsible for 0.6 million deaths every year^[1]. In the United States, colon cancer is among the most commonly diagnosed cancer with an estimated 97220 new cases expected by the end of 2018 and an overall life time risk of about 4.49% for men and 4.15% for women^[2,3]. Despite the decline in overall incidence and mortality of colon cancer over the past 30 years^[4], there continues to be an alarming rise in the number of early-onset colon cancer cases in individuals younger than 50 years^[5]. Using the Surveillance Epidemiology and End Results Program Cancer Registries database, we previously demonstrated that this rise is most significant within the age group 40-44^[6]. About 70%-80% of early-onset colon cancer cases are sporadic, and these patients have aggressive features, are often diagnosed at a more advanced stage, and tend to have poor survival^[5-11].

In 2016, we showed that the secreted frizzled-related protein 4 (*SFRP4*) gene was uniquely over-expressed in early-onset colon cancer patients^[12]. This is a protein coding gene and a member of the *SFRP* family. The cysteine-rich domain of *SFRP4* is homologous to the putative *WNT*-binding site of frizzled proteins, making *SFRP4*, like other *SFRPs*, a modulator of *WNT* signaling^[13,14]. As a *WNT* inhibitor, *SFRP4* prevents cell growth and proliferation and has been implicated in Pyle disease^[15]. *SFRP4* is expressed in mouse embryonic mesenchyme, cardiovascular system, and musculoskeletal system^[16]. Reports show that *SFRP4* plays an essential role in ovarian cancer pathogenicity by inhibiting epithelial-mesenchymal transition (EMT) and cell migration, while promoting cell adhesion^[13].

Although EMT is a typical process in embryonic development, heart valve formation, and other developmental processes, its role in promoting cancer metastasis is well documented^[17,18]. EMT provides a molecular environment that allows epithelial cells to lose polarity and cell-to-cell adhesion and gain migratory and invasive

properties to become mesenchymal cells^[17,18]. The relationship between *SFRP4* and EMT-linked genes in colon cancer pathogenesis and the implication of *SFRP4* overexpression in patient survival has not been investigated previously. In this study, we demonstrate a correlation between *SFRP4* gene expression and the expression of some key EMT-linked genes in colon cancer and show that the overexpression of *SFRP4* associates with poor survival in colon cancer patients.

MATERIALS AND METHODS

Ethics statement

This study does not involve human subjects. It utilizes the publicly available de-identified colon adenocarcinoma (COAD) data set from The Cancer Genome Atlas (TCGA).

TCGA program

A landmark cancer genomics program molecularly characterized over 20000 primary cancer and matched normal samples spanning 33 cancer types. TCGA was formed in 2005 when the U.S. National Cancer and National Human Genome Research Institutes teamed together to support the launch of the project to map comprehensively various cancer genomic changes (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga/history/timeline>). This joint effort brought together researchers from diverse disciplines and multiple institutions. Over the next dozen years, TCGA generated over 2.5 petabytes of genomic, epigenomic, transcriptomic, and proteomic data. The data, which has already led to improvements in our ability to diagnose, treat, and prevent cancer, will remain publicly available for anyone in the research community to use (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>).

As of March 2019, the TCGA data portal contains 365463 pieces of genomic and clinical data for 45 projects; 68 primary sites; 33549 cases; 22872 genes, and 3142246 mutations. The TCGA-COAD project contains 461 cases including two primary sites, colon and rectosigmoid junction, and the following disease types: Adenomas and adenocarcinomas, complex epithelial neoplasms; cystic, mucinous, and serous neoplasms, epithelial neoplasms, and no other specified carcinomas.

Data set

A total of 286 of 462 "Primary Tumor" samples from TCGA-COAD cohort was used in this study for gene expression and Kaplan-Meier survival analyses^[19]. **Table 1** summarizes the gender, age, and tumor stage of samples.

Gene expression

SFRP4 gene expression relative to the EMT genes, Fibronectin (*FN1*), Vimentin (*VIM*), Zinc Finger E-Box Binding Homeobox 1 (*ZEB1*), *ZEB2*, Twist Family BHLH Transcription Factor 1 (*TWIST1*), *TWIST2*, Snail Family Transcriptional Repressor 1 (*SNAI1*), *SNAI2*, N-cadherin (*CDH2*), Claudin 4 (*CLDN4*), *CLDN7*, Tight Junction Protein 3 (*TJP3*), Mucin 1 (*MUC1*), and E-Cadherin (*CDH1*), was performed using the TCGA-COAD dataset as previously described^[20]. Pearson's and Spearman's rank coefficients and two-dimensional correlation scatter plots were generated using the University of California Santa Cruz Cancer Browser Interface (<https://xena-browser.net>)^[21].

Survival analysis

The TCGA-COAD "Primary tumor" dataset was used to perform and visualize Kaplan-Meier overall survival analysis using the University of California Santa Cruz Xena Browser for cancer genomics ([https://xenabrowser.net/datapages/?Cohort=TCGA%20Colon%20Cancer%20\(COAD\)](https://xenabrowser.net/datapages/?Cohort=TCGA%20Colon%20Cancer%20(COAD)))^[22,23]. *P* values were obtained using the Kaplan-Meier Estimator.

RESULTS

Co-expression of *SFRP4* with EMT genes

The role of *SFRP4* in epithelial-mesenchymal transition was investigated in colon cancer by comparing its expression to that of some key EMT-linked genes using 286 colon cancer patients. We observed co-expression and a positive correlation by Pearson's and Spearman's rank coefficient analysis of *SFRP4* with the EMT markers *CDH2*, *FN1*, *VIM*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2*, *SNAI1*, *SNAI2*, Periostin (*POSTN*), Matrix Metalloproteinase 2 (*MMP2*), *MMP9*, *MMP7*, and Collagen 1 (*COL1A1*) (**Figure 1** and **Figure 2**). A higher expression of *SFRP4* and the EMT-linked markers was

Table 1 Gender, age and tumor stage of The Cancer Genome Atlas-colon adenocarcinoma dataset used for analysis

	Percentage
Gender	
Female	46.8
Male	52.4
Age of initial diagnosis	
Average	66.9 ± 13
Minimum	31
maximum	90
Tumor stage	
Stage I	16.2
Stage IA	0.2
Stage II	6.5
Stage IIA	29.9
Stage IIB	2.2
Stage IIC	0.2
Stage III	4.3
Stage IIIA	1.7
Stage IIIB	12.8
Stage IIIC	8.9
Stage IV	10.0
Stage IVA	3.7
Stage IVB	0.4
Not reported	2.4
Discrepant	0.6

observed in samples that were more mesenchymal than epithelial. A negative correlation was observed between *SFRP4* and *CLDN4*, *CLDN7*, *TJP3*, *MUC1*, and *CDH1* (Figure 1 and Figure 3). Based on the Pearson's and Spearman's rank coefficients, *SFRP4* shows the strongest correlation with *CDH2* followed by *MMP2*, and the least correlation with *CLDN7* (Figure 2 and Figure 3). These data suggest a role for *SFRP4* in the EMT molecular pathway.

Colon cancer patients over-expressing SFRP4 have poor overall survival

We analyzed the effect of *SFRP4* overexpression on colon cancer patient survival. Our data showed that colon cancer patients with higher *SFRP4* expression (≥ 8.661) had poor overall survival compared to patients with lower *SFRP4* expression (< 8.661) (Figure 4A). A similar trend was observed when patient cohort was divided into three groups. Patients with intermediate levels of *SFRP4* expression (7.467-10.05) showed survival curve that was between that of patients with higher (≥ 10.05) and lower (< 7.467) expression (Figure 4B). These data demonstrate that *SFRP4* has an impact on colon cancer patient survival.

DISCUSSION

Very little is known about the role of *SFRP4* in cancer pathogenesis, let alone its mechanism of action. Most of the speculations on *SFRP4* mechanism appear to focus on its role as a *WNT* signaling modulator, through which it suppresses epithelial mesenchymal transition, a mechanism that is implicated in cancer metastasis and invasion^[13,17,18]. In ovarian cancer, *SFRP4* regulates metastasis by inhibiting EMT-linked genes, cell proliferation, and migration, while promoting apoptosis and upregulating cell adhesion molecules such as *CDH1*^[13,24]. A Study by Muley *et al*^[25] showed that *SFRP4* can restrict ovarian serous cystadenocarcinoma growth in mice by interfering with endothelial cell function, suggesting *SFRP4* as a potent angiogenesis inhibitor. While these observations indicate that *SFRP4* represses cancer pathogenesis, this is in contrast to our previous observation of *SFRP4* upregulation in early-onset colon cancer^[12] or its strong correlation with epithelial mesenchymal-linked genes

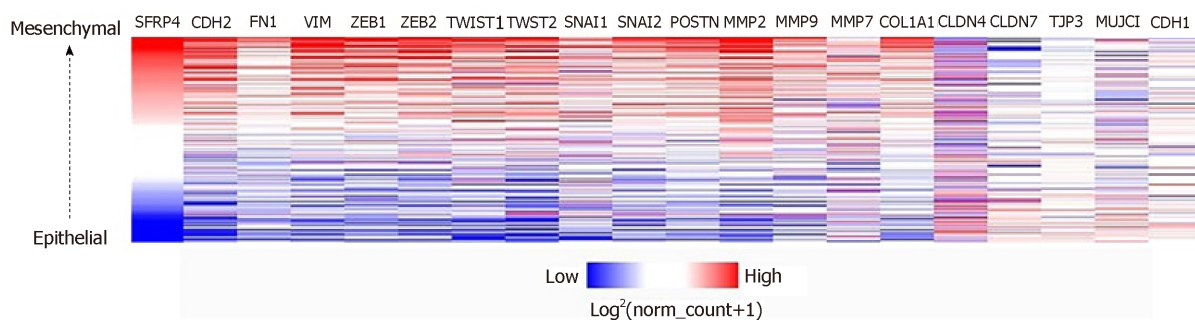


Figure 1 *SFRP4* co-expresses with epithelial mesenchymal transition-linked genes in colon cancer. *SFRP4* and the epithelial mesenchymal transition-linked genes have higher expression in samples that are more mesenchymal (red) compared to the more epithelial samples with lower expression (blue).

observed in this study.

Our previous report on the overexpression of *SFRP4* in early-onset colon cancer informed us of the potential role of *SFRP4* in colon cancer particularly with respect to age of onset^[12]. Our data showed that *SFRP4* co-expresses and correlates positively with the EMT-linked genes *CDH2*, *FN1*, *VIM*, *SNAI1*, *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *POSTN*, *COL1A1*, *MMP2*, *MMP7*, and *MMP9*, many of which promote epithelial mesenchymal transition by repressing proteins that maintain tissue integrity such as *CDH1*^[26,27]. Some of the hallmarks of EMT are cell separation and invasion, characterized by loss of cell-cell adhesion and gain of migratory and invasive properties^[18]. Given the role of *SFRP4* as an EMT inhibitor, overexpression in early-onset colon cancer might be a response mechanism to serve as a check on EMT in the wake of increase expression of EMT genes.

However, overexpression of *SFRP4* may not always correlate with EMT suppression. There is accumulating evidence that *SFRP* family proteins may also have oncogenic potential in certain cancer types^[28,29]. While the binding and sequestering of *WNT* ligand by *SFRPs* inhibit *WNT* signaling^[30-33], binding may also allow *SFRPs* to act as carriers for *WNT* proteins in a diffusion gradient, thus expanding the range and activity of *WNT* signaling. This has been reported in *Xenopus* where *SFRP3* and *Crescent* were shown potentially to activate canonical *WNT* signaling by promoting the diffusion of *WNT8* and *WNT11*^[34]. There have also been reports on the dimerization of the N-terminal cysteine-rich domain of *SFRPs* and Frizzled proteins, hence suggesting *SFRPs* direct activation of *WNT* signaling by mimicking the *WNT* ligand^[35-37]. Additionally, it has been suggested that by antagonizing each other, the *SFRPs* indirectly activate *WNT* signaling, as seen in rat renal organogenesis where *SFRP2* was reported to inhibit *SFRP1* activities^[38]. Also, studies have shown that *WNT* regulation could be concentration dependent with low *SFRP* concentrations activating *WNT* signaling and high concentrations inhibiting it^[39].

Consistent with the concept of *SFRPs* functioning as oncogenes, *SFRP1* has been reported to be over-expressed in basal-like breast cancers and implicated in brain-specific metastases, while *SFRP2* induces mammary tumor formation in canines by blocking apoptosis^[40,41]. Both *SFRP1* and *SFRP2* promote cellular invasion, proliferation, and *in vivo* tumor growth in renal cancer^[42,43], and *SFRP1* in gastric cancer. *SFRP2* enhances *WNT3A* signaling in HEK293 and HSG cells^[44] and activates *WNT* signaling through direct interaction with *FZ4/7* in the mouse^[45]. Interestingly, overexpression of *SFRP4* has also been observed in stomach cancer where it associates with poor patient outcomes and interacts strongly with EMT-linked genes in gliomas^[29]. Taken together, these observations suggest that besides being a *WNT* signaling inhibitor, the *SFRP* family members, including *SFRP4*, also have oncogenic potentials, and their mechanism of action might be context dependent or cancer type dependent.

Consistent with the important role *MMP2* plays in the breakdown of the extracellular matrix and the EMT process, particularly in cell invasion and metastasis, *SFRP4* expression was strongly correlated with *MMP2*. In addition to *CDH1*, *SFRP4* was negatively correlated with many other cell adhesion molecules. Given the role these genes play as gatekeepers of EMT and metastasis, it follows that *SFRP4* is upregulated with EMT following downregulation of cell-adhesion molecules. Overexpression of *SFRP4* is also consistent with overexpression of other EMT regulators we previously reported in early-onset colon cancer, such as cartilage oligomeric matrix protein, (*COMP*) which catalyzes collagen fibrillation to maintain the extracellular matrix^[20,46].

Overexpression of *SFRP4* in colon cancer patients resulted in poor survival that

SEFP4	Correlation													Pearson's rho	spearman's rank rho	
SEFP4																
CDH2	0.8026															
	0.8163															
		MMP2														
MMP2	0.8021	0.8076														
	0.7988	0.8035											FN1			
FN1	0.7146	0.8015	0.777									VIM				
	0.7187	0.8074	0.7519													
VIM	0.7622	0.8362	0.8807	0.8317						ZEB1						
	0.7895	0.8331	0.8739	0.8247												
ZEB1	0.7562	0.7523	0.8281	0.7173	0.8460					COL1A1						
	0.7973	0.7603	0.8141	0.7104	0.8408											
COL1A1	0.7538	0.8285	0.895	0.853	0.8803	0.719				TWIST2						
	0.7561	0.8341	0.9005	0.8247	0.8785	0.7117										
TWIST2	0.7413	0.7682	0.85	0.7302	0.8369	0.7414	0.8127					POSTN				
	0.7442	0.7598	0.8526	0.7241	0.8394	0.7429	0.8162									
POSTN	0.739	0.8153	0.8273	0.8568	0.8664	0.6972	0.9096	0.7589				ZEB2				
	0.7471	0.8172	0.8090	0.8262	0.8547	0.6801	0.9004	0.7550								
ZEB2	0.7366	0.7687	0.8691	0.7834	0.935	0.8784	0.8124	0.7968	0.8129			TWIST1				
	0.7590	0.7582	0.8505	0.7639	0.9254	0.8760	0.8007	0.7939	0.7855							
TWIST1	0.72	0.7096	0.8154	0.7044	0.7759	0.6454	0.8115	0.7701	0.7946	0.7074			SNAI2			
	0.7310	0.7235	0.8390	0.6864	0.7960	0.6667	0.8100	0.8024	0.7998	0.7182						
SNAI2	0.679	0.7365	0.8617	0.789	0.8766	0.7925	0.8485	0.7628	0.8607	0.8685	0.7661			MMP9		
	0.6929	0.7391	0.8448	0.7699	0.8704	0.7945	0.8407	0.7752	0.8479	0.8587	0.7838					
MMP9	0.5666	0.6136	0.6497	0.6447	0.762	0.6141	0.6502	0.6491	0.6259	0.7283	0.6004	0.6914			SNAI1	
	0.5888	0.6082	0.6331	0.6459	0.7477	0.6217	0.6427	0.6412	0.6170	0.7167	0.6053	0.6844				
SNAI1	0.4772	0.5145	0.5125	0.5788	0.5581	0.4376	0.6015	0.5947	0.5999	0.4741	0.5941	0.561	0.367			MMP7
	0.4580	0.5088	0.5150	0.5456	0.5598	0.4310	0.5646	0.5863	0.5756	0.4684	0.5545	0.5409	0.3839			
MMP7	0.2874	0.2491	0.3141	0.2258	0.2487	0.1967	0.2592	0.2852	0.2301	0.1966	0.2716	0.2507	0.1672	0.1574		
	0.2734	0.2576	0.2955	0.2227	0.2503	0.1979	0.2800	0.2658	0.2372	0.1830	0.2747	0.2417	0.1103	0.1891		

Figure 2 Epithelial mesenchymal transition-linked genes with positive correlation with SFRP4 expression.

correlated with the survival analysis observed for colon cancer patients overexpressing *COMP*^[20]. Given the implication of *WNT* signaling in many developmental and disease pathways^[47-49] and the knowledge that *SFRP4* inhibits *WNT* signaling, it is possible that early deaths might be due to secondary complications following overexpression of *SFRP4*, which interferes with the normal functions of neighboring healthy cells. However, it is also possible that early deaths are due primarily to disease independent of *SFRP4*, whose overexpression might only be a consequence of the disease.

Although high *SFRP* expression in colon cancer was observed in this study, others have suggested that *SFRP4* expression in tumor may be contributed by the stroma fraction. This was documented in studies by Vincent *et al*^[29] where a strong correlation between *SFRP4* and the stroma was observed in 14 cancer types, including colorectal. Nevertheless, considering that the stroma constitutes an integral part of the tumor microenvironment, its expression of *SFRP4* will undoubtedly constitute an important disease mechanism for potential targeted therapy.

In summary, we had previously shown that *SFRP4* was over-expressed in early-onset colon cancer^[12] and report here *SFRP4* expression was correlated with EMT-linked genes. These findings highlight *SFRP4* as an important contributor to the pathogenesis of colon cancer, particularly early-onset colon cancer, and therefore as a potential biomarker for the early diagnosis of colon cancer. Considering that *SFRP4* modulates *WNT* signaling during cell proliferation, migration, and EMT, which are all hallmarks of cancer pathogenesis. Overexpression of *SFRP4* in early-onset colon cancer and co-expression with EMT-linked genes provide an important first step towards understanding its molecular mechanism in young patients, particularly in the context of cancer metastasis and cell invasion.

SFRP4	CDRRELATION	Pearson's rho	spearman's rank rho		
SFRP4	CLDN4				
CLDN4	-0.1272 -0.1440		CLDN7		
CLDN7	-0.3534 -0.3379	-0.3426 -0.2413	TJP3		
TJP3	-0.2552 -0.2936	-0.3358 -0.1841	-0.4023 -0.3500	MUC1	
MUC1	-0.1979 -0.1929	-0.0902 -0.1368	0.3505 -0.3597	-0.2712 -0.3729	CDH1
CDH1	-0.0639 -0.0379	0.3877 -0.3219	0.0044 -0.1341	0.3488 0.1588	0.2404 -0.2733

Figure 3 Epithelial mesenchymal transition-linked genes with negative correlation SFRP4 express.

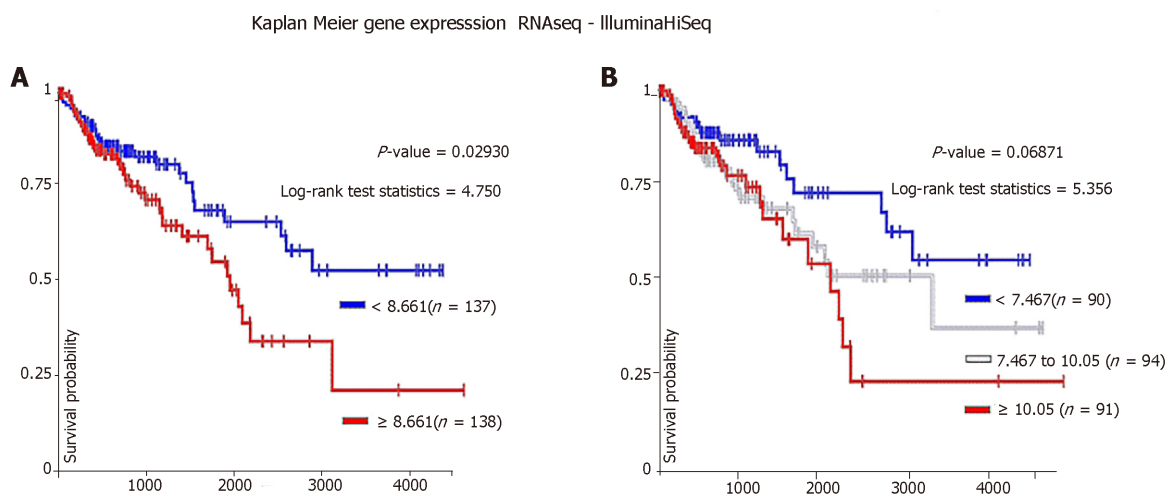


Figure 4 Overall survival of colon cancer patients with low, intermediate or high SFRP4 expression. A: Patients expressing higher levels of SFRP4 (red line) had poor survival compared to those expressing lower SFRP4 levels (blue line); (B) When a three group analysis was performed, patients with higher SFRP4 expression (red line) presented with poor overall survival compared to patients with intermediate (white lines) or low expression (blue line).

ARTICLE HIGHLIGHTS

Research background

Colon cancer affects 1.2 million people around the world and causes about 0.6 million deaths annually. Although the overall incidence and mortality is decreasing, there has been an alarming increase in early-onset cases (< 50 years). These younger patients are often diagnosed at late stages with more aggressive tumors and tend to have poorer survival.

Research motivation

We previously showed that secreted frizzled-related protein 4 (SFRP4), speculated to play an essential role in cancer by inhibiting epithelial mesenchymal transition (EMT), is over-expressed in younger patients.

Research objectives

The aim of our study was to investigate whether there is a correlation between SFRP4 expression and EMT-linked genes in colon cancer and whether SFRP4 over-expression affects patient survival using a larger cohort of patients.

Research methods

Correlation between SFRP4 expression and EMT-linked genes along with survival analysis were performed using the University of California Santa Cruz Cancer browser interface using publicly available The Cancer Genome Atlas-colon adenocarcinoma cohort of colon cancer cases.

Research results

SFRP4 is co-expressed with EMT-linked markers, such as CDH2, FN1, VIM, TWIST1, TWIST2,

SNAI1, *SNAI2*, *ZEB1*, *ZEB2*, *POSTN*, *MMP2*, *MMP7*, *MMP9*, and *COL1A1*, in colon cancer patients. *SFRP4* expression negatively correlated with the EMT-linked suppressors *CLDN4*, *CLDN7*, *TJP3*, *MUC1*, and *CDH1*. Mesenchymal-like samples showed higher expression of *SFRP4* and EMT-linked markers relative to epithelial-like samples. This implicates *SFRP4* in colon cancer EMT mechanism. Additionally, patients with colon tumors over-expressing *SFRP4* presented with significantly poorer overall survival.

Research conclusions

Results of this study suggest a potential role of *SFRP4* in colon cancer aggressiveness, disease progression, and poorer patient survival. Although the role of *SFRPs* including *SFRP4* as *WNT* signaling inhibitors is well documented in the literature, data presented in this manuscript suggest that the *SFRP* family proteins might also activate *WNT* signaling and promote cell proliferation, therefore demonstrating its oncogenic potential and suggesting its mechanism of action might be context dependent.

Research perspectives

More extensive and detailed mechanistic *in vitro* and *in vivo* studies will have to be performed to confirm these initial observations in colon cancer patients.

ACKNOWLEDGEMENTS

We would like to thank the patients without whom this work would not be possible.

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