Original Article

Transcriptional expression, prognostic value and immune infiltration of SFRP family in colorectal cancer: a study based on comprehensive bioinformatics and *in vitro* analyses

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Background: Secreted frizzled-related protein (SFRP) is a crucial regulator of Wnt signaling, involved in multiple biological processes including cell proliferation and metastasis. Despite the accumulation of evidence that indicated that SFRPs are differentially expressed and play a key role in various malignancies, the function of different SFRPs in colorectal cancer (CRC) remains insufficiently studied.

Methods: Multicenter databases, including GEPIA, cBioPortal, UALCAN, Pathway Commons, STRING, TIMER, CCLE, and LinkedOmics, comprehensively analyzed differential expression, prognostic value, genetic alterations, signaling pathways, immune cell infiltration, and associated genes of the SFRP family in CRC patients. Colony formation, wound healing, and transwell assays were performed to further validate *in vitro*.

Results: SFRP family members were differentially expressed in CRC, with each member showing varying degrees of genetic alterations. Except for SFRP5, the remaining members show a significant correlation with immune cells. Interestingly, only SFRP2 significantly correlated with CRC prognosis and stage. Additionally, SFRP2 participated in a number of critical biological processes, including metastasis and cell proliferation. Moreover, cell function assays suggested the elimination of SFRP2 inhibits the proliferation, migration, and invasion of HCT116 cells.

Conclusions: The differential expression of SFRP2 is closely associated with the prognosis of CRC patients. In addition, abnormal expression of SFRP2 has a significant impact on the progression of CRC, including proliferation, migration, and invasion. SFRP2 may become a novel prognostic factor for CRC.

Keywords: Bioinformatics analysis; secreted frizzled-related protein (SFRP) family; colorectal cancer (CRC); biomarker; prognosis

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Introduction

As of 2020, colorectal cancer (CRC) remains the third leading cause of cancer-related death among males and females in the world (1). Although there is a declining trend in CRC incidence in some high-incidence countries, mainly attributed to early screening (e.g., colonoscopy) and the removal of precancerous lesions (polyps), it may miss early cancerous lesions (adenomas) (2,3). Histopathological diagnosis is still the most accurate method available. Among malignant tumors in China, CRC ranks among the top five in both incidence and mortality, and approximately 60% of patients are diagnosed in the middle and late stages (4). It is necessary to discover some more accurate tumor indicators, which can provide new strategies for early diagnosis of CRC.

In all, five members of the glycoprotein-secreting secreted frizzled-related protein (SFRP) family were identified: SFRP1-5. The five members of this family share a similar signal peptide and a frizzled cysteine domain. The signal peptide is essential in secreting SFRP2 and is probably absent in the released mature protein (5). A high degree of overlap exists between the frizzled domain in SFRP family proteins and the domain of Wnt ligand, resulting in mutual binding between the two proteins, which regulates the activity of the Wnt pathway (6).

There has been a correlation between an overactive Wnt signaling pathway and cancer progression, including tumor growth and metastatic processes (7,8). Moreover, abnormal expression of SFRPs in different tumors has been confirmed by numerous studies. For instance, SFRP1 is over-expressed in breast cancer and metastatic renal carcinoma and has been similarly demonstrated in gastric cancer cells (9-11). Emerging evidence suggests that downregulation of SFRP4 expression in ovarian cancer leads to greater metastases and that SFRP4 expression levels directly correlate with patient prognosis (12). Furthermore, the frizzled protein secreted by SFRP5 is involved in histone modification, and down-regulated SFRP5 can act as a tumor suppressor gene in renal carcinoma (13).

In this study, through the screening of database analysis results, only SFRP2 was found to have a statistically significant impact on the prognosis for patients with CRC. Moreover, overexpression of SFRP2 resulted in poor overall survival (OS) and disease-free survival (DFS). However, whether the abnormal expression of SFRP2 can affect the proliferation, migration, and invasion of CRC cells has been poorly studied. Thus, we performed further *in vitro* analysis to validate this issue. The results demonstrated that descending regulation of SFRP2 expression could dramatically suppress the proliferation, migration, and invasion of HCT116 cells. Taken together, our findings provide new perspectives on improving the prognosis for patients with CRC. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-152/rc).

Highlight box

Key findings

- SFRP2 may represent a new prognostic factor for CRC.

What is known and what is new?

- SFRP2 is dysregulated in a variety of cancers, including melanoma, glioma, cervical cancer, breast cancer, and gastric cancer.
- There is very little information in the literature about the correlation between the expression of the SFRP family and CRC. This manuscript is the first study to combine a multicenter database to analyze the expression and prognostic value of the entire SFRP family in CRC.

What is the implication, and what should change now?

- This manuscript reports that SFRP2 may represent a novel prognostic factor in CRC. The findings of this manuscript need to be validated in more CRC cells.

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

GEPIA

The GEPIA database integrates cancer data from The Cancer Genome Atlas (TCGA) with normal tissue data from GTEx, enabling dynamic analysis of gene expression profile data (14). In the present study, we investigated the expression of the SFRP family in pan-cancer and in different stages of CRC using GEPIA, as well as the prognosis of survival in CRC.

cBioPortal

cBioPortal is a visual cancer genomics database that includes data on somatic mutations, DNA copy number alterations, messenger RNA (mRNA) and protein expression, and DNA methylation (15). The types of genomic data integrated into

cBioPortal include somatic mutations, DNA copy-number alterations (CNAs), mRNA and microRNA (miRNA) expression, DNA methylation, protein enrichment networks, and phosphorylated protein enrichment.

UALCAN

UALCAN is a user-friendly and interactive web resource for the analysis of cancer genomics data. UALCAN contains TCGA transcriptomic data, proteomic data, miRNA, and lncRNA data that can help tumor researchers find potential prognostic markers. In the present study, the results of the analysis of SFRP family expression and clinical characteristics of CRC patients were obtained by UALCAN.

Pathway Commons

Pathway Commons (http://www.pathwaycommons.org) is a publicly accessible pathway database that brings together multiple species from numerous sources, including biochemical reactions, complex assembly, transport and catalytic events, as well as interactions of protein complexes, DNA, RNA, and small molecule compounds. Various genes associated with each of the SFRP family members were analyzed by PCViz, which is a pathway commons network visualizer tool.

STRING

The STRING database is a searchable database of known protein-protein interactions and predicted protein-protein interactions. The database contains a large number of networks of protein interactions from multiple species. Users can access the network that interacts with this protein by directly entering the name of the protein. In this study, the STRING database was used to investigate the network of proteins with potential interactions with the SFRP family.

Tumor immune estimation resource (TIMER)

The TIMER database uses RNA-Seq expression profiling data to detect the infiltration of 6 types of immune cells in tumor tissues and provide quantitative analysis (16). By selecting the "gene module", entering the SFRP family genes and selecting the tumor type, the correlation of each gene in the family with the immune infiltration of colon and rectal cancer can be visualized.

LinkedOmics

LinkedOmics is a multi-omics database that integrates global proteomics data into selected TCGA tumor samples. Genomic and proteomic data from all 32 TCGA cancer types and massive cancer cohorts are included. (17). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, associated gene analysis and co-expressed gene analysis in this study were generated by LinkedOmics to visualize the results.

Cell culture and lentivirus transfection

HCT116 cells were obtained from the cell bank of the Chinese Academy of Science. RPMI-1640 medium (PAN-Biotech, Adenbach, Bavaria) with 10% fetal bovine serum (Hyclone, UT, USA) was used to culture HCT116 cells. The small interference RNA (siRNA) was synthesized by Genechem (Shanghai, China). The target sequence was as follows: SFRP2 5'-GAGGAGAUGAACGACAUCATT-3'. The control siRNA sequence was 5'-UUCUCCGAACG UGUCACGU-3'.

RT-qPCR

Total RNA was extracted using the RNA kit (Promega, Madison, USA). cDNA was synthesized by reverse transcription using the PrimeScript™ kit (Takara, Japan). The PCR results were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Electrophoresed proteins were obtained by protein lysis extracts (Solarbio, Beijing, China). The concentration of extracted proteins was detected using the BCA kit (Solarbio, Beijing, China). The amount of protein sampled was 30 μg per lane. PVDF membranes after protein transfer were incubated in primary antibody overnight at 4 ℃, followed by secondary antibody incubation at room temperature for 1 hour. Electrophoresis results were detected using enhanced chemiluminescence (ECL) reagents.

Cell function assays (colony formation, wound healing, and transwell)

The detailed methodology has been described in our

previous study (18).

Statistical analysis

The database has been generated for all statistical analyses and differences between control and transfected groups in the *in vitro* validation were tested using a *t*-test. The P value less than 0.05 was considered statistically significant.

Results

Distinct expression of SFRP family in pan-cancer

Transcripts data from the GEPIA database of 31 solid tumors were used to analyze the differential expression of the SFRP family (*Figure 1*). The expression of the SFRP family in CRC was the focus of our study. As shown in *Figure 2*, SFRP1, SFRP2, SFRP3, and SFRP5 were lowly expressed in colon and rectal cancer tissue, while SFRP4 was highly expressed in colon and rectal cancer tissue.

Based on the mRNA expression level, we then assessed the association between the SFRP family and the pathological stage of CRC. Intriguingly, except for SFRP2 and SFRP4, none of the remaining SFRP members showed significant variability in expression (*Figure 3*).

Prognostic value of the SFRP family in CRC patients

We further investigated the relationship between SFRP mRNA expression and OS and DFS in CRC patients using the GEPIA database. Surprisingly, with the exception of SFRP2, the remaining SFRP family members were not statistically significant with OS (*Figure 4A-4E*). The same situation was presented in the relationship with DFS (*Figure 5A-5E*). According to the UALCAN findings, there was a strong correlation between the expression of SFRP2 and the individual cancer stages, histological subtypes, metastatic status, and *TP53* mutation status (*Figure 6A-6H*). Given these findings, we speculated that SFRP2 may be a potential prognostic marker for CRC.

Genetic alterations and PPI analysis in patients with CRC

Analysis of genetic alterations suggested at least two alteration types for colon adenocarcinoma and up to six alteration types for mucinous adenocarcinoma of the colon and rectum (*Figure 7A*). Twenty-one percent of SFRPs were mutated in a sample of 630 CRC patients (*Figure 7B*). In addition, SFRP1, SFRP2, SFRP3, SFRP4, and SFRP5 were altered in 7%, 4%, 4%, 3%, and 3% of CRC samples, respectively (*Figure 7B*).

The genes associated with each SFRP member are visually displayed in *Figure 7C*. Furthermore, the STRING results revealed that there were 57 SFRP-linked nodes in the built-up PPI network (*Figure 7D*). Most of these protein nodes are associated with the Wnt pathway. To better visualize the potential genes interacting with SFRP family, a gene network was constructed by the Pathway Commons database.

Immune cell infiltration of SFRPs in colon and rectal cancer patients

T lymphocyte is a key component of the body's defense system, capable of recognizing heterologous substances and clearing them in a timely manner. Emerging evidence indicated that tumor progression is associated with T-cell-mediated cellular metabolism, and disruption of T-cell metabolism can lead to abnormal proliferation of tumor cells (19). The results in *Figure 8* showed that the expression of SFRP1 was significantly correlated with the infiltration of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils and dendritic cells in colon and rectal cancer. SFRP2 was positively correlated with the infiltration of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils and dendritic cells in colon cancer patients, but not significantly in rectal cancer patients. The expression of SFRP3 was positively correlated with the infiltration of B cells, $CD4^+$ T cells, macrophages, and dendritic cells into the two types of cancer. Regarding SFRP4, infiltration of all immune cells except B and $CD8^+$ T cells was markedly associated with SFRP4 expression. However, SFRP5 was not associated with any of these immune cells except $CD4^+$ T cell (COAD), dendritic cell (COAD) and macrophages.

KEGG pathway analysis of co-expressed genes associated with SFRP2 in CRC

Combined with previous results, SFRP2 showed potential to be a prognostic marker in CRC. Sequencing data from 379 CRC patients in the LinkedOmics database were extracted. According to the findings in *Figure 9A*, SFRP2 was positively associated with 10,611 genes and negatively associated with 9,217 genes. Furthermore, 50 significant genes had positive and negative correlations with SFRP2, respectively (*Figure 9B,9C*). Of these, the highest positive

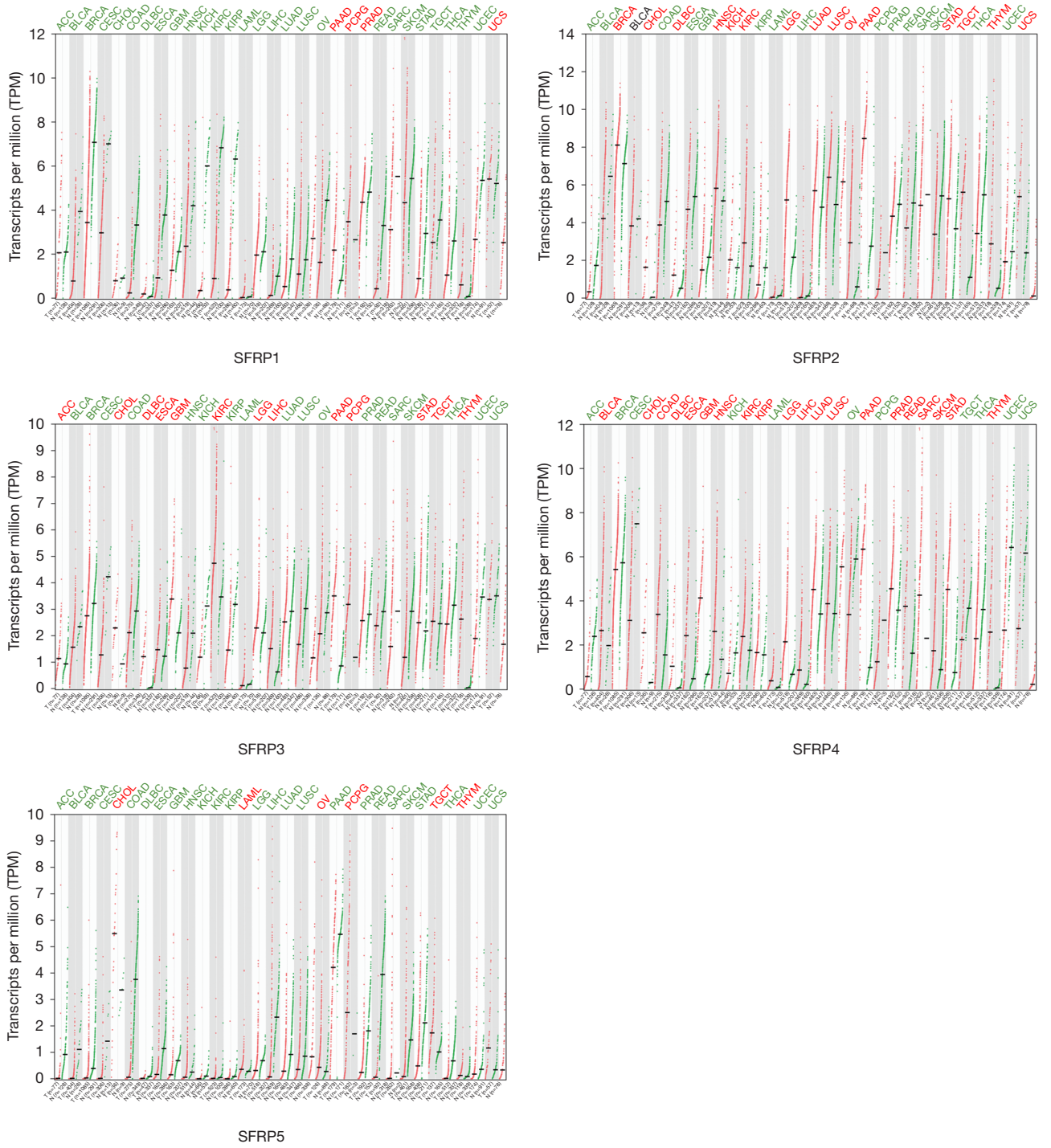


Figure 1 Expression of SFRP family members in various types of cancer (GEPIA). Scatter plots indicated that SFRP1, SFRP2, SFRP3 and SFRP5 were lowly expressed compared to normal tissues, while SFRP4 was expressed at higher levels than the former. SFRP, secreted frizzled-related protein; TPM, transcripts per million.

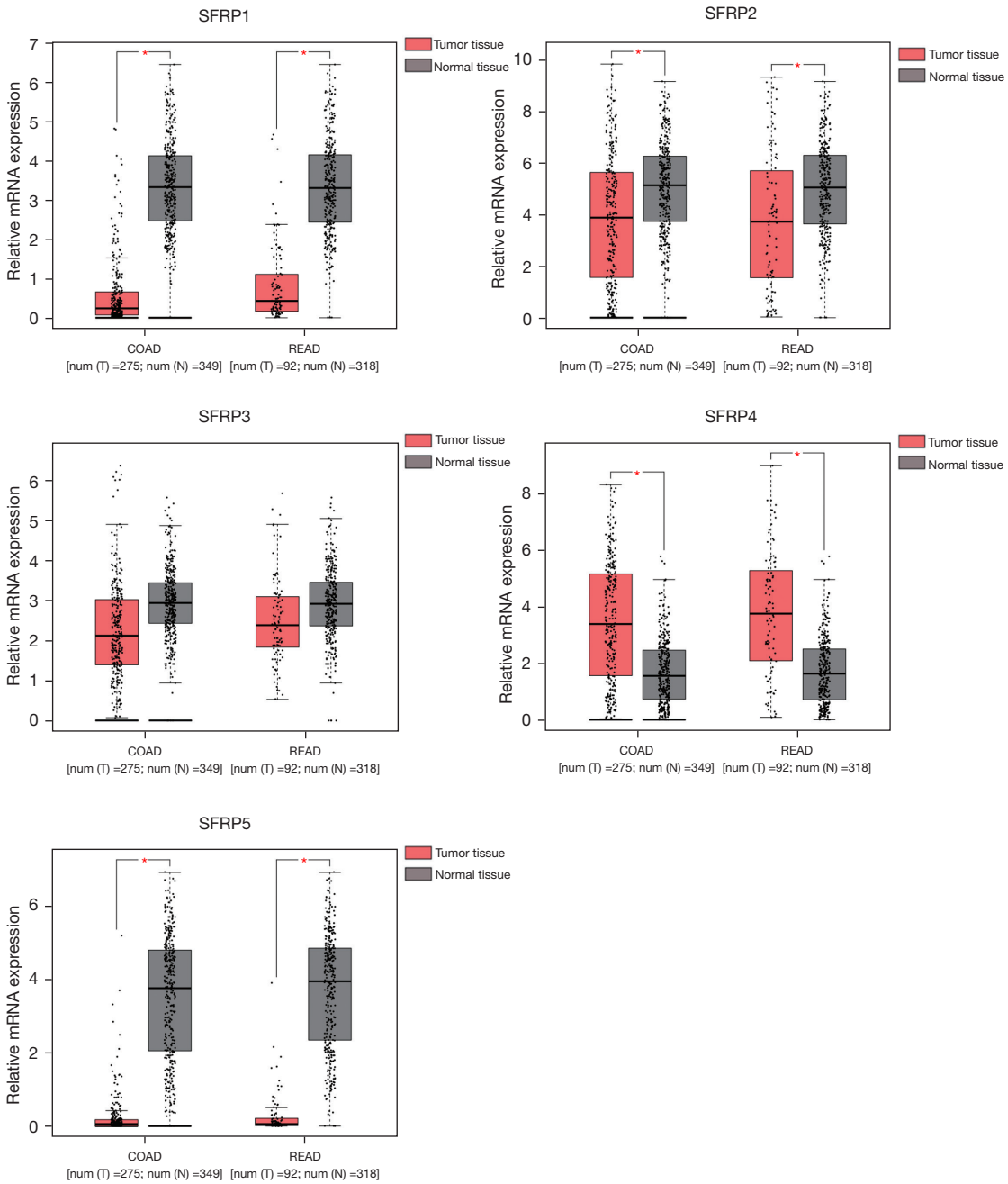


Figure 2 Expression analysis of SFRP family members in CRC and normal tissues (GEPIA). The mRNA expression levels of each SFRP family member in CRC and corresponding normal tissues were compared in the box plot. *, P<0.05. mRNA, messenger RNA; SFRP, secreted frizzled-related protein; CRC, colorectal cancer; COAD, colon adenocarcinoma; READ rectum adenocarcinoma.

association was observed for SFRP2 and GAS1 (Pearson correlation =0.87, P=3.711e-121), while the highest negative association was observed for C6orf136 (Pearson correlation =0.47, P=1.496e-22). Through KEGG pathway analysis, SFRP2 showed a positive correlation with several major cellular functional processes, such as cell growth, migration, invasion, and angiogenesis (*Figure 9D*). In addition, as shown in *Figure 9E,9F*, each biological process and molecular function category of SFRP2 was represented by red and green bars.

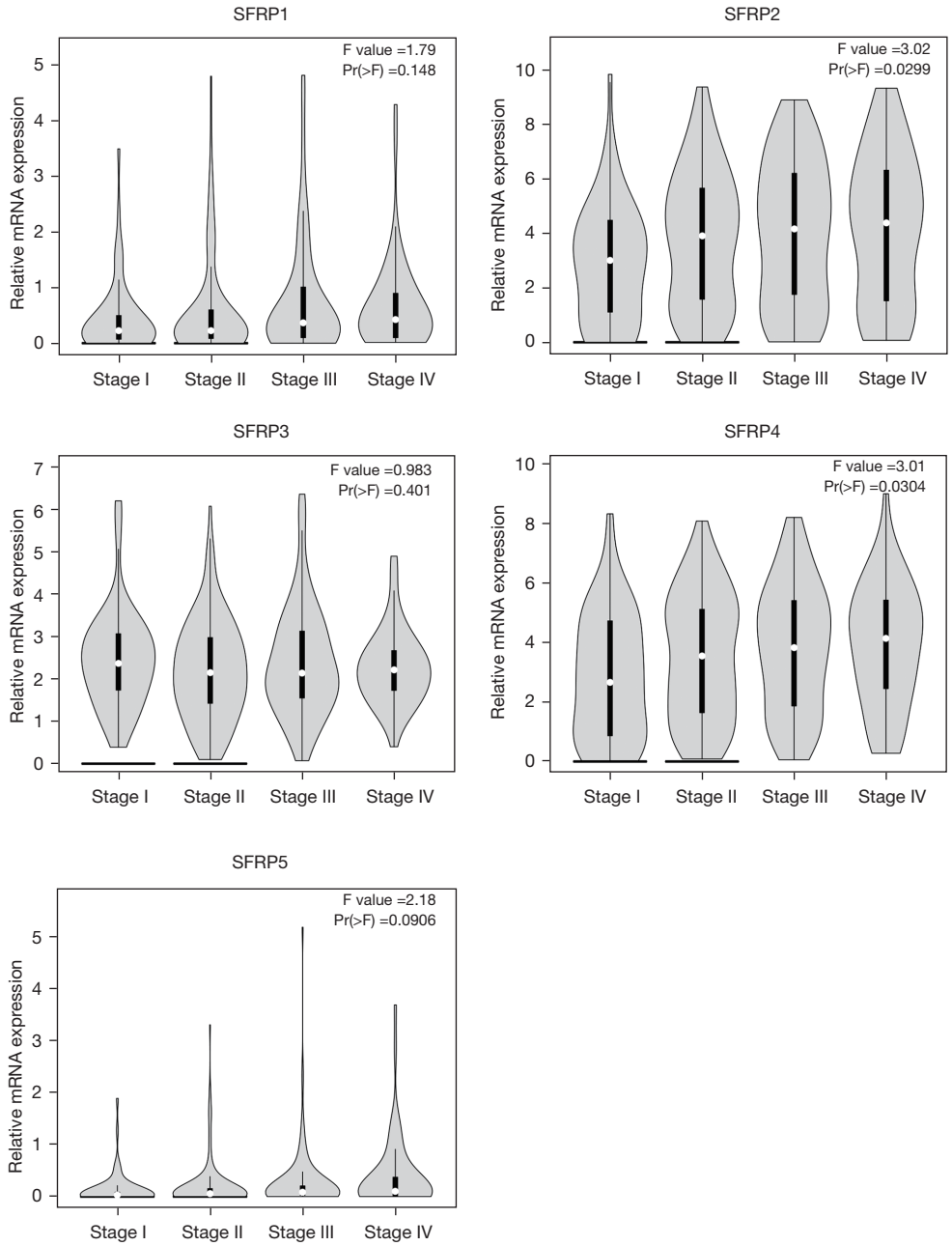


Figure 3 Association between mRNA expression of SFRPs and tumor stage in CRC patients (GEPIA). The expression of SFRP2 and SFRP4 was significantly correlated with the pathological stage of CRC patients (P<0.05). mRNA, messenger RNA; SFRP, secreted frizzled-related protein; CRC, colorectal cancer.

Knockdown of SFRP2 inhibits CRC cell proliferation, migration, and invasion

First, the mRNA expression data of SFRP2 were downloaded directly from the cancer cell line encyclopedia (CCLE) website (https://www.betastasis.com/tissues/cancer_cell_line_encyclopedia/gene_expression_barplot). As shown in *Figure 10A*, among the 57 CRC cell lines, HCT116, HT29, NCH508 and SW116 cells showed the highest expression. Therefore, we selected one of the most common HCT116 cells for further *in vitro* analysis.

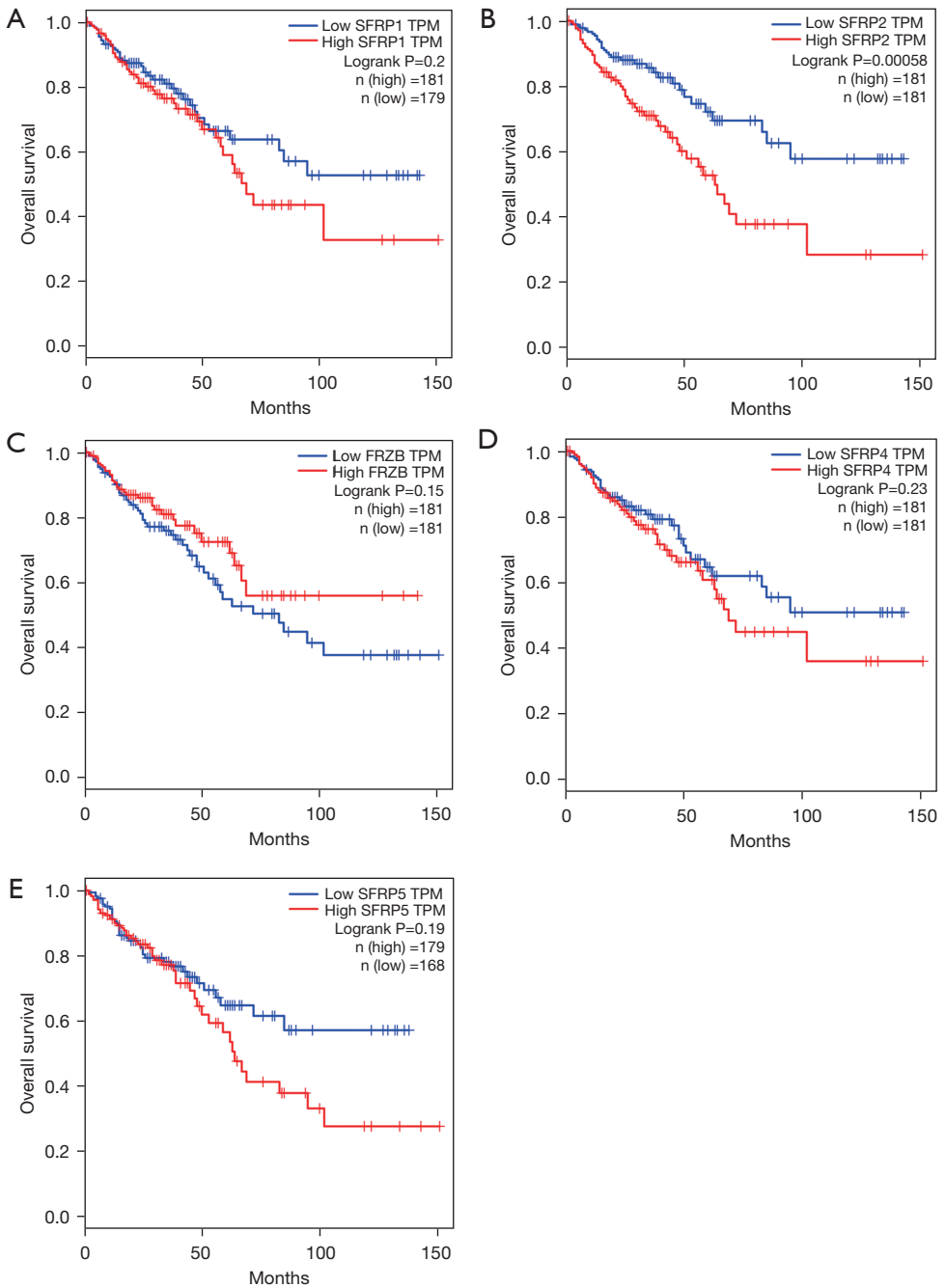


Figure 4 (A-E) Prognostic value of mRNA expression for each SFRP family member in CRC (GEPIA). CRC patients with high expression of SFRP2 were significantly associated with shorter OS (P<0.05). OS, overall survival. FRZB frizzled-related protein; TPM transcripts per million; CRC colorectal cancer; SFRP secreted frizzled-related protein.

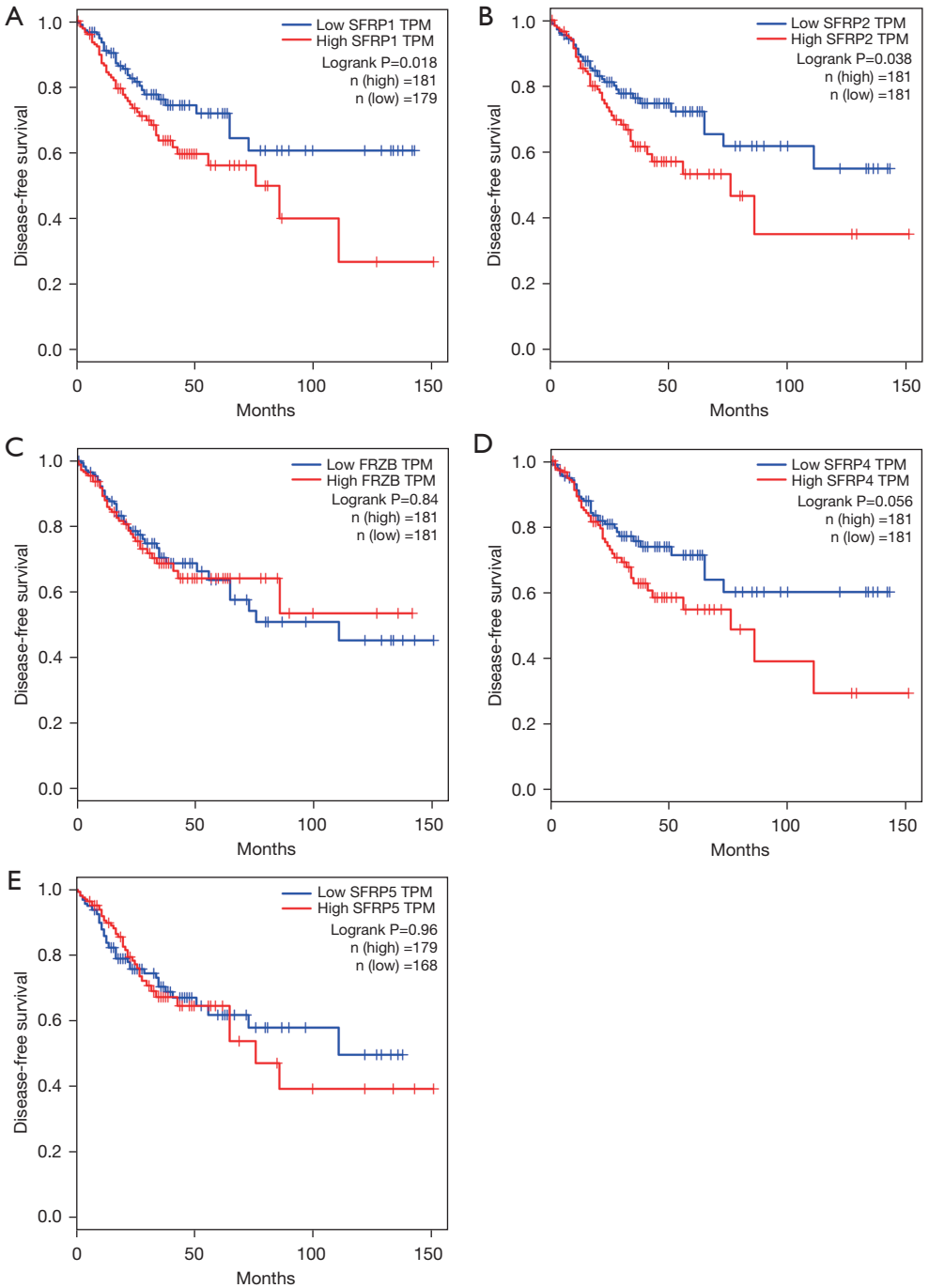


Figure 5 (A-E) Prognostic value of mRNA expression for each SFRP family member in CRC (GEPIA). CRC patients with high expression of SFRP2 were significantly associated with shorter DFS (P<0.05). DFS, disease-free survival; FRZB, frizzled-related protein; TPM, transcripts per million; CRC, colorectal cancer; SFRP, secreted frizzled-related protein.

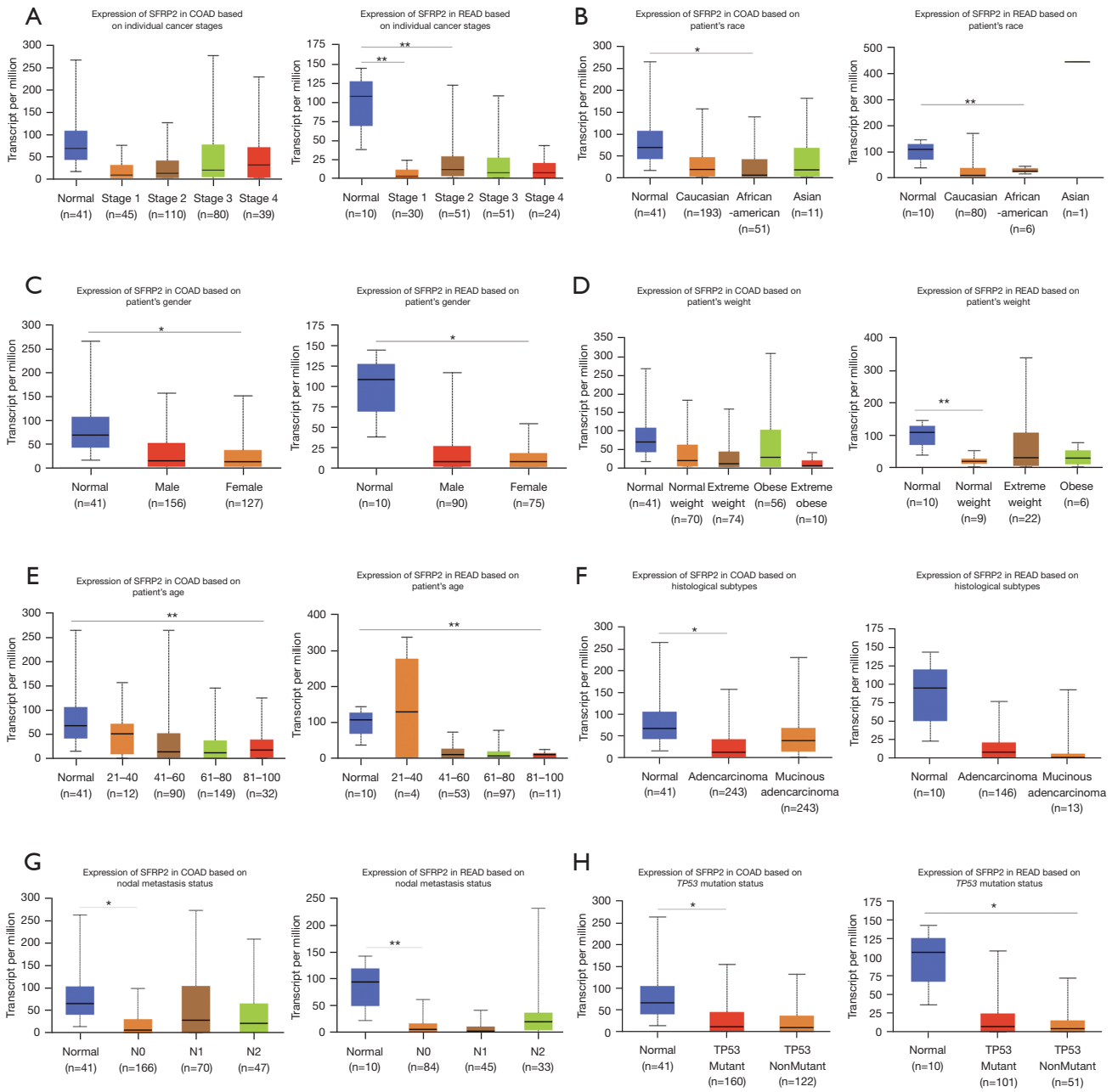


Figure 6 (A-H) SFRP2 transcript levels in subgroups of CRC patients, stratified by individual cancer stages, race, gender, and other criteria (UALCAN). Compared with normal tissues, SFRP2 was lowly expressed in all clinical subtypes shown in (A-H) and had a significant correlation with these clinical characteristics. *, P<0.05; **, P<0.01. COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; CRC, colorectal cancer; SFRP, secreted frizzled-related protein.

After lentiviral transfection, both mRNA and protein expression levels of SFRP2 were significantly suppressed (*Figure 10B,10C*). To validate the previous KEGG results, cell colony formation, wound healing and transwell assays were used to validate the effect of knockdown of SFRP2 on the proliferation, migration and invasion ability of HCT116 cells, respectively. The results showed that the proliferation, migration and invasion ability of HCT116 cells were significantly inhibited after knockdown of SFRP2 (*Figure 10D-10F*). Taken together, these data suggest that

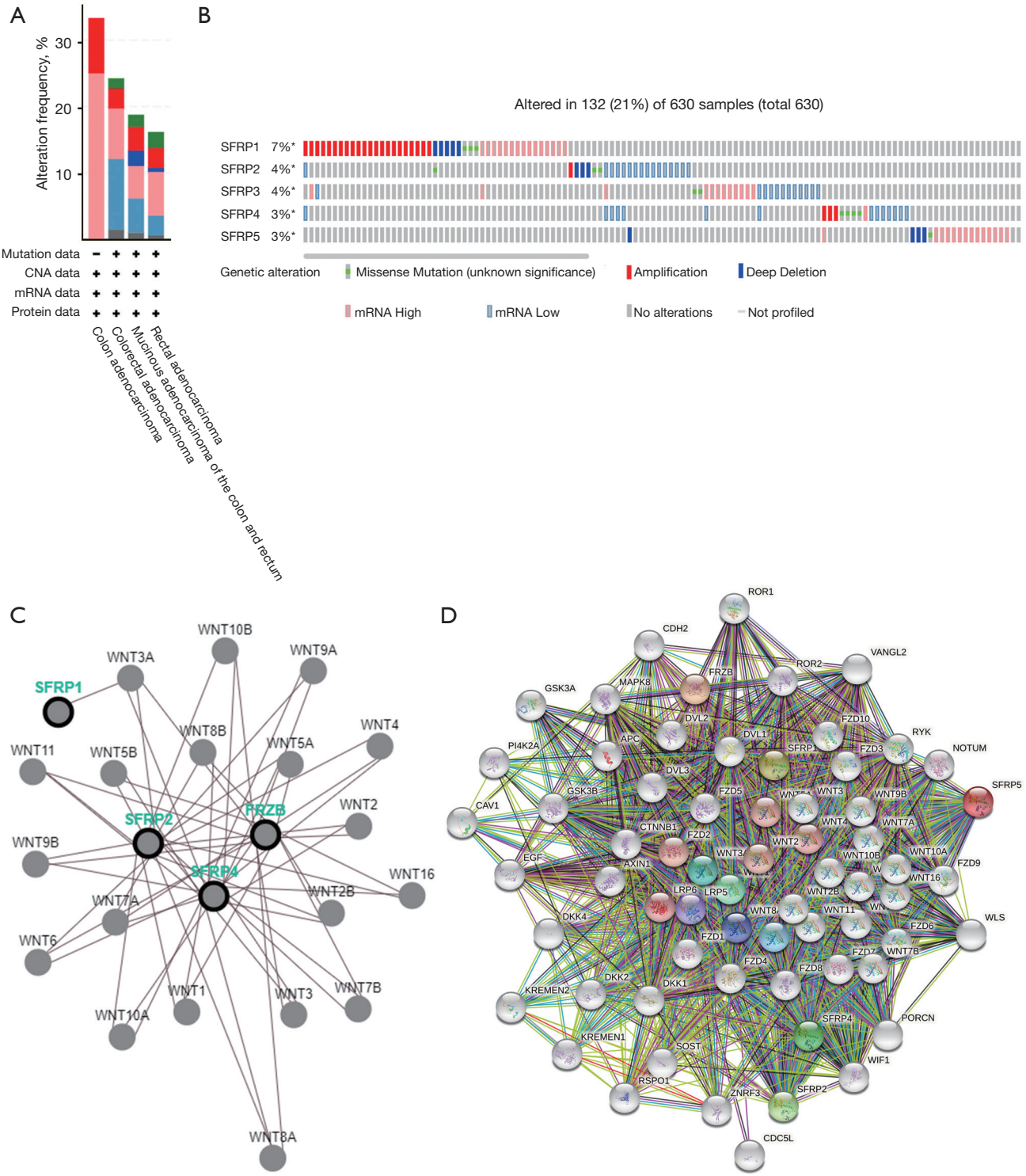
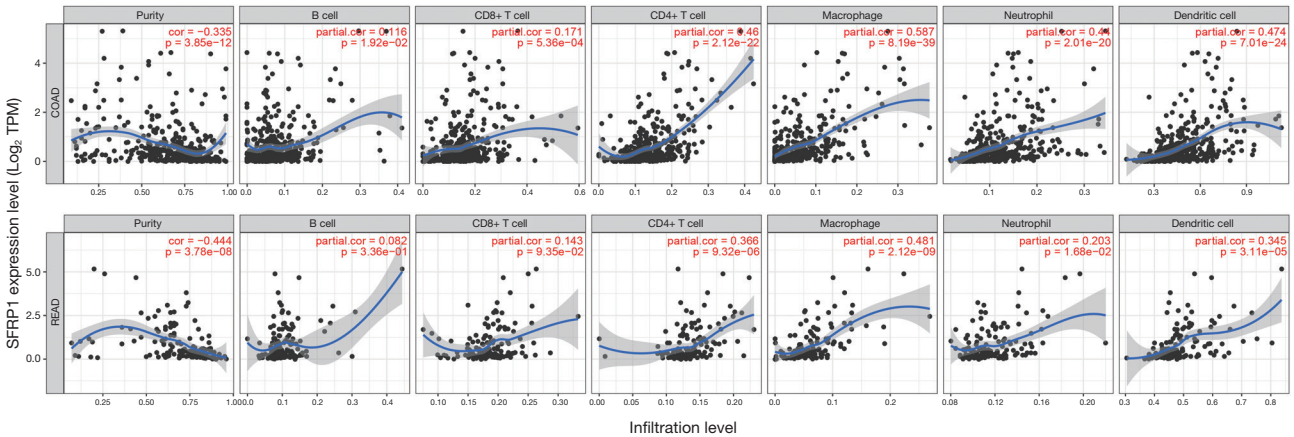
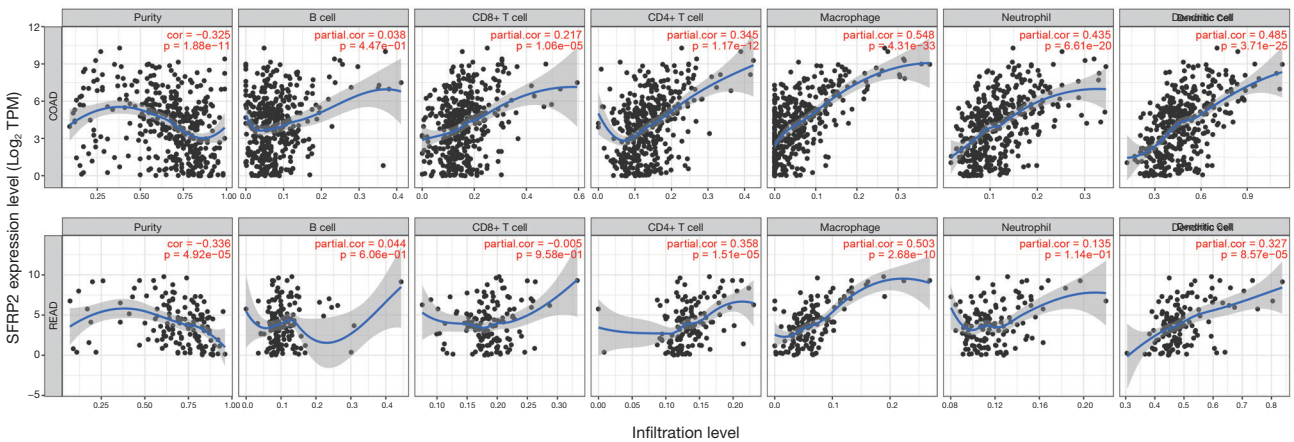
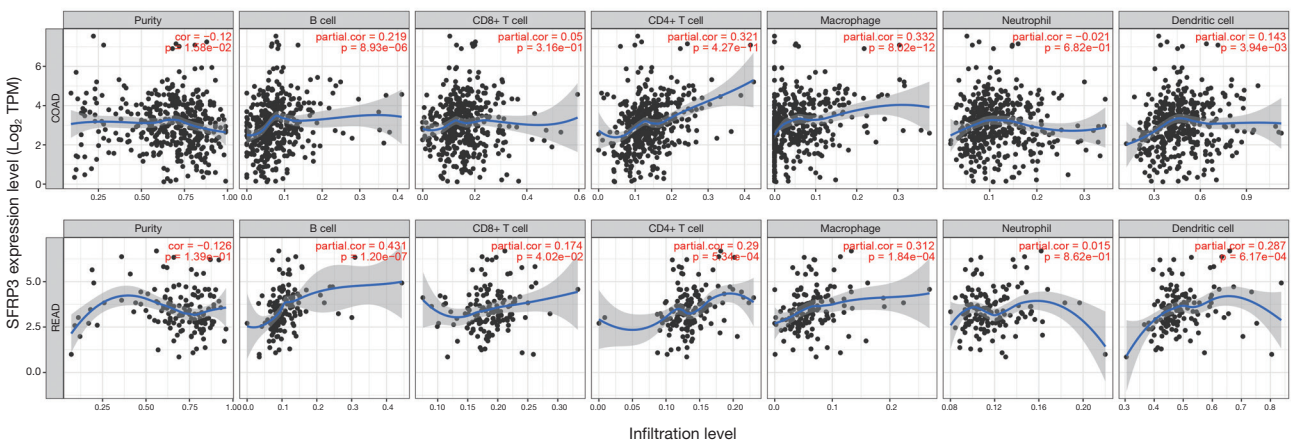


Figure 7 Mutation and expression analysis of *SFRP* gene in CRC (cBioPortal, Pathway Commons and STRING). (A,B) Summary of alterations in different expressed SFRPs in CRC. SFRPs were altered in 132 of the 630 CRC patient samples, accounting for 21%. (C,D) Protein-protein interaction network of the SFRP family. *, P<0.05. SFRP, secreted frizzled-related protein; CRC, colorectal cancer; CNA, copy number alteration.







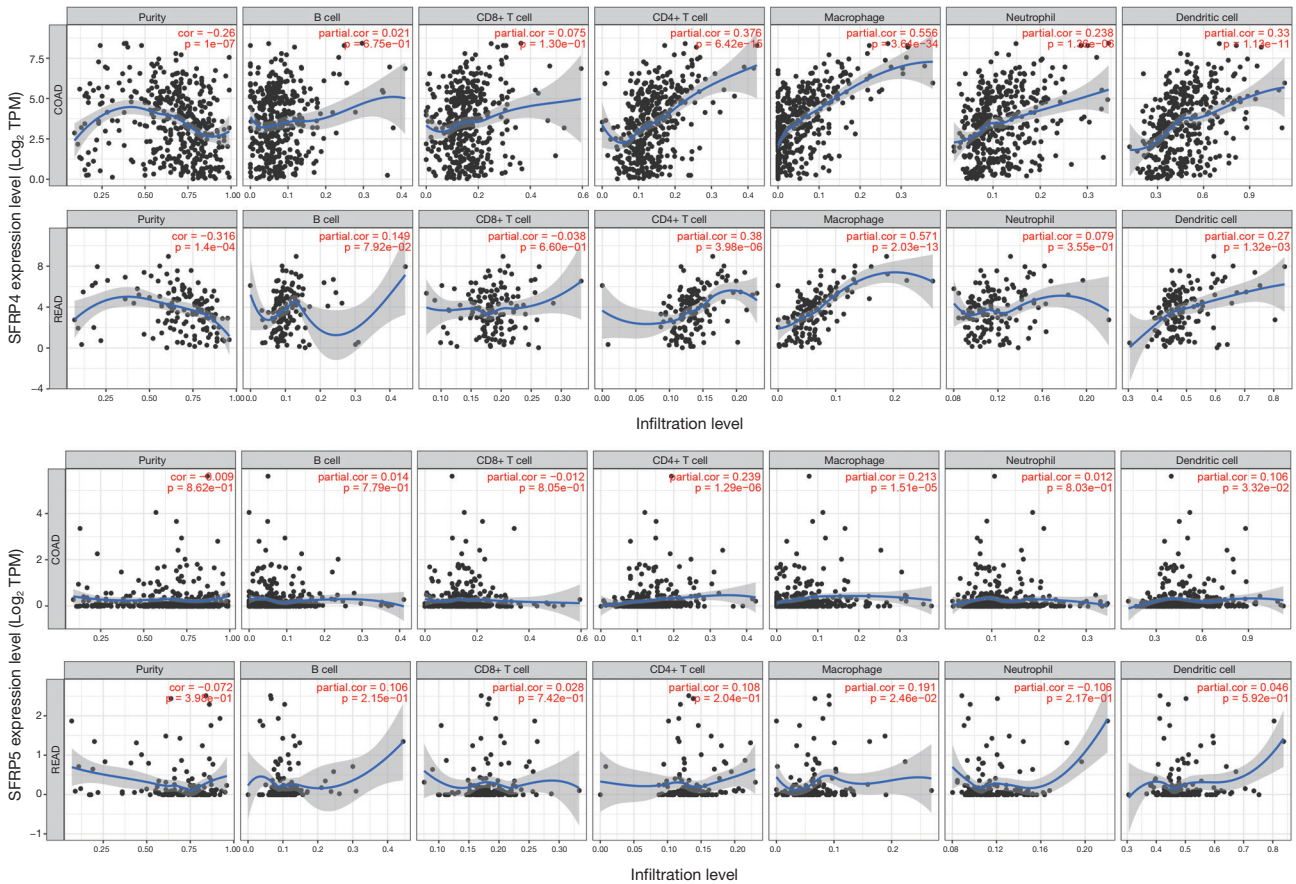


Figure 8 Association between differentially expressed SFRPs and immune cell infiltration (TIMER). Association between immune cell abundance and SFRP1-5 expression. TPM, transcripts per million; SFRP, secreted frizzled-related protein.

SFRP2 may be involved in the progression of CRC.

Discussion

Prior studies that have noted the dysregulation of SFRP2 in various cancers, including melanoma, glioma, prostate cancer, breast cancer, and gastric cancer (20-24). However, there is very little information in the literature regarding the correlation between the SFRP family and CRC transcriptome expression, biological function, and prognostic value. One interesting finding in our research is that Only SFRP2 showed significant associations with pathological staging and survival outcomes in CRC patients. SFRP2 was found to be capable of regulating the progression of CRC in further *in vitro* experiments. Emerging evidence has indicated that approximately 35% of CRC patients are caused by mutations in tumor suppressors or oncogenes (25). DNA methylation analysis revealed that SFRP1 and SFRP2 have a high mutation status in CRC patient samples.

In 1997, SFRP2 was first identified in cultures of mouse embryonic cells and located on human chromosome 4q31.3 (26,27). Accumulating evidence suggests that SFRP2 binds to Wnt3a, activating Wnt signaling, and that exposure to Wnt3a and SFRP2 modulates cell proliferation (28). In addition, several *in vivo* studies have further confirmed that the absence of SFRP2 resulted in reduced Wnt activity when examining the levels of activated β-catenin in the gut of $SFRP2^{-/-}$ mutant mice (29). These data indicated that SFRP2 is a positive regulator of Wnt signaling. By controlling the Wnt pathway's activity, SFRP2 can also affect tumor metastasis in addition to cell proliferation (30). Indeed, one of the major causes of the current difficulty in treating advanced tumors is the formation of metastases. Emerging evidence suggests that SFRP2 expression is distinctly higher in metastatic osteosarcoma compared to non-

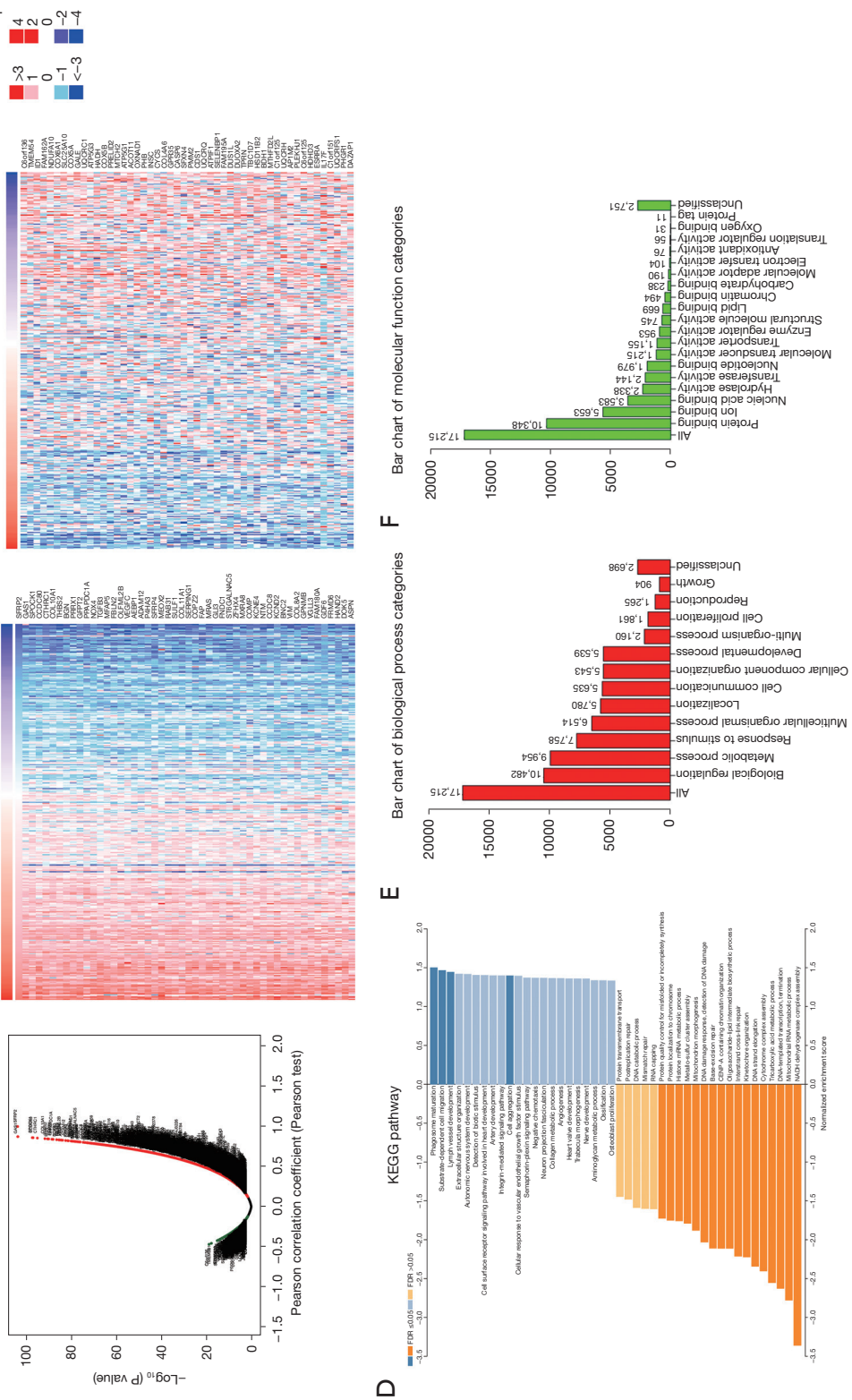


Figure 9 Differentially expressed genes and related biological processes associated with SFRP2 in CRC (LinkedOmics). (A) Pearson test was used to analyze the correlation between SFRP2 and differentially expressed genes in CRC. (B,C) Heatmap analysis showing genes positively and negatively correlated with SFRP2 in CRC (Top 50). Red represents positively correlated genes, and green represents negatively correlated genes. (D) KEGG analysis showing functional pathways associated with SFRP2. (E,F) Bar chart showing the categories of biological processes and molecular functional categories associated with SFRP2. KEGG, Kyoto encyclopedia of genes and genomes; CRC, colorectal cancer; SFRP, secreted frizzled-related protein.

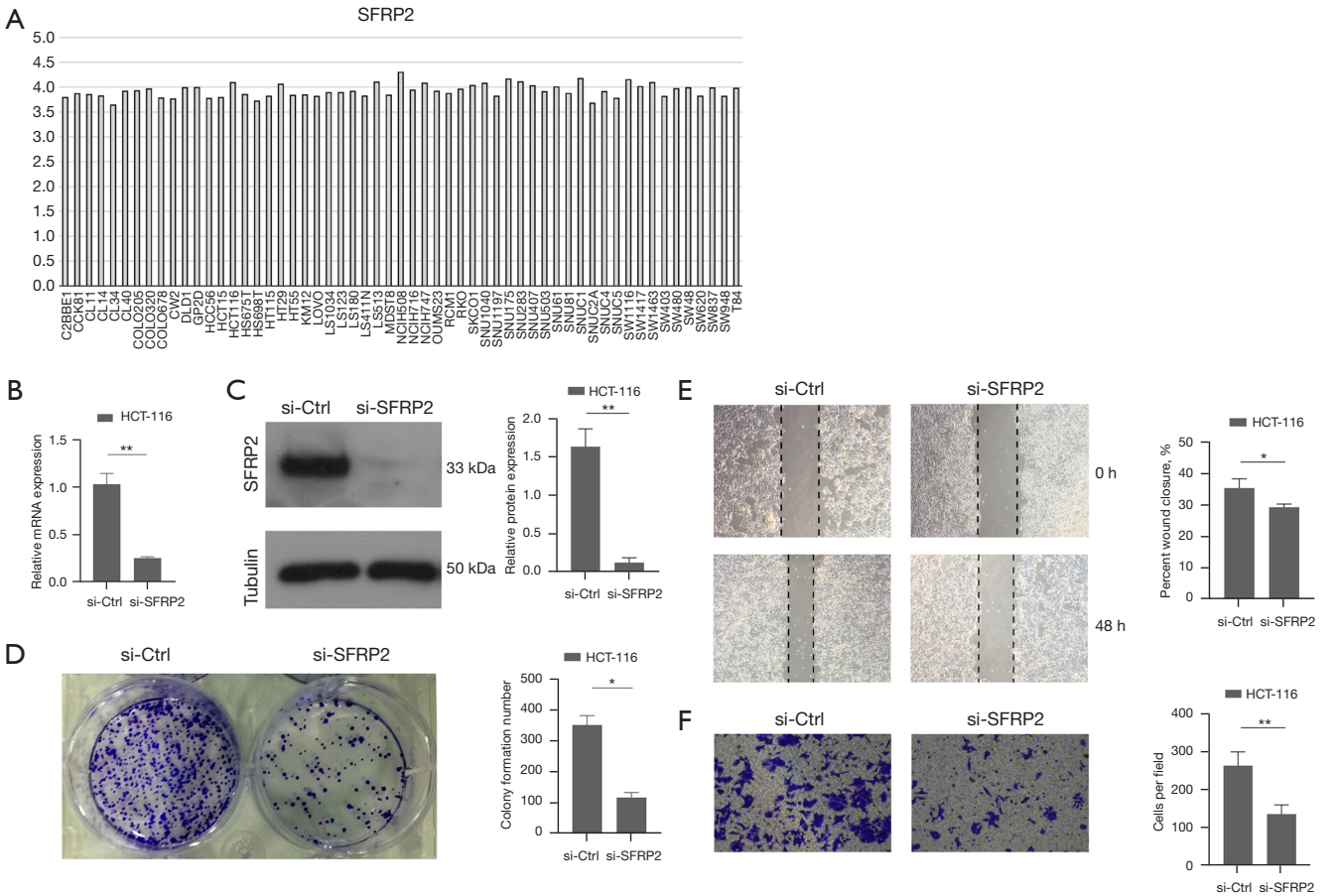


Figure 10 SFRP2-knockdown inhibits CRC cell proliferation, migration and invasion. (A) The mRNA level of SFRP2 in different CRC cell lines from CCLE analysis. (B) qPCR validation of the mRNA expression of SFRP2 in HCT116 cells transduced with the siRNA. (C) Western blot validation of SFRP2 protein expression in HCT116 cells after transfection with siRNA. (D) Colony formation assay of HCT116 cells showing that knockdown of SFRP2 inhibited cell proliferation. Crystal violet staining. (E) Wound healing assay by inverted microscopy showed that knockdown of SFRP2 inhibited cell migration, original magnification: ×200. (F) Transwell assay showing that knockdown of SFRP2 inhibited cell migration, original magnification: ×200. *, P<0.05; **, P<0.01. CCLE, cancer cell line encyclopedia; CRC, colorectal cancer; SFRP, secreted frizzled-related protein.

metastatic osteosarcoma cell lines and tissue samples (31). Furthermore, SFRP2 promotes metastatic growth in breast cancer and melanoma as well (21,22).

For the first time, our study combined transcriptome, mutation, mRNA, protein, and immune infiltration expression of the SFRP family in CRC from multiple databases of GEPIA, cBioPortal, Pathway Commons, STRING, TIMER, CCLE, and LinkedOmics. A further investigation of SFRPs in CRC was conducted through GEPIA. Nevertheless, only SFRP2 showed a significant correlation in both OS and DFS, suggesting that SFRP2 may be a major prognostic marker in CRC.

Transcriptome analysis revealed that SFRPs were all genetically altered to varying degrees in CRC. These findings could elucidate some of the origins of CRC. Then we found that SFRPs are mainly connected to proteins in the Wnt pathway. Moreover, the tumor microenvironment (TME) is receiving increasing attention from researchers as it may provide the conditions for tumors to undergo immune escape (32). In the present work, TIMER was used to analyze the associations between six major immune cells and SFRPs. Our results showed that except for SFRP5, the remaining SFRPs showed significant association with these immune cells. Combining the data from the previous

analysis data, we further focused on the gene co-expression and signaling pathway of SFRP2. Through KEGG analysis, we found that SFRP2 is mainly involved in the biological process of cell proliferation, which is consistent with the previously mentioned studies. Proliferation, migration, and invasion were all dramatically reduced in HCT116 cells when SFRP2 expression was knocked down. This finding is consistent with the studies reported by Techavichit *et al.* (31), Kaur *et al.* (21) and Montagner *et al.* (22).

Inevitably, there are several limitations existing in our study. All clinical data in the study were obtained from databases, and domestic multicenter clinical samples should be added to future analyses. The HCT116 cells used in the study were APC wild-type, and CRC cells of the APC mutant lines should be added for future experiments. In addition, the specific mechanism of how SFRP2 regulates the Wnt pathway needs to be further explored.

Conclusions

We combined multiple databases to comprehensively study the expression, prognosis, and immune infiltration of the SFRP family in CRC, and finally screened SFRP2 for *in vitro* validation. The results showed that the elimination of SFRP2 influenced the process of proliferation, migration, and invasion of CRC, suggesting the possibility of SFRP2 as a new CRC marker.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-152/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-152/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-152/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-152/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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