ISSN 1948-5204 (online)

World Journal of *Gastrointestinal Oncology*

World J Gastrointest Oncol 2023 December 15; 15(12): 2049-2241

Published by Baishideng Publishing Group Inc

World Journal of Gastrointestinal Oncology

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AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

INDEXING/ABSTRACTING

The *WJGO* is now abstracted and indexed in PubMed, PubMed Central, Science Citation Index Expanded (SCIE, also known as SciSearch®), Journal Citation Reports/Science Edition, Scopus, Reference Citation Analysis, China Science and Technology Journal Database, and Superstar Journals Database. The 2023 edition of Journal Citation Reports® cites the 2022 impact factor (IF) for *WJGO* as 3.0; IF without journal self cites: 2.9; 5-year IF: 3.0; Journal Citation Indicator: 0.49; Ranking: 157 among 241 journals in oncology; Quartile category: Q3; Ranking: 58 among 93 journals in gastroenterology and hepatology; and Quartile category: Q3. The *WJGO*'s CiteScore for 2022 is 4.1 and Scopus CiteScore rank 2022: Gastroenterology is 71/149; Oncology is 197/366.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Xiang-Di Zhang*; **Production Department Director:** *Xu Guo*; **Editorial Office Director:** *Jia-Ru Fan*.

NAME OF JOURNAL
World Journal of Gastrointestinal Oncology

ISSN
ISSN 1948-5204 (online)

LAUNCH DATE
February 15, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Monjur Ahmed, Florin Burada

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-5204/editorialboard.htm

PUBLICATION DATE
December 15, 2023

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World J Gastrointest Oncol 2023 December 15; 15(12): 2150-2168

DOI: 10.4251/wjgo.v15.i12.2150

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Basic Study

Association between heat shock factor protein 4 methylation and colorectal cancer risk and potential molecular mechanisms: A bioinformatics study

Wen-Jing Zhang, Ke-Lin Yue, Jing-Zhai Wang, Yu Zhang

Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): E

P-Reviewer: Duraes LC, United States; El-Arabey AA, Egypt; Rotondo JC, Italy; Shinozaki M, Japan

Received: July 28, 2023
Peer-review started: July 28, 2023
First decision: September 26, 2023
Revised: October 16, 2023
Accepted: November 17, 2023
Article in press: November 17, 2023
Published online: December 15, 2023

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Abstract

BACKGROUND

We previously demonstrated that heat shock factor protein 4 (HSF4) facilitates colorectal cancer (CRC) progression. DNA methylation, a major modifier of gene expression and stability, is involved in CRC development and outcome.

AIM

To investigate the correlation between *HSF4* methylation and CRC risk, and to uncover the underlying molecular mechanisms.

METHODS

Differences in β values of *HSF4* methylation loci in multiple malignancies and their correlation with *HSF4* mRNA expression were analyzed based on Shiny Methylation Analysis Resource Tool. *HSF4* methylation-related genes were identified by LinkedOmics in CRC, and Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses were performed. Protein-protein interaction network of *HSF4* methylation-related genes was constructed by String database and MCODE algorithm.

RESULTS

A total of 19 CpG methylation loci were identified in *HSF4*, and their β values were significantly increased in CRC tissues and exhibited a positive correlation with *HSF4* mRNA expression. Unfortunately, the prognostic and diagnostic

performance of these CpG loci in CRC patients was mediocre. In CRC, there were 1694 *HSF4* methylation-related genes; 1468 of which displayed positive and 226 negative associations, and they were involved in regulating phenotypes such as immune, inflammatory, and metabolic reprogramming. *EGFR*, *RELA*, *STAT3*, *FCGR3A*, *POLR2K*, and *AXIN1* are hub genes among the *HSF4* methylation-related genes.

CONCLUSION

HSF4 is highly methylated in CRC, but there is no significant correlation between it and the prognosis and diagnosis of CRC. *HSF4* methylation may serve as one of the ways in which HSF4 mediates the CRC process.

Key Words: Colorectal cancer; DNA methylation; Prognosis; Diagnosis; Bioinformatics; Heat shock factor protein 4

Core Tip: Colorectal cancer (CRC) is a common malignant tumor of the gastrointestinal tract with clinical manifestations of diarrhea, constipation, and abdominal pain. We previously demonstrated that heat shock factor protein 4 (HSF4) accelerates the malignant biological behavior of CRC cells *in vivo* and in vitro. This study reveals that HSF4 is highly methylated and associated with *HSF4* overexpression in CRC. Although the diagnostic and prognostic value of *HSF4* methylation is poor, it may be involved in the process of CRC by mediating the expression of *HSF4* or related genes. Combined with the finding of our previous study, the present study suggests that the high expression of *HSF4* mRNA and protein and its oncogenic effects are likely to be associated with *HSF4* methylation.

Citation: Zhang WJ, Yue KL, Wang JZ, Zhang Y. Association between heat shock factor protein 4 methylation and colorectal cancer risk and potential molecular mechanisms: A bioinformatics study. *World J Gastrointest Oncol* 2023; 15(12): 2150-2168
URL: https://www.wjgnet.com/1948-5204/full/v15/i12/2150.htm
DOI: https://dx.doi.org/10.4251/wjgo.v15.i12.2150

INTRODUCTION

Colorectal cancer (CRC) is a malignant tumor of the digestive tract that occurs in the rectum, cecum, and entire colon, with symptoms such as abdominal pain, difficulty passing stool, constipation, or diarrhea[1]. According to the latest statistics from the World Health Organization[2], CRC has become the third most common malignancy worldwide after lung cancer and breast cancer, with about 200000 new cases occurring worldwide each year, of which, 916000 die from CRC. According to the American Society of Clinical Oncology[3], the 5-year survival rate for patients with CRC is approximately 65%. Nevertheless, most CRC patients have already developed distal metastases by the time they receive a definitive diagnosis, which leads to a shrinking 5-year survival rate to 14%[3]. Consequently, the search for new biomarkers will facilitate the timely diagnosis of CRC and provide new insights into the mechanisms of CRC occurrence and development.

DNA methylation is a process of chemical modification of DNA that affects biological processes such as gene expression, cell differentiation and development[4-6]. In epigenetics, DNA methylation is an important marker of cellular genetic information and is widely applied in cancer prediction and diagnosis[7,8]. For CRC, the United States Food and Drug Administration currently approves SEPT9 (blood samples) and a combination of *NDRG4* and *BMP3* (stool samples) as commercially available biomarkers related to methylation[9]. In addition, *APC*, *SFRP1*, *SFRP2*, *SDC2*, *MGMT*, *VIM* and *NDRG4* are methylation-related candidate markers of CRC[10]. Mechanistically, DNA methylation can inhibit gene transcription or activate gene expression, thereby affecting protein synthesis to mediate the cancer process. For instance, teashirt zinc finger homeobox 3 (TSHZ3) promoter methylation effectively suppresses TSHZ3 expression, which facilitates CRC growth and metastasis[11]. Heparanase 2 (HPSE2) is highly methylated in CRC and is associated with poor patient prognosis, and high methylation of *HPSE2* reduces *HPSE2* expression, which inhibits the p53/p21 signaling cascade and facilitates proliferation of CRC cells *in vivo* and *in vitro*[12]. Heat shock response (HSR), an ancient cellular self-protective response, helps tumor cells to survive and proliferate smoothly under the stimulation of adverse microenvironment, oxidative stress and other stressors[13]. Heat shock factor protein 4 (HSF4), a member of the heat shock transcription factor family, plays an important role in HSR by preventing abnormal protein folding and aggregation to maintain intracellular protein homeostasis[13,14]. HSF4 has been identified as a cancer-promoting factor in lymphoma [15], breast cancer[16], and cervical cancer[17]. Our previous study demonstrated that *HSF4* is significantly upregulated in CRC, which predicts poor patient prognosis, and that it promotes CRC progression by enhancing the activity of c-MET and downstream ERK1/2 and AKT signaling pathways[18]. Nevertheless, whether DNA methylation is involved in HSF4-mediated CRC progression remains to be investigated.

This study investigated the correlation between *HSF4* methylation and *HSF4* expression, and its prognostic and diagnostic value in CRC, and aimed to identify the potential molecular mechanisms associated with *HSF4* methylation through bioinformatics analysis. The aim was to provide a theoretical basis and a novel perspective for *HSF4* as a methylation-related biomarker for future CRC diagnosis and treatment.

Table 1 Basic information of the dataset in this study

Web	Sample source	Sample type	Platform	Samples number
SMART	TCGA_COAD	Tissue	Methylation 450K	Normal = 34, tumor = 288
SMART	TCGA_32 cancer types	Tissue	Methylation 450K	Normal = 676, tumor = 8604
LinkedOmics	TCGA_COADREAD	Tissue	Methylation 27K	Tumor = 233

MATERIALS AND METHODS

Differential analysis of HSF4 methylation and its prognostic and diagnostic value

The Shiny Methylation Analysis Resource Tool (SMART) APP is an interactive and user-friendly web application for comprehensive analysis of DNA methylation in the The Cancer Genome Atlas (TCGA) project, with data from TCGA (https://portal.gdc.cancer.gov/)[19]. The level of methylation at each CpG loci of *HSF4* was assessed using the β value, which is the ratio of the methylation of the allele to the intensity of unmethylation, ranging from 0 to 1. In this study, we analyzed differences in the β values of 19 methylation probes associated with *HSF4* in 33 malignancies by SMART, including COAD, READ, BRCA, LAML, LGG, LIHC, BLCA, CESC, CHOL, KIRP, SKCM, LUAD, ACC, DLBC, KIRC, PCPG, OV, ESCA GBM, STAD, UCEC, UCS, HNSC, TGCT, THCA, THYM, KICH, PRAD, SARC, LUSC, MESO, PAAD, and UVM. Wilcoxon rank sum test was performed for difference analysis of β values, and data was adjusted using the Benjamini-Hochberg method. In addition, β values of 19 *HSF4*-related methylation probes were analyzed differentially in COAD stages and their correlation with HSF4 mRNA expression based on SMART. The differential analysis of β values in COAD stages was performed based on ANOVA, and the correlation between β values of each probe and *HSF4* mRNA expression was performed based on Pearson. The COAD dataset in SMART was extracted and *HSF4* methylation in prognostic and diagnostic value of COAD was assessed by survival (https://cran.r-project.org/web/packages/survival/index.html) and pROC[20]/timeROC[21] R packages, respectively. Kaplan-Meier survival curves, Receiver operating characteristic (ROC) curves and time-dependent ROC curves were visualized with the ggplot2 R package[22]. Patient information is shown in Table 1.

Identification of HSF4 methylation-related genes and their enrichment analysis

LinkedOmics is a publicly available portal that includes three analysis modules, LinkFinder, LinkInterpreter and LinkCompare, to support users in performing multi-omics analysis in cancer, with data from TCGA (https://www.cancer.gov/ccg/ research/genome-sequencing/tcga) and Clinical Proteomic Tumor Analysis Consortium (https://proteomics.cancer.gov/programs/cptac)[23]. In this study, genes associated with *HSF4* methylation were identified at COAD through LinkedOmics. *HSF4* methylation-associated genes were identified by Spearman and subjected to correction by the Benjamini-Hochberg method. Finally, *HSF4* methylation-related genes were displayed by volcano plot and heatmap. Enrichment analysis of *HSF4* methylation-related genes was performed by hypergeometric distribution algorithm based on Gene Ontology (GO)[24] and Kyoto Encyclopedia of Genes and Genomes (KEGG)[25] databases, and presented by bubble and histogram plots. The above results was visualized with the ggplot2 R package[22].

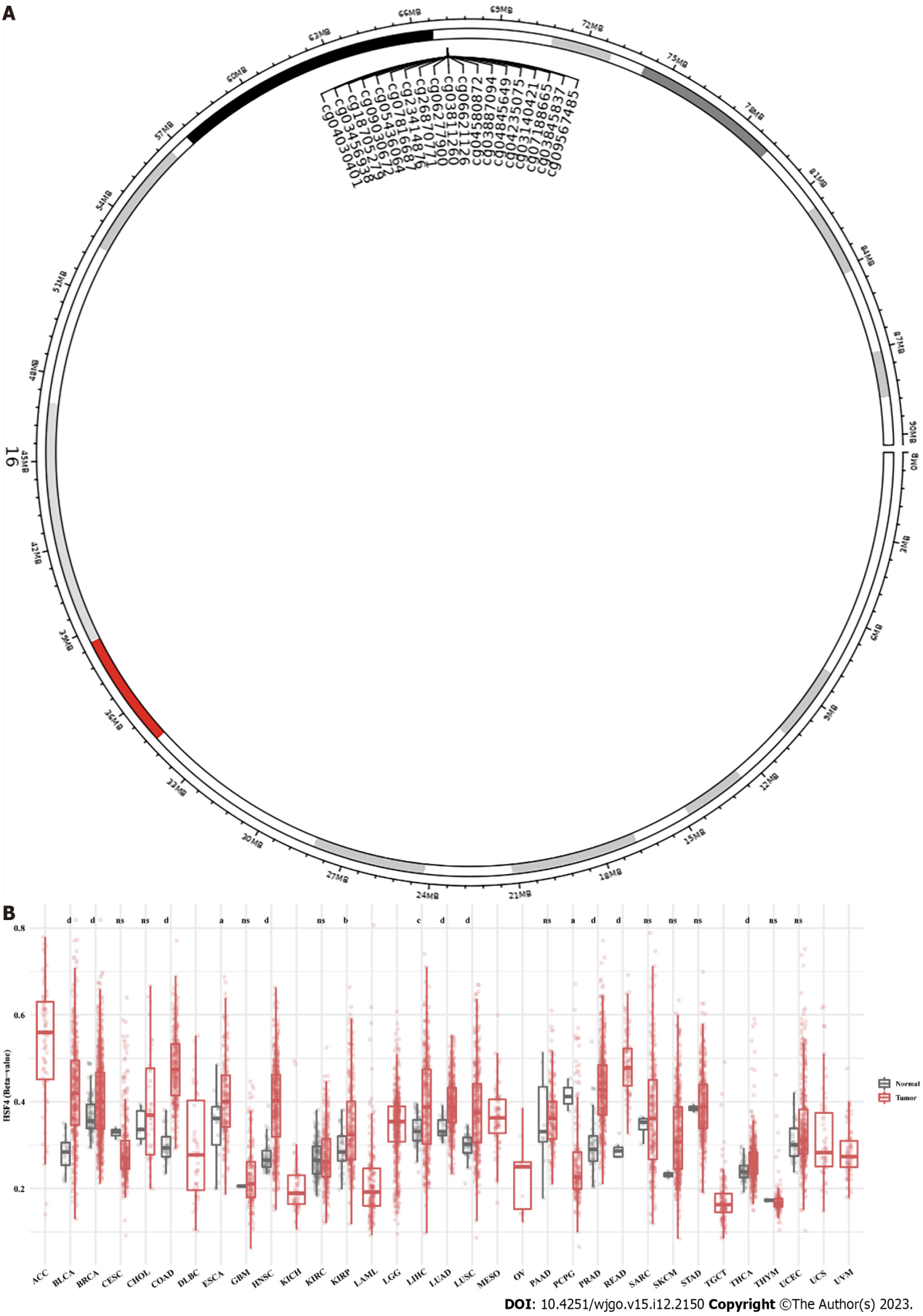
Protein-protein interaction network construction for HSF4 methylation-associated genes

The protein-protein interaction (PPI) network construction for *HSF4* methylation-related genes was based on the String database[26], CytoScape software[27] and the MCODE plugin[28]. Briefly, *HSF4* methylation-related genes obtained from LinkedOmics were extracted, and the interactions of these genes were predicted from the String database. The minimum required interaction score of the String database was set to highest confidence. The interactions were imported into CytoScape software (version:3.8.2) for visualization and clustering analysis of the PPI network was performed by the MCODE algorithm. The parameters of MCODE are degree cutoff = 2, node density cutoff = 0.1, node score cutoff = 0.2, K-core = 2, Max depth = 100.

RESULTS

Identification of HSF4 methylation levels

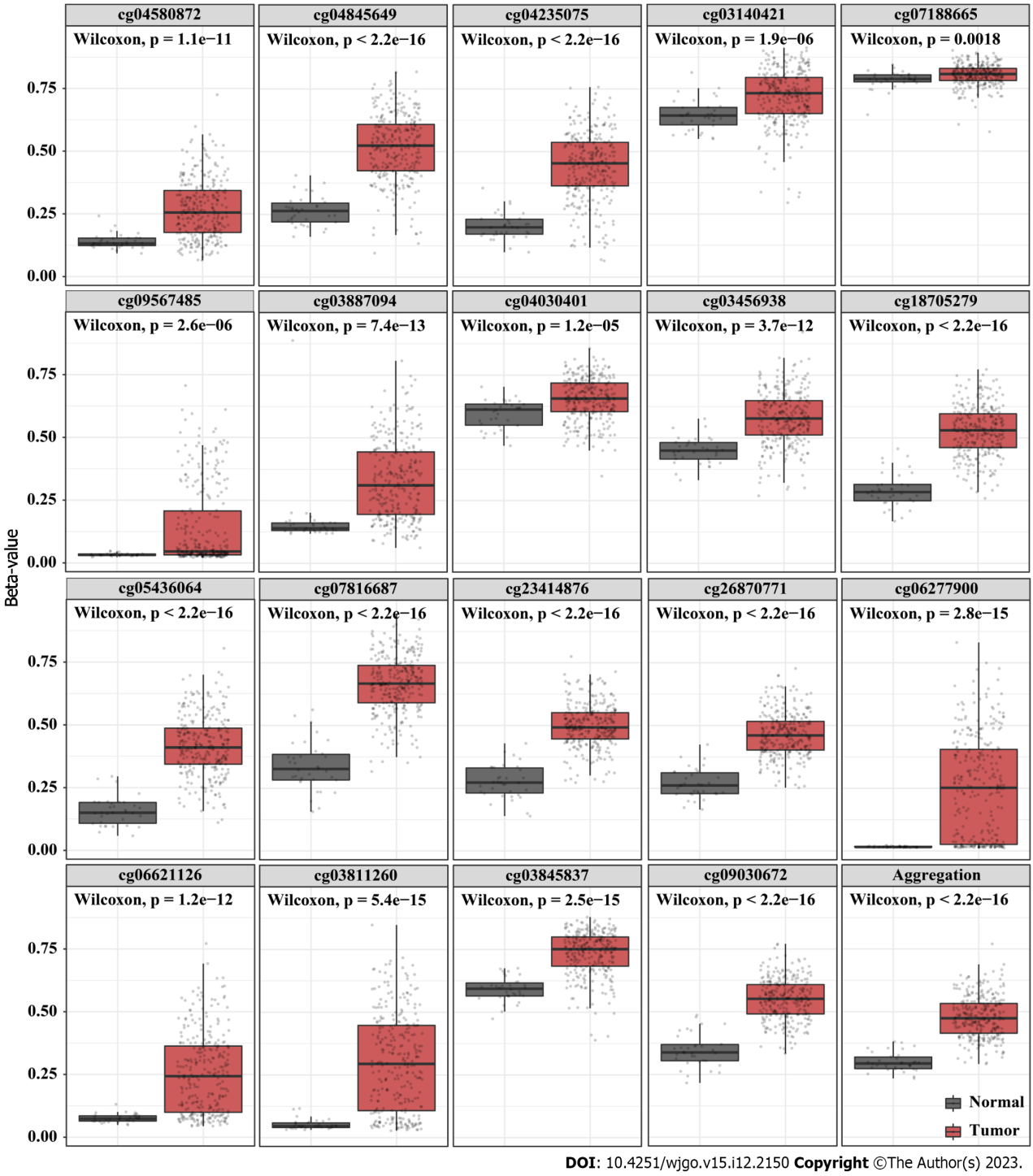
HSF4 is located on chromosome 16 with 19 CpG loci, with 14 on CpG island, three on N Shore and two on S Shore (Figure 1). Differential analysis revealed that β values of *HSF4* CpG-aggregation methylation were significantly enhanced in most malignancies, including COAD, and READ (Figure 1B). Similarly, the β values of each CpG site were significantly higher in most malignant tumors than in the corresponding paracancerous tissues (Supplementary Figure 1). It is notable that all CpG loci of *HSF4* had significantly elevated β values in these malignancies only in COAD (Supplementary Figure 1 and Figure 2). In READ, only two probes, cg07188665 and cg09567485, exhibited no significant difference in β values. We analyzed the methylation levels of HSF4 CpG loci in different tumor stages. The β values of cg06277900, cg03811260, cg04580872, cg06621126, cg03887094 and cg09567485 probes were significantly different at various stages of COAD (Supplementary Figure 2). Therefore, we further explored the correlation between *HSF4* methylation and *HSF4* expression. In COAD, the β values of the probes displayed a significant positive correlation with *HSF4* expression, except



DOI: 10.4251/wjgo.v15.i12.2150

Figure 1 Pan-cancer analysis of heat shock factor protein 4 methylation levels. A: Schematic representation of the distribution of eat shock factor protein 4 (*HSF4*) methylated CpG loci. B: Differential analysis of the β values of 19 CpG methylation loci of *HSF4* in multiple malignancies. [a]*P* < 0.05, [b]*P* < 0.01, [c]*P* <

0.001, and [d]*P* < 0.0001; ns: No significant difference.



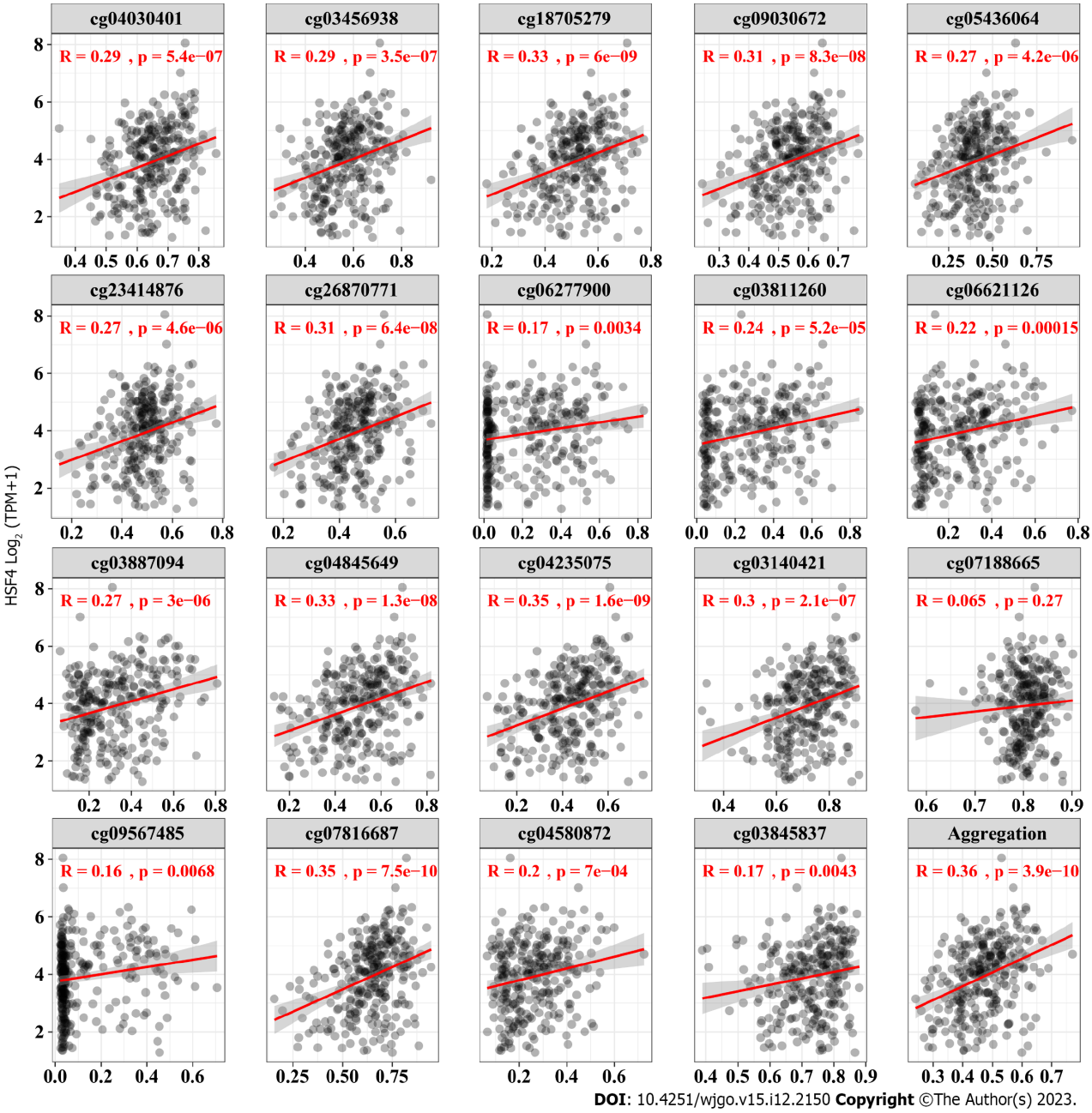
DOI: 10.4251/wjgo.v15.i12.2150

Figure 2 Differential analysis of β values of 19 probes related to heat shock factor protein 4 methylation in colon adenocarcinoma and paracancerous tissues. The β values of all 19 probes were significantly increased in the tissues of colon adenocarcinoma (COAD) patients. Black is paracancerous tissue, and red is COAD tissue.

for cg07188665 (Figure 3). Combined with our previous findings, we believe that HSF4 promotes the CRC process at least through DNA methylation.

HSF4 methylation correlates poorly with CRC prognosis and diagnosis

In view of the differences in *HSF4* methylation in CRC, we further analyzed the prognostic and diagnostic value of *HSF4* methylation. Kaplan-Meier curves indicated no significant difference in survival among COAD patients with high and



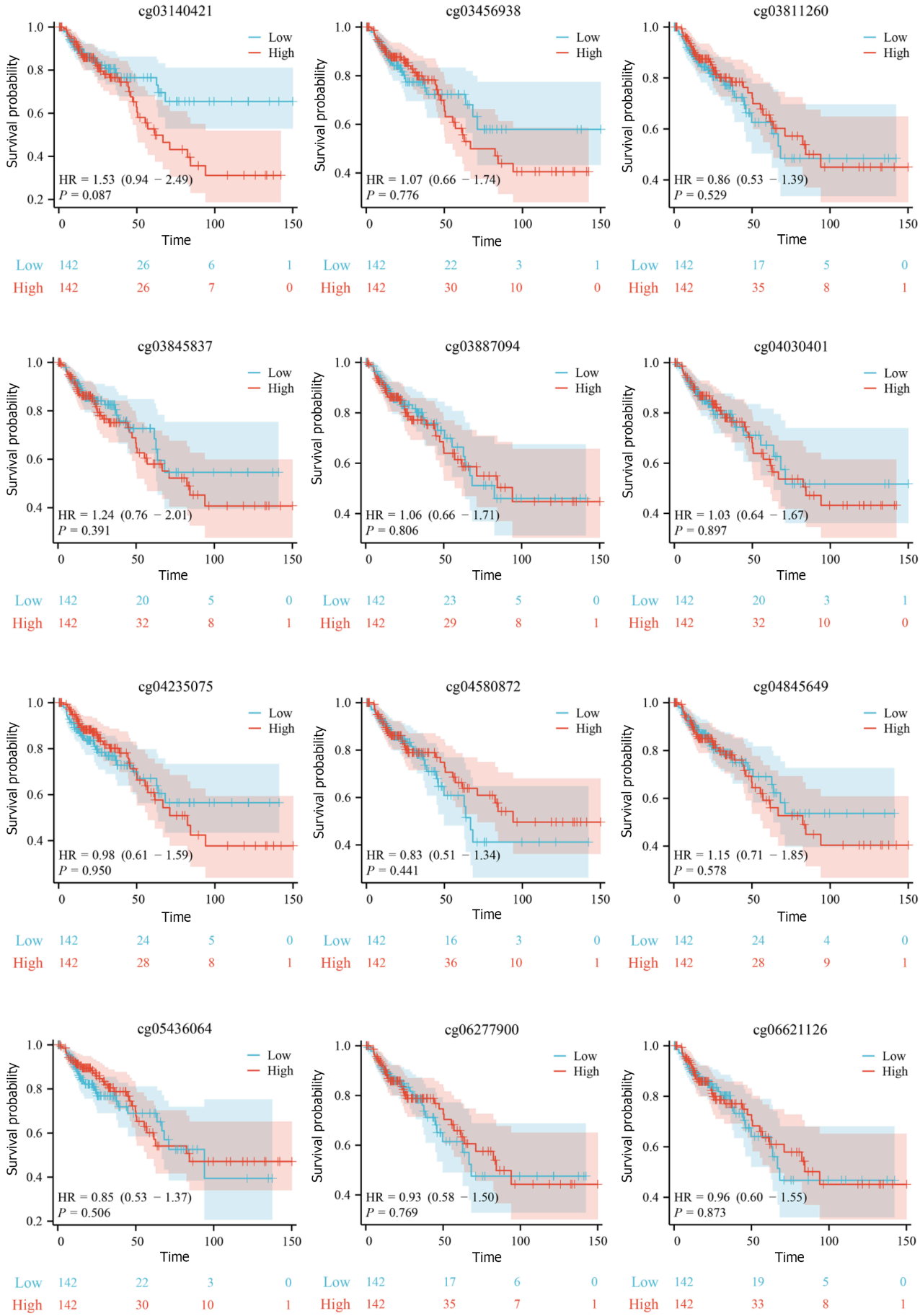
DOI: 10.4251/wjgo.v15.i12.2150

Figure 3 Correlation analysis of heat shock factor protein 4 expression and heat shock factor protein 4 methylation levels. The β values of all 19 probes exhibited a significant positive correlation with the expression of eat shock factor protein 4 (*HSF4*) mRNA. The x-axis is the β value of 19 probes, and y-axis is $\log_2$ (TPM + 1) of *HSF4* mRNA.

low methylation levels for each CpG loci (Figure 4). Nevertheless, most patients with hypermethylated CpG loci had better prognosis. The ROC curve revealed that the area under the curve (AUC) of each CpG loci ranged from 0.498 to 0.574 in COAD patients, suggesting the mediocre diagnostic value of *HSF4* methylation in COAD patients (Figure 5A). The time-dependent ROC curves suggested that the AUC of each CpG loci was greater with increasing time (Figure 5B). The above results indicated that the performance of *HSF4* methylation as a prognostic and diagnostic biomarker in CRC was ordinary, which may have been caused by relatively low accumulation of single genes.

Identification of HSF4 methylation-related genes in CRC and their functional enrichment analysis

We analyzed the genes associated with *HSF4* methylation in CRC by LinkedOmics. The expression of 1468 genes was positively correlated with *HSF4* methylation levels, and expression of 226 genes was negatively correlated with *HSF4* methylation levels in the COAD cohort (Figure 6A). The heatmap illustrated the top 50 genes with absolute correlation coefficients (Figures 6B and C). To further understand the functions and pathways involved in these genes, we performed GO and KEGG enrichment analysis. GO identified that the proteins encoded by these genes were mainly extracellular matrix, and associated with processes such as positive regulation of mitogen-activated protein kinase cascade, tumor necrosis factor superfamily cytokine production, neutrophil mediated cytotoxicity, and chemokine activity (Figure 6D). KEGG enrichment revealed that *HSF4* methylation-related genes were involved in pathways including chemokine


cg03140421
Survival probability
Time
Low
High
HR = 1.53 (0.94 − 2.49)
P = 0.087
Low 142 26 6 1
High 142 26 7 0
cg03456938
HR = 1.07 (0.66 − 1.74)
P = 0.776
Low 142 22 3 1
High 142 30 10 0
cg03811260
HR = 0.86 (0.53 − 1.39)
P = 0.529
Low 142 17 5 0
High 142 35 8 1
cg03845837
HR = 1.24 (0.76 − 2.01)
P = 0.391
Low 142 20 5 0
High 142 32 8 1
cg03887094
HR = 1.06 (0.66 − 1.71)
P = 0.806
Low 142 23 5 0
High 142 29 8 1
cg04030401
HR = 1.03 (0.64 − 1.67)
P = 0.897
Low 142 20 3 1
High 142 32 10 0
cg04235075
HR = 0.98 (0.61 − 1.59)
P = 0.950
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High 142 28 8 1
cg04580872
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P = 0.441
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cg04845649
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P = 0.578
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High 142 28 9 1
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P = 0.506
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P = 0.769
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P = 0.873
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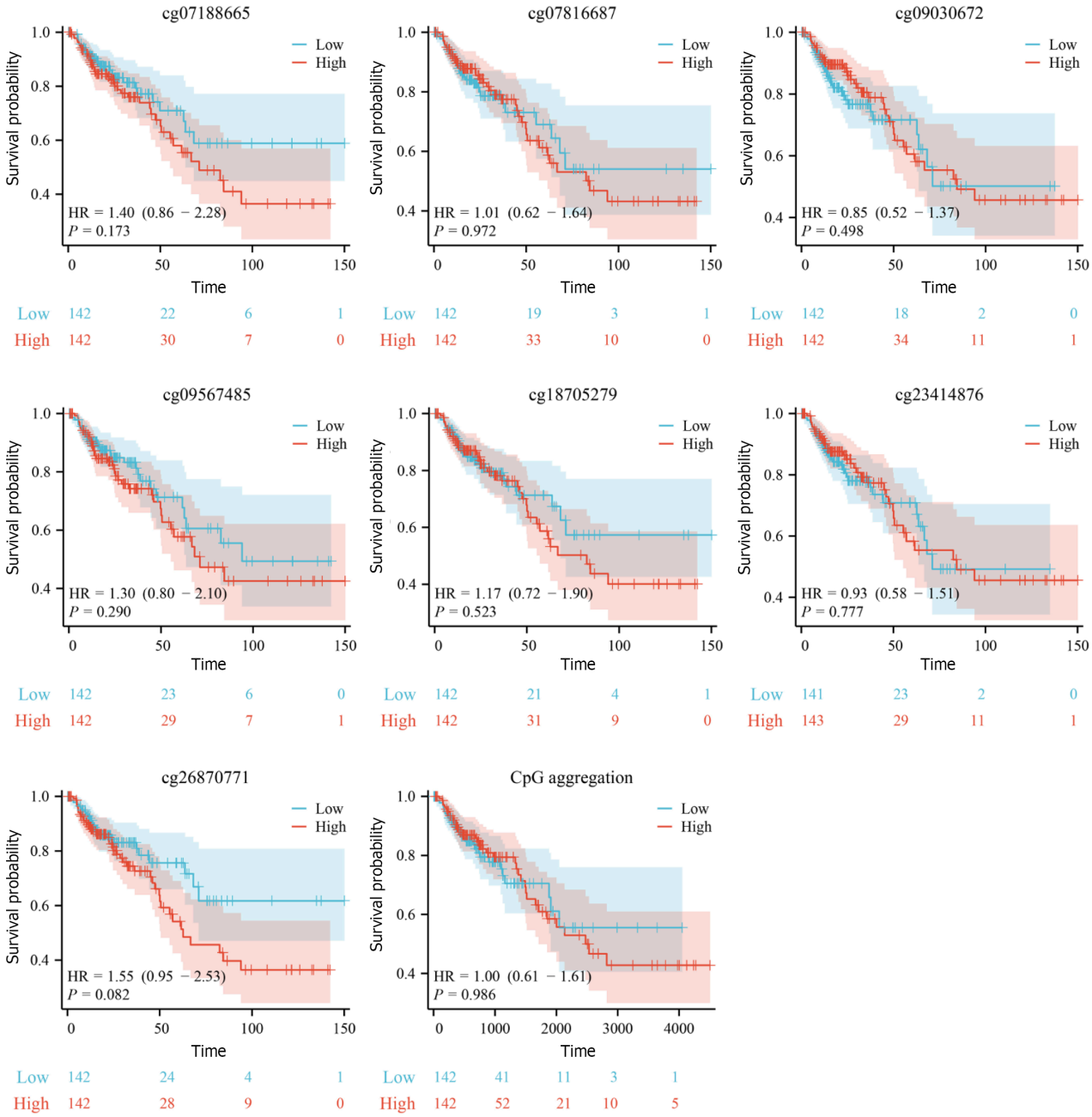
High 142 33 8 1



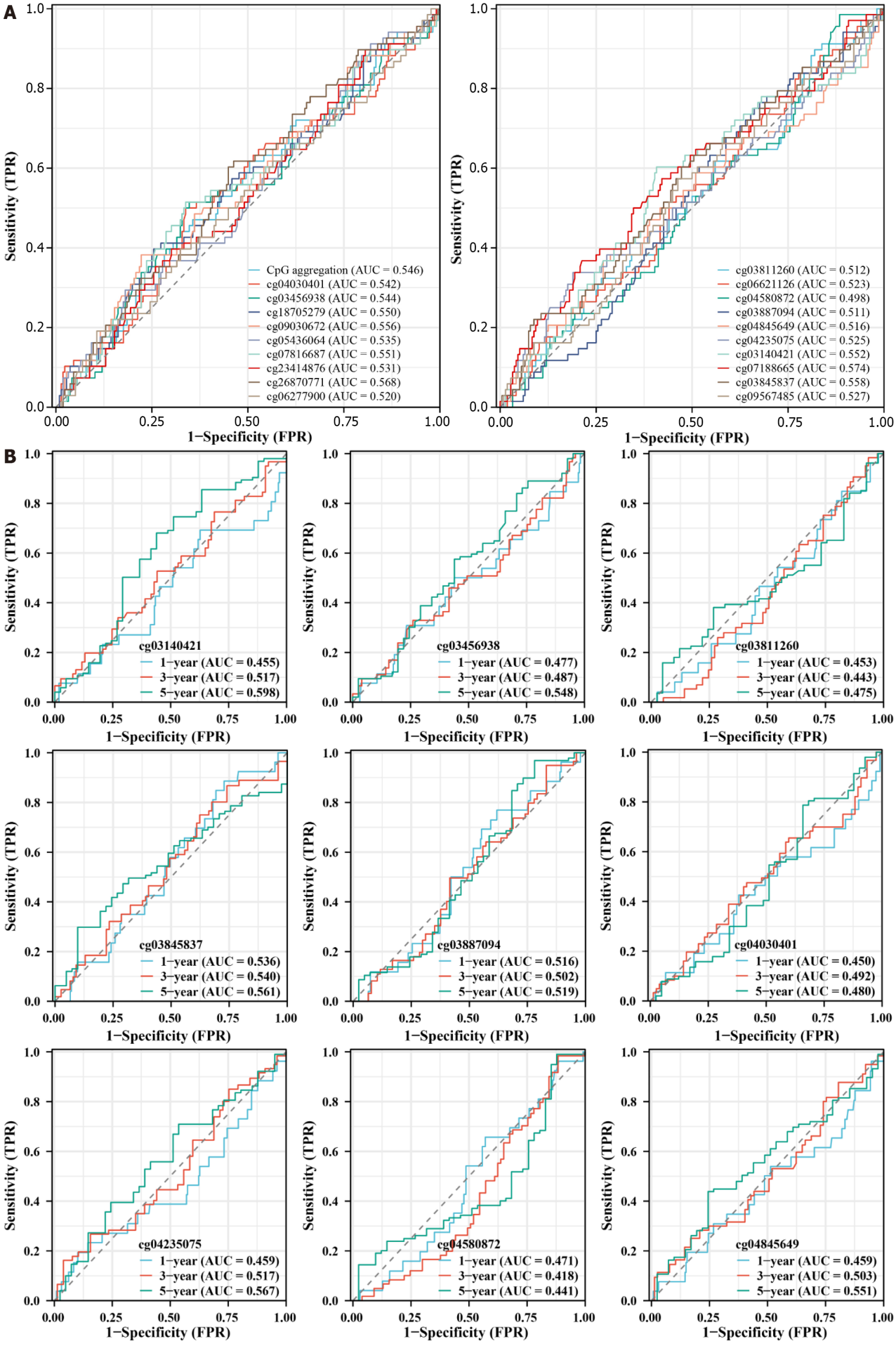
DOI: 10.4251/wjgo.v15.i12.2150 **Copyright** ©The Author(s) 2023.

Figure 4 Correlation analysis of heat shock factor protein 4 methylation and prognosis of patients with colon adenocarcinoma. Kaplan Meier survival curves illustrating the survival of colon adenocarcinoma patients with high and low beta values for the 19 probes. The blue curve represents the cohort with low β values, and the red curve stands for the cohort with high β values.

signaling pathway, calcium signaling pathway, glycosphingolipid biosynthesis - lacto and neolacto series, inflammatory bowel disease and inflammatory bowel disease (Figure 6E). It is suggested that *HSF4* methylation mediates the phenotypic involvement of immune, inflammatory, and metabolic reprogramming in the CRC process.

PPI network of HSF4 methylation-associated genes in CRC

To identify the hub genes in *HSF4* methylation-related genes, we constructed a relevant PPI network based on the String database and the MCODE algorithm. The PPI network constructed for *HSF4* methylation positively correlated genes contained 422 nodes and 702 edges, and 22 clustering networks were obtained (Figure 7A). The top 20 genes in this network with the highest number of edges are displayed in Figure 7B, where *EGFR*, *RELA*, *STAT3*, *ESR1*, and *F2* had the highest number of edges. The top 10 interworking networks with clustering scores are illustrated in Figure 7C. The network consisting of *NUP98*, *SUMO3*, *IPO8*, and *HSPA6* had the highest clustering score, which contained 11 nodes and 35 edges (Figure 7C). In the same way, the network constructed for negatively associated genes contained 110 genes, 122 interactions and five clusters (Figure 8A). The edge numbers TOP5 of *FCGR3A*, *POLR2K*, *AXIN1*, *CCL2* and COPS5 had eight, seven, six, five and five edges, respectively (Figure 8B). The five clustering networks composed of genes and interactions are shown in Figure 8C. It is suggested that these genes are involved in *HSF4* methylation mediation of the


A
Sensitivity (TPR)
1−Specificity (FPR)
CpG aggregation (AUC = 0.546)
cg04030401 (AUC = 0.542)
cg03456938 (AUC = 0.544)
cg18705279 (AUC = 0.550)
cg09030672 (AUC = 0.556)
cg05436064 (AUC = 0.535)
cg07816687 (AUC = 0.551)
cg23414876 (AUC = 0.531)
cg26870771 (AUC = 0.568)
cg06277900 (AUC = 0.520)
cg03811260 (AUC = 0.512)
cg06621126 (AUC = 0.523)
cg04580872 (AUC = 0.498)
cg03887094 (AUC = 0.511)
cg04845649 (AUC = 0.516)
cg04235075 (AUC = 0.525)
cg03140421 (AUC = 0.552)
cg07188665 (AUC = 0.574)
cg03845837 (AUC = 0.558)
cg09567485 (AUC = 0.527)
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1−year (AUC = 0.455)
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5−year (AUC = 0.475)
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3−year (AUC = 0.540)
5−year (AUC = 0.561)
cg03887094
1−year (AUC = 0.516)
3−year (AUC = 0.502)
5−year (AUC = 0.519)
cg04030401
1−year (AUC = 0.450)
3−year (AUC = 0.492)
5−year (AUC = 0.480)
cg04235075
1−year (AUC = 0.459)
3−year (AUC = 0.517)
5−year (AUC = 0.567)
cg04580872
1−year (AUC = 0.471)
3−year (AUC = 0.418)
5−year (AUC = 0.441)
cg04845649
1−year (AUC = 0.459)
3−year (AUC = 0.503)
5−year (AUC = 0.551)

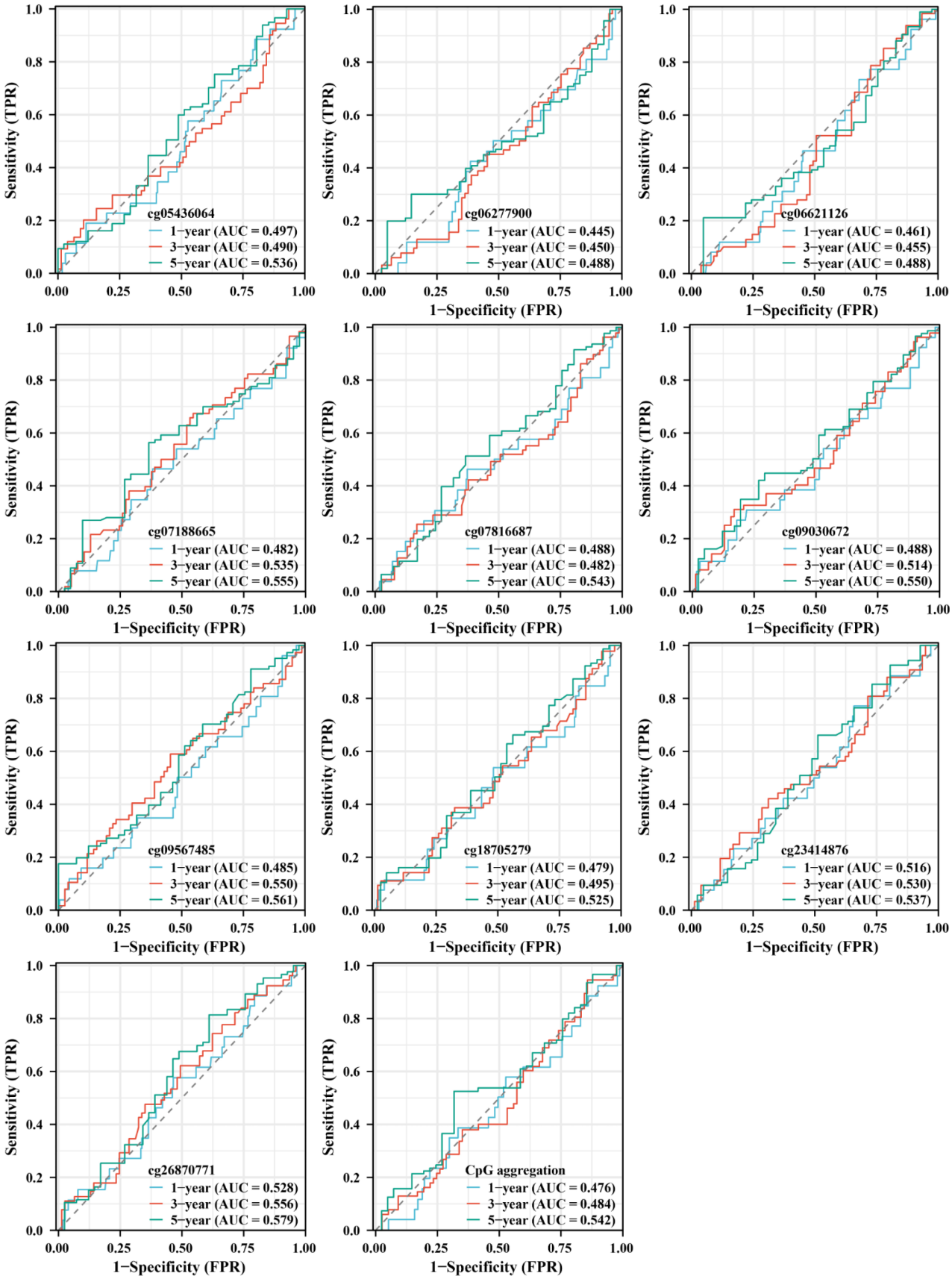

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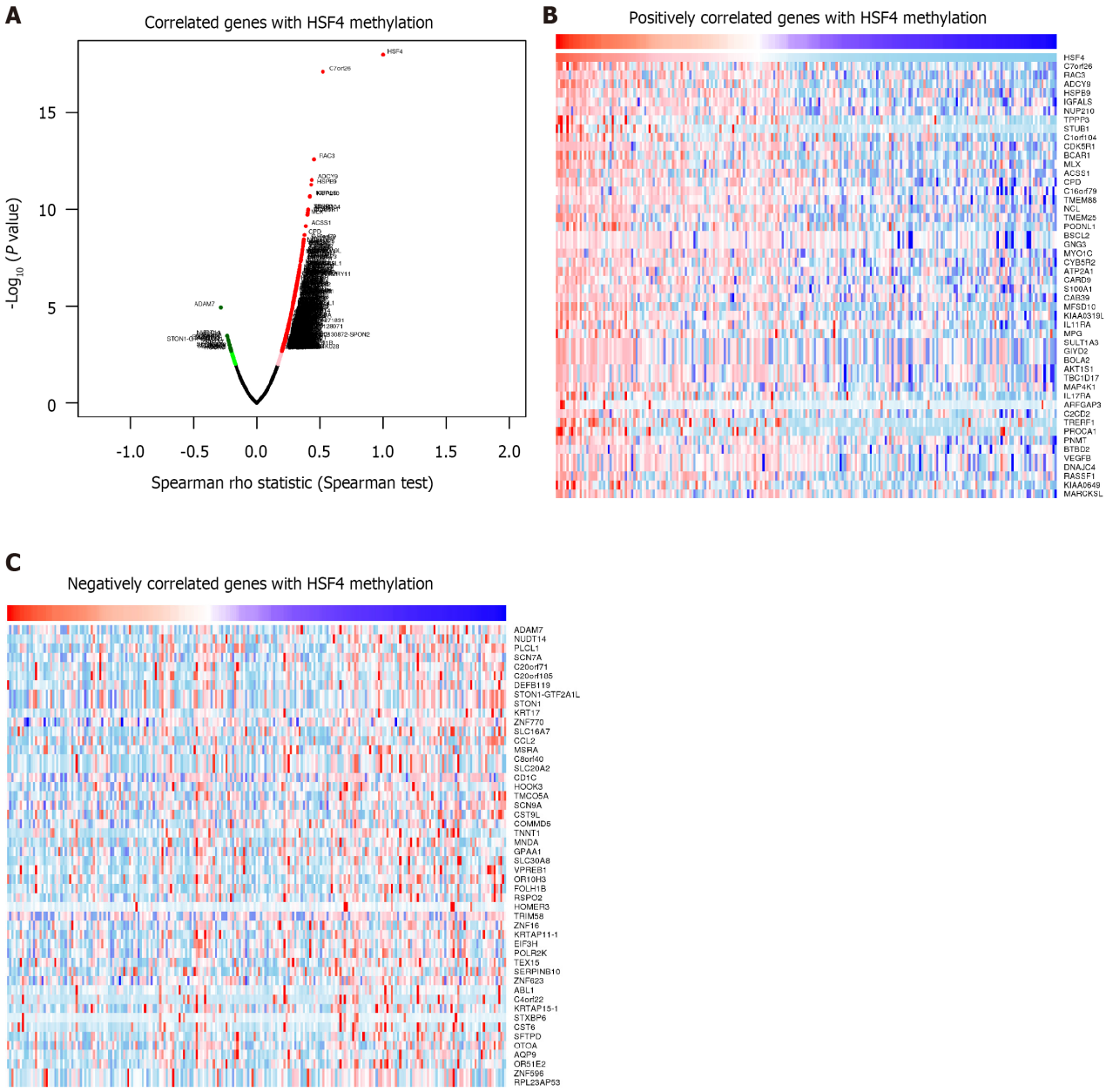
Figure 5 Correlation analysis of heat shock factor protein 4 methylation and diagnosis in patients with colonic adenocarcinoma. A: Receiver operating characteristic (ROC) curves exhibiting the diagnostic value of 19 heat shock factor protein 4 (*HSF4*) methylation-associated probes in colon adenocarcinoma patients. B: Time-dependent ROC curves displaying the area under the curve of *HSF4* methylation at 1, 3 and 5 years. AUC: Area under the curve; TPR: True positive rate; FPR: False positive rate.


A
Correlated genes with HSF4 methylation
-Log10 (P value)
Spearman rho statistic (Spearman test)
B
Positively correlated genes with HSF4 methylation
C
Negatively correlated genes with HSF4 methylation
D
positive regulation of MAPK cascade
regulation of cellular response to growth factor stimulus
tumor necrosis factor superfamily cytokine production
myeloid leukocyte mediated immunity
neutrophil mediated cytotoxicity
collagen−containing extracellular matrix
cell−cell junction
apical plasma membrane
sarcoplasm
sarcoplasmic reticulum
signaling receptor activator activity
growth factor binding
chemokine activity
CCR chemokine receptor binding
transforming growth factor beta binding
BP
CC
MF
P adj
0.003
0.002
0.001
Counts
20
40
60
Gene ratio

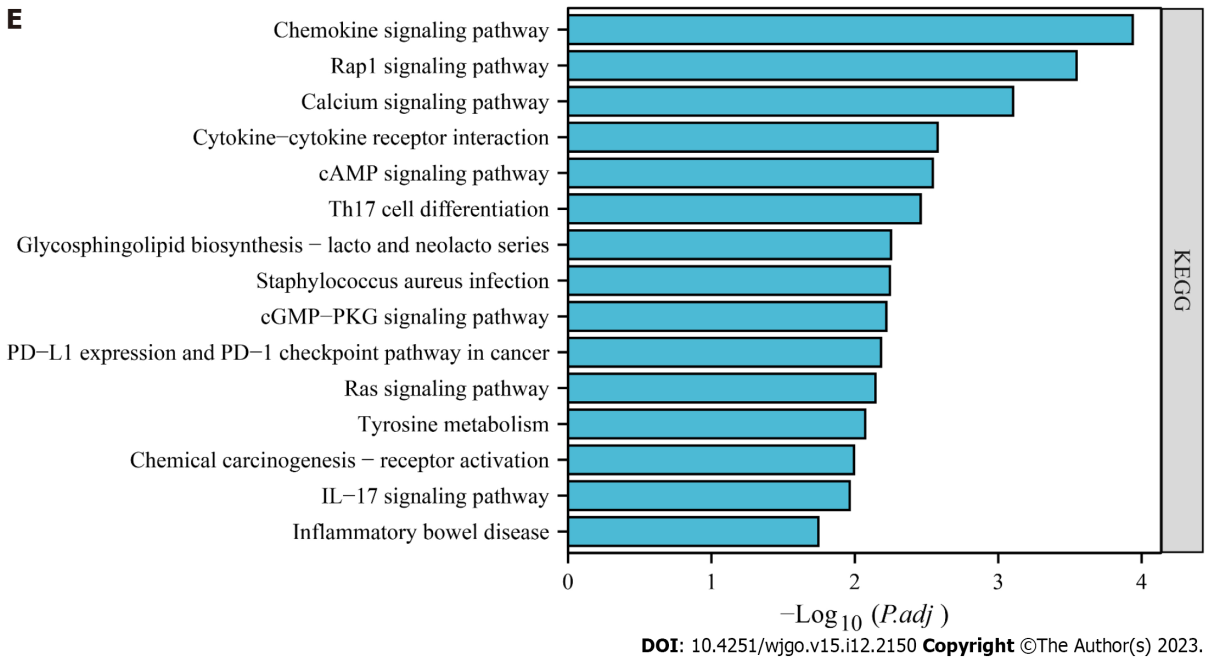


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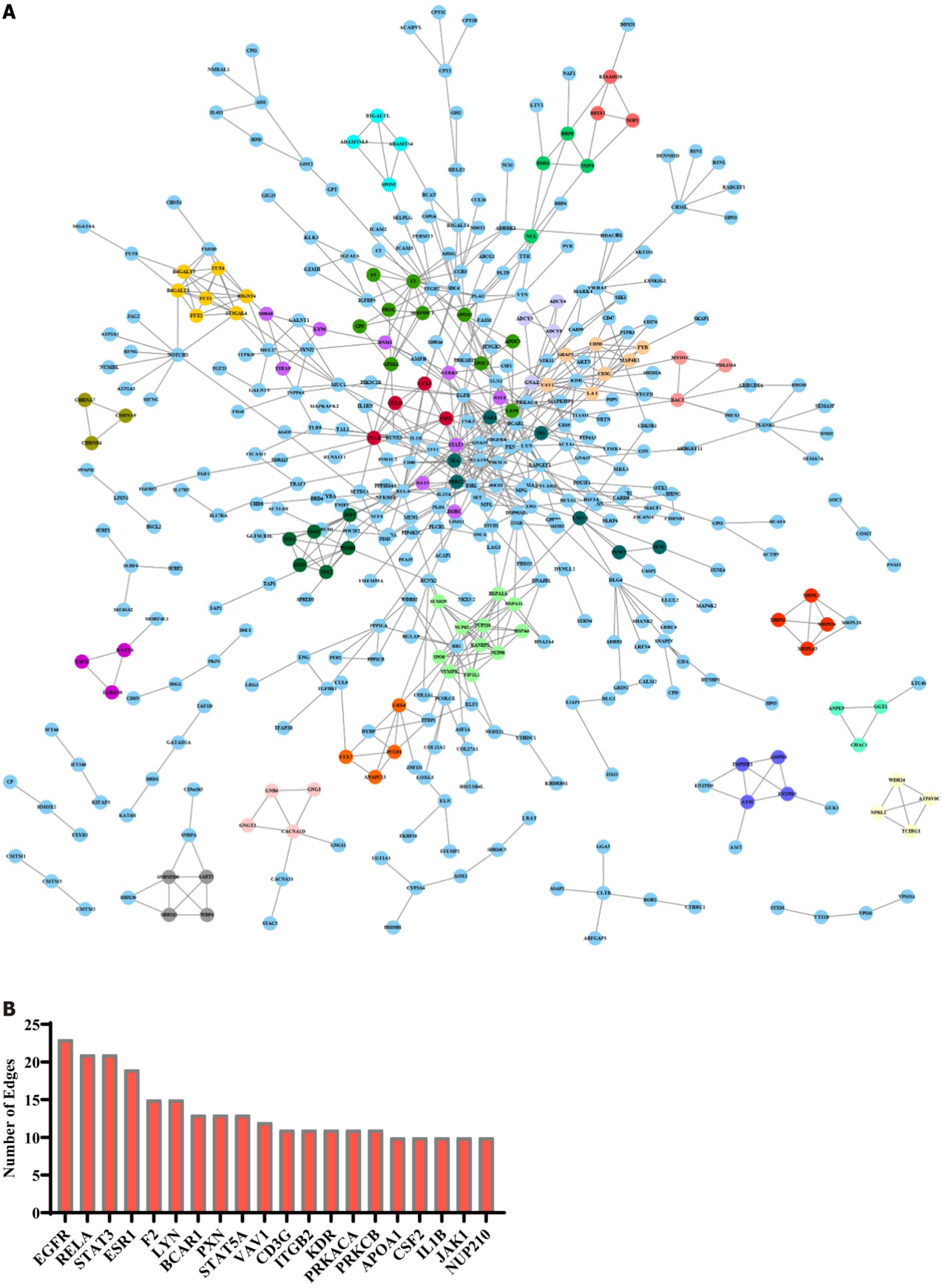
Figure 6 Identification of heat shock factor protein 4 methylation-related genes and their enrichment analysis in colorectal cancer. A: Volcano plot showing genes positively and negatively associated with heat shock factor protein 4 (*HSF4*) methylation in colorectal cancer. B, C: Expression profiles of the top 50 genes ranked by absolute correlation coefficient of *HSF4* methylation-related genes. B is the expression profile of genes positively associated with *HSF4* methylation; C is the expression profile of genes negatively associated with *HSF4* methylation. D: Bubble plots exhibiting the GO enrichment results of all *HSF4* methylation-related genes. E: Possible pathways involved in *HSF4* methylation-related genes obtained by Kyoto Encyclopedia of Genes and Genomes enrichment analysis. HSF4: Heat shock factor protein 4; BP: Biological process; CC: Cell component; MF: Molecular function; PD-L1: Programmed cell death-Ligand 1; PD-1: Programmed death 1; IL-17: Interleukin-17.

CRC process.

DISCUSSION

CRC is the second leading cause of cancer-related deaths worldwide. CRC is the outcome of progressive accumulation of a series of mutations and epigenetic changes in the rectum, cecum, and colon, leading to the development of colorectal adenoma and invasive adenocarcinoma. DNA methylation, one of the major epigenetic modifications, has been partially identified as a commercial diagnostic and prognostic biomarker for CRC. We have previously identified *HSF4* as an oncogenic gene in CRC[18]. Therefore, we tapped the diagnostic and prognostic value of *HSF4* and its possible molecular mechanisms in CRC. Unfortunately, *HSF4*, like most single-gene markers[29-32], has a mediocre diagnostic and prognostic value for its methylation levels in CRC. As in previous studies[29-32], this may be due to the small sample size analyzed or the insufficient accumulation of single gene methylation. Therefore, we analyzed the role of *HSF4* methylation in CRC at the molecular mechanism level. It is noteworthy that we identified 1694 genes associated with *HSF4* methylation, and their possible involvement in immune, inflammatory, and metabolic reprogramming. In addition, the constructed PPI network demonstrated that *EGFR*, *RELA*, *STAT3*, *ESR1*, *FCGR3A*, *AXIN1*, *CCL2*, and *COPS5* are hub genes among *HSF4* methylation-related genes.

Most of these hub genes have been demonstrated to be involved in the CRC process and have been applied as therapies for CRC. For instance, EGFR is a transmembrane receptor that plays a regulatory role in tumor cell function by binding to EGFs, promoting cell proliferation, differentiation, and survival[33]. Currently, monoclonal antibodies against EGFR, such as cetuximab or panitumumab, are utilized in the clinical treatment of patients with metastatic CRC[34,35]. RELA, also known as p65 or nuclear factor (NF)-κB p65, is known to be a key transcription factor in tumors, and it mediates immune and inflammatory responses to facilitate cancer cell survival and metastasis, which leads to it being a key target in tumor therapy[36,37]. Similarly, FCGR3A belongs to the Fc γ receptor family, which is mainly expressed on the surface of natural killer cells, monocytes, and macrophages and plays an important role in antibody-mediated immune responses[38]. Polymorphisms in *FCGR3A* are associated with progression-free survival in patients with metastatic CRC treated with cetuximab[39,40]. COPS5, also known as CSN5 or JAB1, is one of the constituent proteins of the COP9 signalosome, is a nuclear-plasmid transmembrane protein with multiple functions, and is involved in the regulation of various cellular processes such as cell proliferation, differentiation, apoptosis, and DNA replication and repair[41]. It has been demonstrated that COPS5 plays a role as a pro-cancer factor in CRC by regulating Wnt and PI14K/AKT pathways[42-44]. Some of these hub genes have also been proven to be related to HSF family proteins. For example, HSR-induced activation of HSP1 is regulated by the NF-κB, which activates transcription of HSPA1A[45]. In turn, HSP1 inhibits the activation of NF-κB pathway[46,47]. Stephanou and Latchman[48] showed that the activation of *STAT3* alone


A
B
Number of Edges
25
20
15
10
5
0
EGFR
RELA
STAT3
ESR1
F2
LYN
BCAR1
PXN
STAT5A
VAV1
CD3G
ITGB2
KDR
PRKACA
PRKCB
APOA1
CSF2
IL1B
JAK1
NUP210

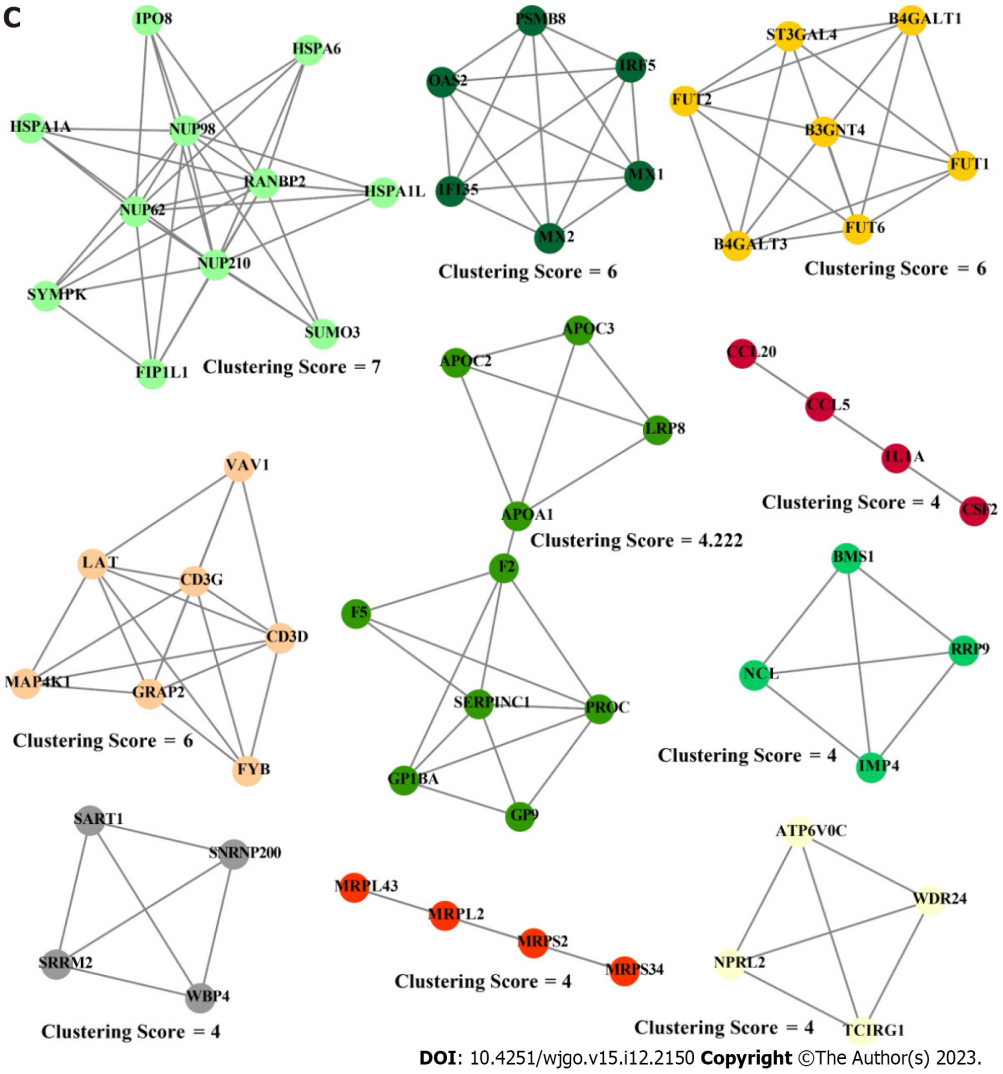


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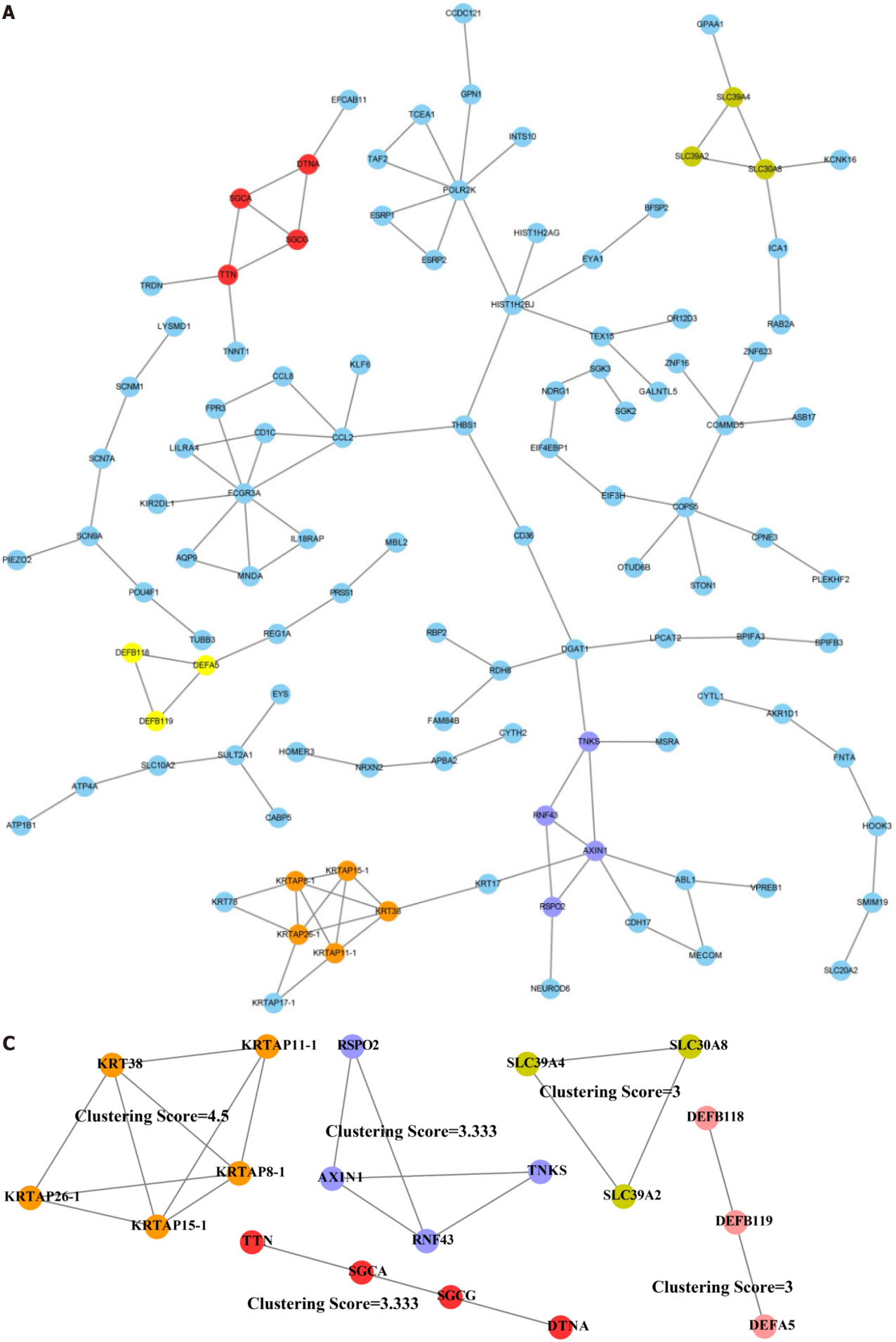
Figure 7 Protein-protein interaction network construction of heat shock factor protein 4 methylation positively associated genes. A: Protein-protein interaction (PPI) network of heat shock factor protein 4 methylation positively related genes constructed based on String database and MCODE algorithm. B: Top 20 genes in PPI network in terms of edge number. C: The top 10 clustering networks in terms of clustering scores obtained by the MCODE algorithm.

facilitates the mobilization of the HSP promoter. Nevertheless, whether *HSF4* methylation-mediated alterations in HSF4 expression are crosstalk with these hub genes in CRC remains to be further investigated.

Unfortunately, there were some limitations to this study. For instance, the sample size was small, and a larger sample might reveal satisfactory diagnostic and prognostic values[49,50]. Exploring the correlation between *HSF4* methylation and CRC subtypes or *HSF4*-related gene methylation combinations may improve the value of *HSF4* in CRC[9,49]. It is essential to verify *HSF4* methylation in CRC tissues by methylation sequencing or microarrays, which could support the findings of this study[51]. Although we demonstrated that *HSF4* methylation levels exhibited a positive correlation with *HSF4* mRNA expression, *in vivo* and *in vitro* experiments are lacking for validation. In the same way, the molecular mechanisms associated with *HSF4* methylation remain to be explored *in vivo* and *in vitro*. The tumor immune microenvironment (TME) consists of immune cells, blood vessels, and extracellular matrix, and has a dual role in the growth and metastasis of tumor cells[52,53]. HSF family proteins, especially HSF1, have been demonstrated to mediate tumor cell associated immune responses in the TME[54,55]. This predicts that the function of HSF4 in CRC-associated TME is also worthy of investigation.

CONCLUSION

In conclusion, this study reveals that HSF4 is highly methylated in CRC and is associated with *HSF4* overexpression. Although *HSF4* methylation has poor diagnostic and prognostic value, it may be involved in the CRC process by mediating expression of *HSF4* or related genes with potential mechanisms. Combined with our previously described findings[18], the present study believes that high expression of *HSF4* mRNA and protein and its oncogenic effects are most probably due to *HSF4* methylation (Figure 9). Specific mechanisms need to be confirmed by more *in vivo* and *in vitro*


A
CCDC121
GPAA1
GPN1
SLC39A4
EFCAB11
TCEA1
INTS10
TAF2
SLC39A2
SLC30A8
KCNK16
DTNA
POLR2K
SGCA
ESRP1
BFSP2
HIST1H2AG
SGCG
ESRP2
EYA1
ICA1
TRDN
TTN
HIST1H2BJ
LYSMD1
OR12D3
RAB2A
TEX15
TNNT1
ZNF623
KLF6
SGK3
ZNF16
SCNM1
CCL8
NDRG1
GALNTL5
FPR3
SGK2
CD1C
CCL2
THBS1
COMMD5
ASB17
LILRA4
EIF4EBP1
SCN7A
KIR2DL1
FCGR3A
EIF3H
COPS5
SCN9A
IL18RAP
CD36
CPNE3
PIEZO2
MBL2
AQP9
MNDA
OTUD6B
STON1
PLEKHF2
POU4F1
PRSS1
TUBB3
REG1A
RBP2
DGAT1
LPCAT2
BPIFA3
BPIFB3
DEFB118
RDH8
DEFA5
EYS
CYTL1
AKR1D1
DEFB119
FAM84B
CYTH2
TNKS
MSRA
FNTA
HOMER3
SULT2A1
SLC10A2
NRXN2
APBA2
ATP4A
RNF43
HOOK3
ATP1B1
CABP5
AXIN1
KRTAP15-1
KRTAP8-1
ABL1
VPREB1
KRT17
KRT78
KRT38
RSPO2
SMIM19
CDH17
KRTAP26-1
KRTAP11-1
MECOM
SLC20A2
KRTAP17-1
NEUROD6
C
KRTAP11-1
RSPO2
SLC30A8
KRT38
SLC39A4
Clustering Score=4.5
Clustering Score=3
Clustering Score=3.333
DEFB118
KRTAP8-1
AXIN1
TNKS
KRTAP26-1
SLC39A2
KRTAP15-1
DEFB119
TTN
RNF43
SGCA
Clustering Score=3
SGCG
Clustering Score=3.333
DTNA
DEFA5

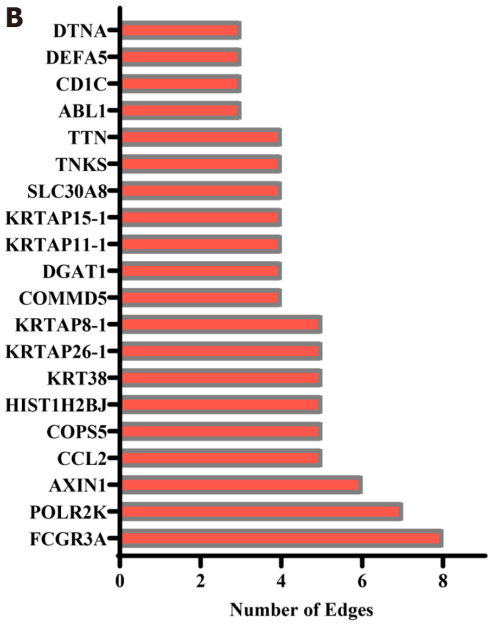


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Figure 8 Protein-protein interaction network construction of heat shock factor protein 4 methylation negatively associated genes. A: Representative images of the protein-protein interaction (PPI) network of heat shock factor protein 4 methylation negatively associated genes. B: The bar chart displaying edge number of each gene in the PPI network. C: Gene composition and interactions of clustering networks obtained by the MCODE algorithm.

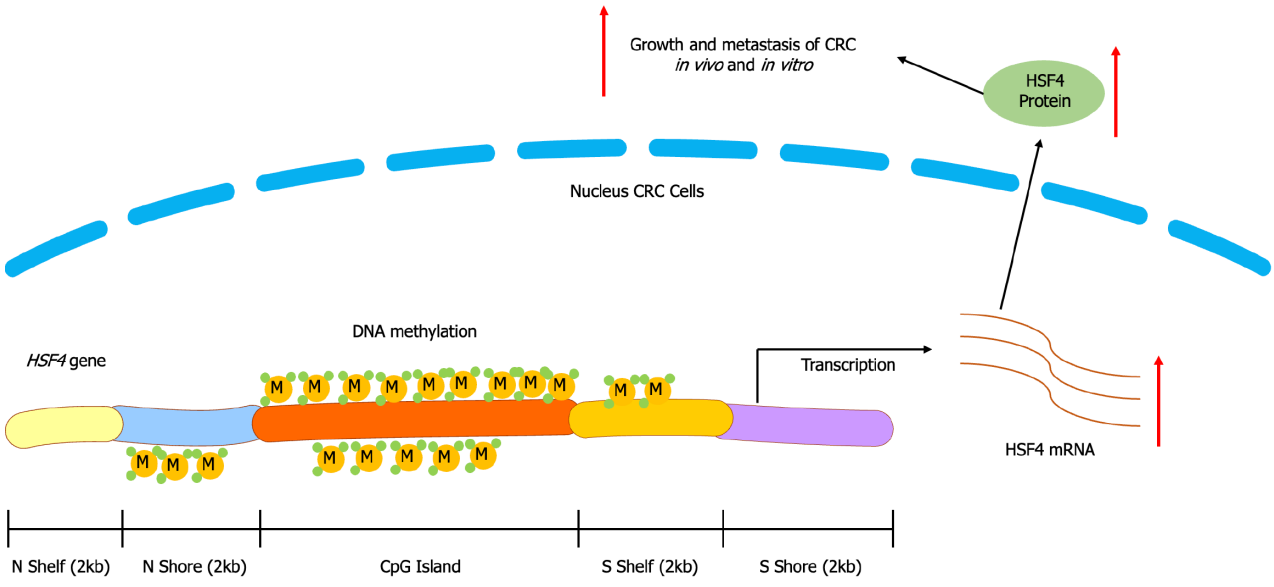

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Figure 9 Diagrammatic representation of this study. CRC: Colorectal cancer; HSF4: Heat shock factor protein 4.

experiments.

ARTICLE HIGHLIGHTS

Research background

DNA methylation is involved in the regulation of gene expression and has been implicated in development and outcome of colorectal cancer (CRC).

Research motivation

We previously demonstrated that heat shock factor protein 4 (HSF4) expression is abnormally high, and contributes to the

malignant biological behavior of CRC *in vivo* and *in vitro*. However, the correlation of *HSF4* methylation with *HSF4* expression and prognosis of CRC patients, and other potential molecular mechanisms need to be further investigated.

Research objectives

The present study was proposed to investigate the correlation between *HSF4* methylation and CRC risk, and to uncover the underlying molecular mechanisms.

Research methods

Identification of *HSF4* methylation sites, and analysis of the differences in β values of *HSF4* methylation sites and their correlation with *HSF4* mRNA expression were performed using Shiny Methylation Analysis Resource Tool Web. The genes associated with *HSF4* methylation were identified by LinkedOmics Web for CRC, and Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses were performed to reveal the functions and signaling that these associated genes may be involved in. The String database and MCODE algorithm were performed to construct protein-protein interaction (PPI) networks of *HSF4* methylation-related genes.

Research results

The *HSF4* gene had 19 CpG methylation sites, and their β-values were significantly higher in CRC tissues, positively correlating with *HSF4* mRNA expression. The β value of the *HSF4* methylation site was not associated with the prognosis of CRC patients. Notably, there are 1694 genes in CRC tissues whose expression is associated with *HSF4* methylation and which are involved in immune, inflammatory, and metabolic reprogramming. EGFR, STAT3 and AXIN1 are hub genes in the PPI network constructed by these *HSF4* methylation-related genes.

Research conclusions

The *HSF4* gene is highly methylated in CRC, and is associated with the overexpression of *HSF4* mRNA. *HSF4* methylation may be involved in the process of CRC by mediating the expression of *HSF4* or related genes.

Research perspectives

The finding will provide a theoretical basis and a new perspective on *HSF4* as a methylation-related biomarker for future CRC diagnosis and treatment.

FOOTNOTES

Author contributions: Zhang WJ and Zhang Y conceived and designed the experiments; Zhang WJ, Yue KL, and Wang JZ analyzed the data; Zhang Y contributed to the data curation; Zhang WJ wrote-original draft preparation; Yue KL, Wang JZ, and Zhang Y participated in the writing-review and editing.

Supported by National Natural Science Foundation of China, No. 82260601; Joint Foundation of Kunming Medical University and Yunnan Provincial Science and Technology Department, No. 202201AY070001-256; Grant for Clinical Medical Center of Yunnan Provincial Health Commission, No. 2021LCZXXF-XH03; and Young Academic Talents Cultivation Foundation of Yunnan Province, No. 202205AC160070.

Institutional review board statement: This study did not involve any animal and human experimentation.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

Country/Territory of origin: China

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Zhang XD

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