

Expression and Gene Regulation Network of Ubiquitin Specific Peptidase 5 in Colorectal Cancer

F. XING AND X. K. HU*

Department of Radiotherapy and Oncology, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150086, China

Xing *et al.*: Role of Ubiquitin Specific Peptidase 5 in Colorectal Cancer

Colorectal cancer is one of the most commonly occurred cancers, although deubiquitination enzymes are related to tumor, the work of ubiquitin specific peptidase 5 in colorectal cancer is still not fully elucidated. Ubiquitin specific peptidase 5 expressions and prognosis was evaluated by analyzing a public dataset from the cancer genome atlas, gene expression omnibus database and a literature review. The biological function of ubiquitin specific peptidase 5 was investigated by gene set enrichment analysis. Ubiquitin specific peptidase 5 interaction proteins were shown by biological general repository for interaction datasets. Ubiquitin specific peptidase 5 is highly expressed and might be potentially used as a diagnostic and prognostic biomarker in colorectal cancer. Functional network analysis suggested that ubiquitin specific peptidase 5 is associated with proteasome, ribosome, deoxyribonucleic acid replication, cell cycle, spliceosome, etc. We discovered the correlation of ubiquitin specific peptidase 5 with several cancer related kinases, E2F transcription factors family and micro ribonucleic acids. Additionally, we presented about the ubiquitin specific peptidase 5 interaction proteins through protein-protein interaction network. Our results revealed information about ubiquitin specific peptidase 5 expression and its potential regulatory networks in colorectal cancer.

Key words: Ubiquitin specific peptidase 5, colorectal cancer, gene function, gene set enrichment analysis

Colorectal Cancer (CRC) has been recognized as a cancer with high incidence and mortality^[1,2]. In the past decades, several new therapeutic drugs were introduced to CRC, but prolonged survival is limited in advanced stage patients. Recently, increasing research evidence showed that multiple molecular pathways involved in the occurrence and development of CRC. Among which, Ubiquitin-Proteasome System (UPS) plays a significant role^[3,4].

UPS is an important way for the degradation of proteins in eukaryotic cells. Ubiquitination enzymes and Deubiquitination (DUB) enzymes regulates targeted protein expression at post-transcriptional level. Ubiquitin Specific Peptidase (USP) family is an important member of DUBs. Numerous studies have shown that aberrantly expressed USP family are associated with tumor cell proliferation, migration and invasion^[3,5]. USP5 is also known as a deubiquitinating enzyme belonging to the USP family, which has been confirmed to be associated

with multiple physiological processes and pathological conditions^[6]. USP5 plays pivotal roles by targeting substrates, such as p53^[7], Forkhead box M1 (FOXO1)^[8], c-Maf^[9] and beta (β)-catenin^[10]. Upregulation of USP5 expression has been found in a variety of cancers and related to poor prognosis^[10-14]. Previous research studies mentioned that high USP5 expression related to tumor cell growth^[15] and brigatinib^[16] resistance in CRC. However, there are few articles and insufficient evidence about the role of USP5 in CRC.

In our study, we sought to unveil the clinical role of USP5 in CRC by bioinformatics analysis based on The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO) and literature reviews. Then we used Gene Set Enrichment Analysis (GSEA) to explore the potential in depth mechanism of USP5, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), kinase targets, transcription factor targets and micro Ribonucleic Acid (miRNA) targets.

*Address for correspondence

E-mail: h06425@hrbmu.edu.cn

MATERIALS AND METHODS

USP5 expression in CRC based on GEO datasets:

The microarray profiles of CRC were obtained from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) from January 2010 to June 2020. The search strategy formulated was as follows: (colon OR rectal OR colorectal) AND (cancer OR tumor OR neoplasm OR malignancy OR carcinoma). Inclusion criteria were listed as *Homo sapiens* of CRC and normal tissues; the total number of cancer and healthy samples were >50; the expression data of USP5 could be provided or calculated in both CRC and normal tissues. Series which does not meet eligibility criteria were excluded.

USP5 expression levels were calculated in both CRC and normal tissues. Unpaired student's t test in GraphPad prism v7.04 (GraphPad Software, California, USA) was performed to analyze the differences in USP5 expression. Receiver Operator Characteristic (ROC) curve were used to identify diagnostic value. Means and Standard Deviations (SD) were extracted to estimate USP5 expression level in cancer and control groups by using Review Manager 5.3. Heterogeneity across studies was analyzed by Chi-square (χ^2) test and I^2 statistics. $I^2 > 50\%$ or $p < 0.05$ was considered heterogeneous and random-effects model was used.

USP5 messenger RNA (mRNA) expression in CRC based on TCGA datasets:

TCGA microarray data based on RNA-sequencing (RNA-seq) were obtained by The University of Alabama Cancer (UALCAN) data analysis portal (<http://ualcan.path.uab.edu/>)^[17]. We used this interactive web-portal to analyze the differential expression of USP5 in CRC and normal tissues.

Immunohistochemistry (IHC) of USP5 protein expression:

To show USP5 protein expression, IHC datasets were retrieved from The Human Protein Atlas database (<https://www.proteinatlas.org/>)^[18].

Survival analysis:

TCGA survival data was downloaded by using OncoLnc (<http://www.oncolnc.org/>)^[19]. GSE87211 microarray including survival data was obtained from GEO database. The best cut-off was analyzed

by X-tile^[20], which is a bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Kaplan-Meier survival curves were drafted by GraphPad.

Literature search and study for USP5 in CRC:

Related studies were selected by comprehensively searching on PubMed and Web of Science from January 2010 to June 2020. The search strategy was made as follows, (colon OR rectal OR colorectal) AND (cancer OR tumor OR neoplasm OR malignancy OR carcinoma) AND (USP5 OR ubiquitin specific peptidase 5). Publications fulfilled one of the following inclusion criteria were considered eligible, which includes the difference of USP5 expression between CRC and normal tissues were shown; USP5 expression and clinical data of colorectal patients were shown. Studies were excluded if they met any one of the following conditions which includes reviews, case report, conference abstracts, letters and comments; results based on TCGA data since we had evaluated USP5 expression in TCGA data by ourselves.

Bioinformatics analyses of USP5:

LinkedOmics (<http://www.linkedomics.org/>) is a publicly available portal including multi-omics data from TCGA, displaying correlated genes of USP5^[21]. The website also used GSEA to perform analyses of GO, KEGG pathways, candidate target genes of protein kinases, transcriptional factors and miRNAs.

The Protein-Protein Interaction (PPI) network was constructed by Biological General Repository for Interaction Datasets (BioGRID) (<https://thebiogrid.org/>)^[22]. BioGRID is a classic database in studying protein interactions by searching 72 408 publications for 1 871 024 protein and genetic interactions, 28 093 chemical associations and 874 796 post translational modifications from major model organism species.

RESULTS AND DISCUSSION

A total of 3068 microarrays from the GEO database were identified, among which 12 microarrays such as GSE9348, GSE23878, GSE25070, GSE28000, GSE41258, GSE44076, GSE44861, GSE73360, GSE74602, GSE89076, GSE106582 and GSE117606 met the entry criteria (fig. 1). Eight microarrays which include GSE9348, GSE23878, GSE41258, GSE44076, GSE73360, GSE74602, GSE89076 and GSE106582 demonstrated that the expression of

USP5 was significantly higher in clinical CRC than normal tissues, whereas no significant difference was found in other four GEO datasets such as GSE25070, GSE28000, GSE44861 and GSE117606 (Table 1 and fig. 2). USP5 had shown a significant diagnostic value in seven microarrays which include GSE9348, GSE23878, GSE44076, GSE73360, GSE74602, GSE89076 and GSE106582 (fig. 3). 821 tumor

samples and 583 normal samples were used in this meta-analysis. The pooled Standard Mean Difference (SMD) of USP5 was 0.58 with 95 % Confidence Interval (CI) (0.23-0.92) by random-effects model ($p < 0.0001$, $I^2 = 88\%$) (fig. 4A) and the results of funnel plot were shown in fig. 4B.

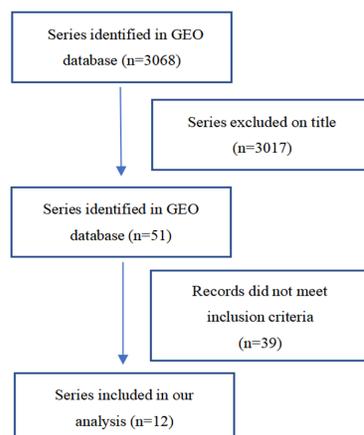


Fig. 1: Flow chart of study selection for GEO dataset

TABLE 1: CHARACTERISTICS OF STUDIES BASED ON GEO DATASET

GEO series	Contributor	Year	Samples	Normal (n)	Cancer (n)	Stage	Platform
GSE9348	Hong <i>et al.</i> ^[23]	2010		12	70	Early stage	GPL570 (HG-U133_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array
GSE23878	Uddin <i>et al.</i> ^[24]	2010		24	35		
GSE25070	Hinoue <i>et al.</i> ^[25]	2011		26	26		GPL6883 Illumina HumanRef-8 v3.0 gene expression beadchip
GSE28000	Jovov <i>et al.</i> ^[26]	2012		34	81		GPL1708 Agilent-012391 Whole Human Genome Oligo Microarray G4112A (Feature number version) GPL4133 Agilent-014850 Whole Human Genome Microarray 4×44K G4112F (Feature number version)
			Tissue				
GSE41258	Sheffer <i>et al.</i> ^[27]	2012		55	184	I-IV	GPL96 (HG-U133A) Affymetrix Human Genome U133A Array
GSE44076	Sole <i>et al.</i> ^[28]	2014		98	98	II	GPL13667 (HG-U219) Affymetrix Human Genome U219 Array
GSE44861	Ryan <i>et al.</i> ^[29]	2014		55	57		GPL3921 (HT_HG-U133A) Affymetrix HT Human Genome U133A Array
GSE73360	Condorelli <i>et al.</i> ^[30]	2016		31	54		GPL17586 (HTA-2_0) Affymetrix Human Transcriptome Array 2.0 (Transcript (gene) version)
GSE89076	Satoh <i>et al.</i> ^[31]	2017		36	36	I-IV	GPL16699 Agilent-039494 SurePrint G3 Human GE v2 8×60K Microarray 039381 (Feature number version)

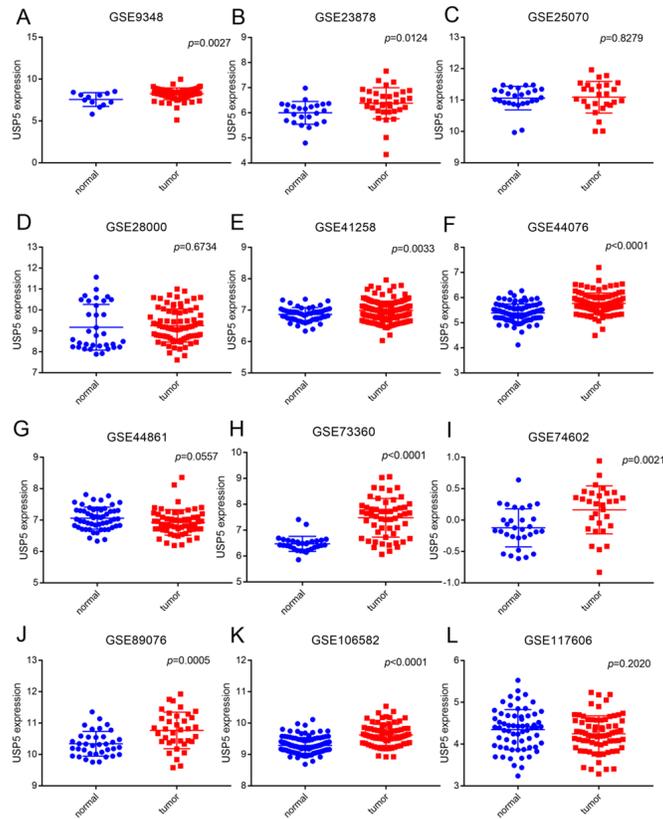


Fig. 2: The expression data of USP5 in CRC in multiple microarrays from GEO, (A) GSE9348; (B) GSE23878; (C) GSE25070; (D) GSE28000; (E) GSE41258; (F) GSE44076; (G) GSE44861; (H) GSE73360; (I) GSE74602; (J) GSE89076; (K) GSE106582 and (L) GSE117606

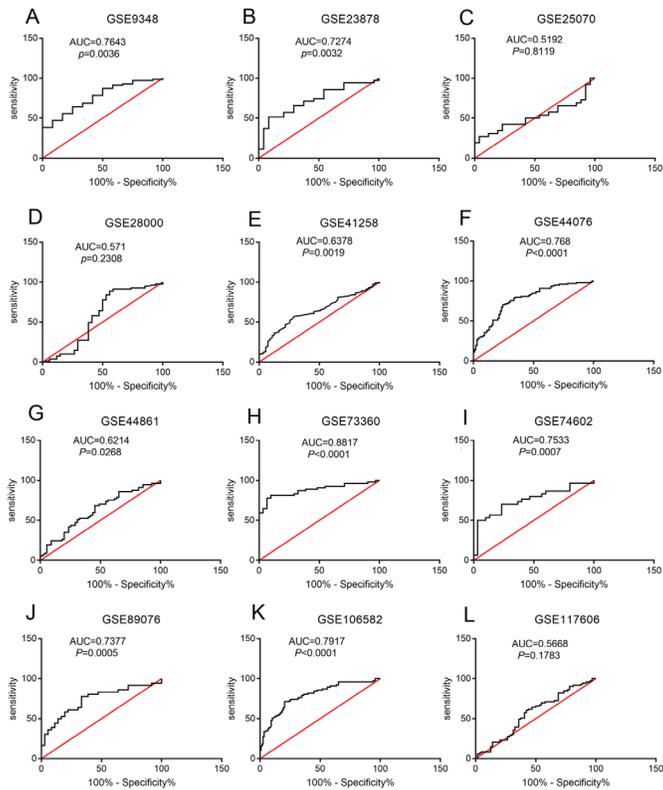


Fig. 3: The ROC curve of USP5 for CRC in microarrays, (A) GSE9348; (B) GSE23878; (C) GSE25070; (D) GSE28000; (E) GSE41258; (F) GSE44076; (G) GSE44861; (H) GSE73360; (I) GSE74602; (J) GSE89076; (K) GSE106582 and (L) GSE117606

Note: (—) Sensitivity % and (- -) Identity %

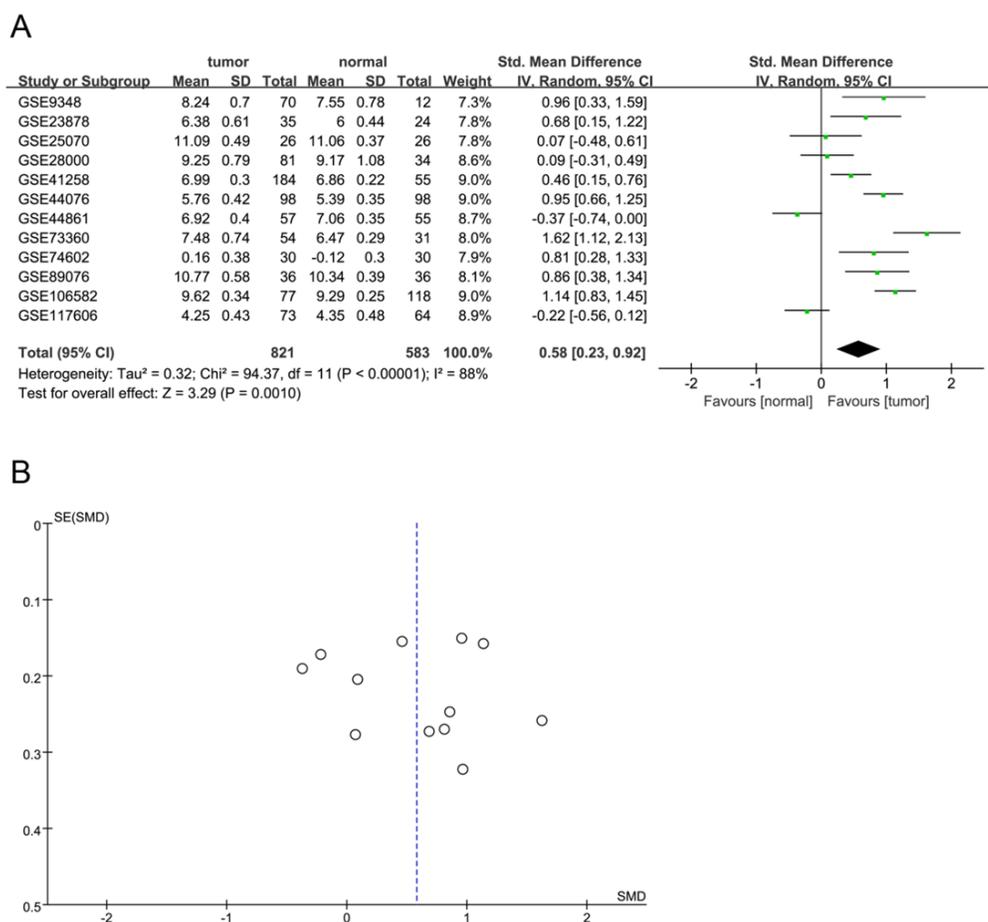


Fig. 4: The meta-analysis of USP5 expression data in microarrays from GEO database, (A) Forest plot and (B) Funnel plot of the combined SMD for USP5 expression between CRC and normal control group by the random effects model

We next analyzed USP5 mRNA expression based on TCGA data by UALCAN. The differential expression in colon adenocarcinoma and rectum adenocarcinoma were shown respectively (fig. 5A and fig. 5B), consistent with GEO database results.

The Human Protein Atlas database was used to present USP5 protein expression and IHC results revealed that USP5 is highly expressed in CRC (fig. 5C and fig. 5D).

Kaplan-Meier survival analysis was performed by using TCGA and GEO database. As shown in fig. 6, USP5 high expression was correlated with poor Overall Survival (OS), which indicated that USP5 expression might be a potential indicator of poor clinical prognosis in patients with CRC.

We explored USP5 expression in CRC based on literature data (fig. 7) where, only one study met the selection criteria. USP5 was considered highly expressed in CRC as a result of quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR), immunoblotting and immunohistochemistry

of cell lines. Also, high USP5 expression was correlated with tumor stage and poor prognosis.

USP5 co-expression genes were obtained based on LinkedOmics (fig. 8A) and a total of 5699 genes co-expressed with USP5 were statistically significant with False Discovery Rate (FDR)<0.05. Top 50 positively correlated significant genes were displayed in fig. 8B. USP5 expression presented a significant correlation with Triosephosphate Isomerase 1 (TPI1), Prohibitin-2 (PHB2) and Nicotinamide Adenine Dinucleotide (NADH) Ubiquinone oxidoreductase subunit A9 (NDUFA9).

GO analysis showed that USP5 expression correlated genes located mainly in mitochondrial, ribosomal, chromosomal and spliceosomal complexes. They participated in cell cycle regulation, Deoxyribonucleic Acid (DNA) replication and RNA processing (fig. 9A-fig. 9C). KEGG pathway analysis showed enrichment in proteasome, ribosome, spliceosome, cell cycle and several metabolic pathways (fig. 9D and fig. 9E).

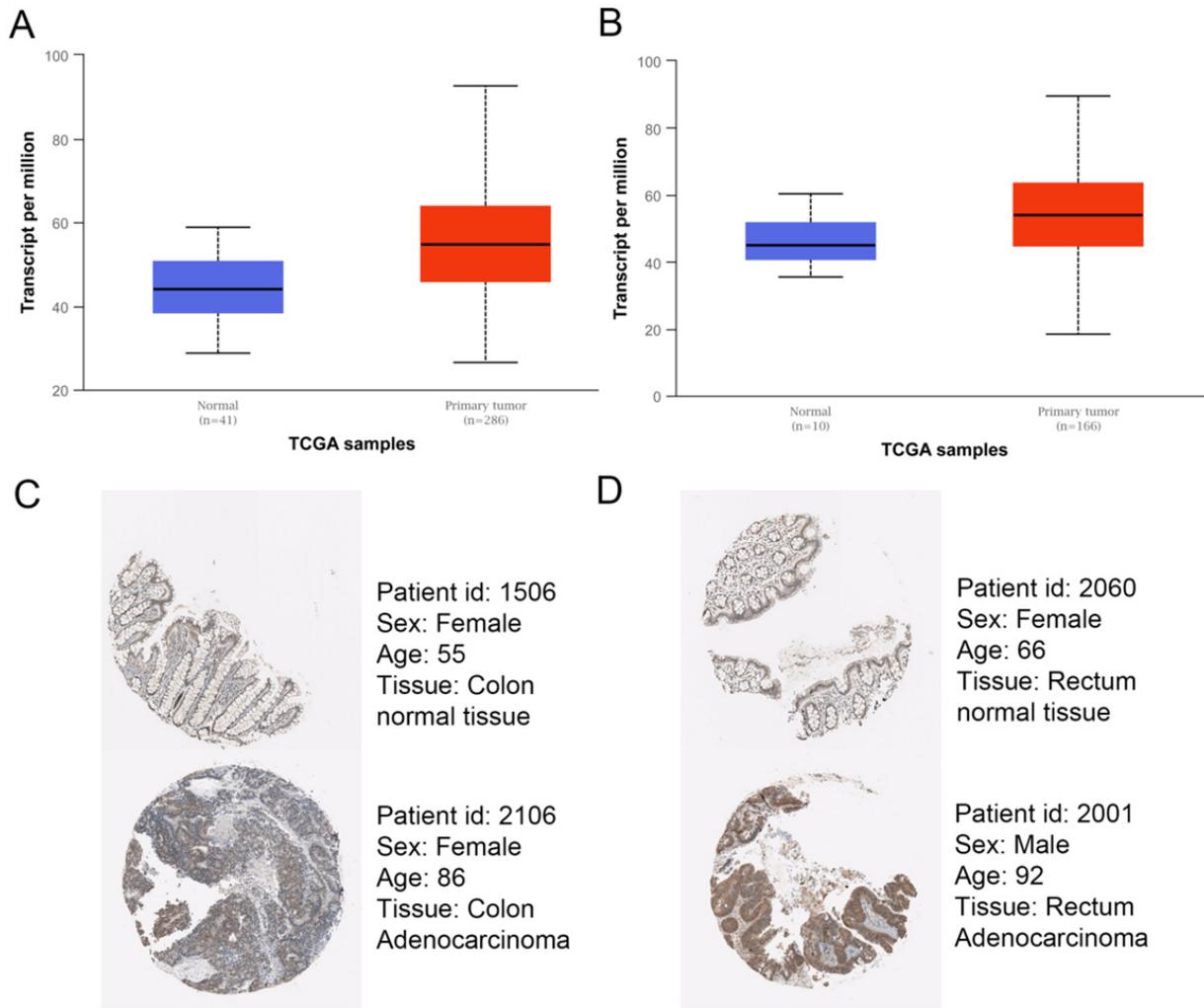


Fig. 5: mRNA and protein expression of USP5 in CRC, (A) USP5 differential expression in colon adenocarcinoma based on TCGA by UALCAN; (B) USP5 differential expression in rectum adenocarcinoma based on TCGA by UALCAN; (C) USP5 protein expression by The Human Protein Atlas database in colon tissue and (D) USP5 protein expression by The Human Protein Atlas database in rectum tissue

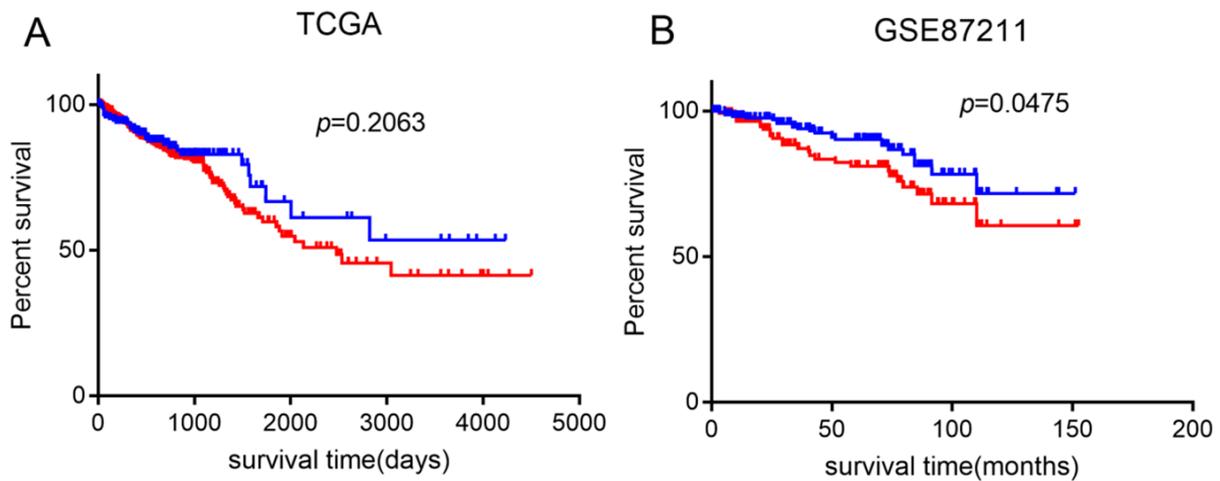


Fig. 6: The relationship of USP5 and prognosis in CRC, Kaplan-Meier survival curves of USP5 based on (A) TCGA and (B) GEO
Note: (—) Low and (—) High

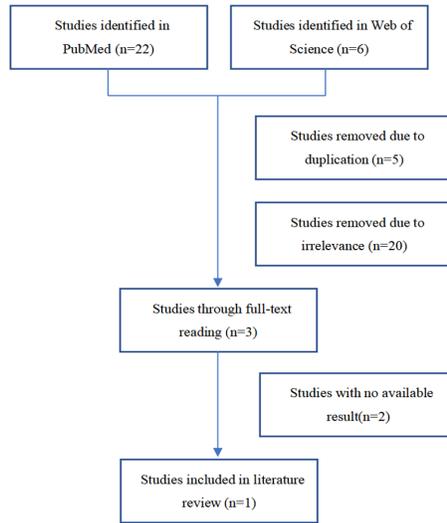


Fig. 7: Flow chart of literature search and study selection

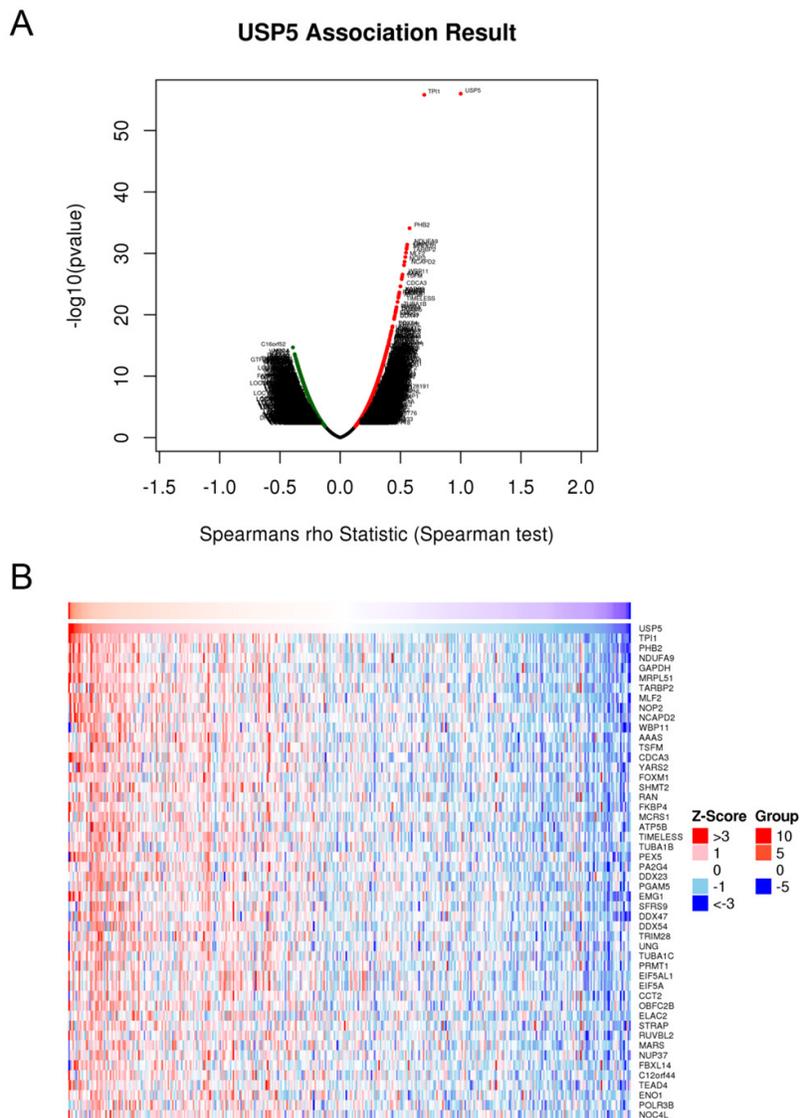


Fig. 8: Differentially expressed genes in correlation with USP5 in CRC, (A) Correlations between USP5 and differentially expressed genes in CRC and (B) Heat maps showing positively correlated genes with USP5 in CRC (TOP 50)

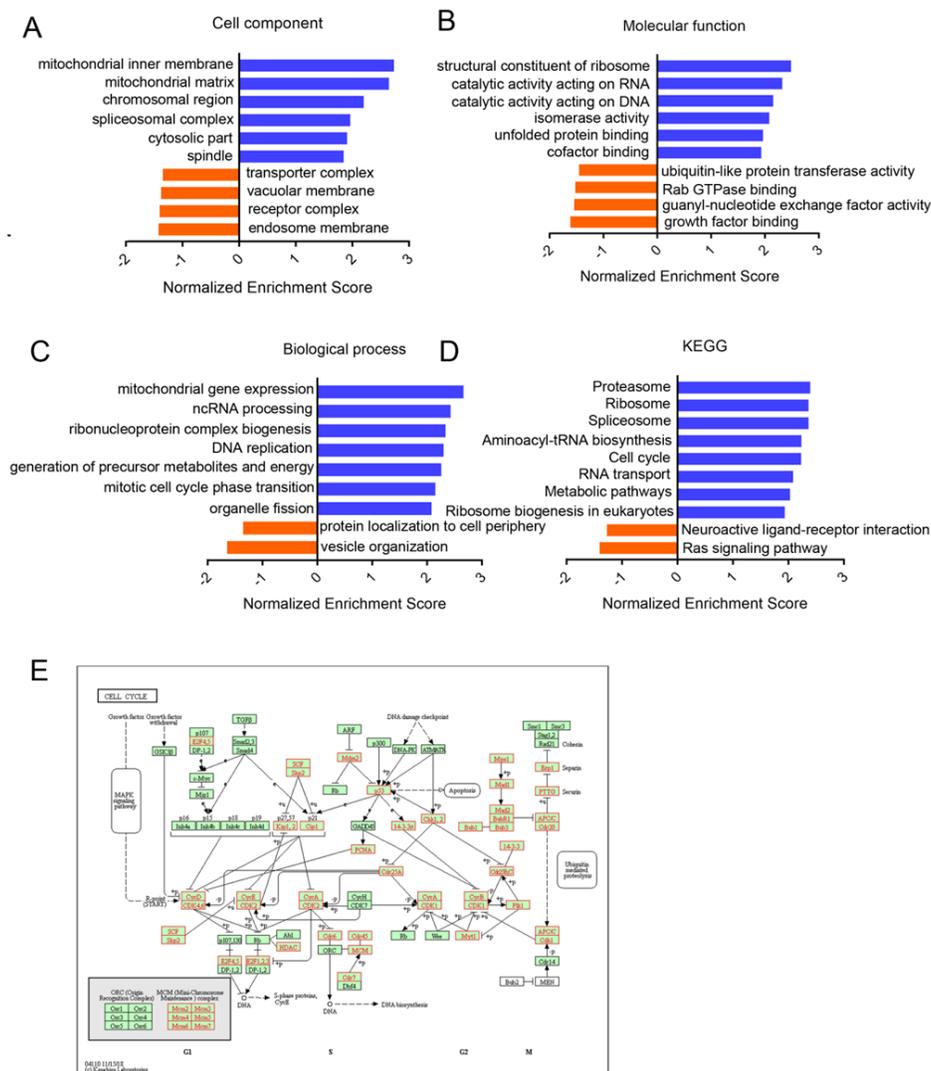


Fig. 9: Significantly enriched GO annotations and KEGG pathways of USP5 in CRC. (A) Cellular components; (B) Molecular functions; (C) Biological processes; (D) KEGG pathway analysis and (E) KEGG pathway annotations of cell cycle, in which red marked nodes are associated with the LeadingEdgeGene

By using GSEA, we analyzed kinase, transcription factor targets and miRNA networks of related genes in order to further explore the targets of USP5 in CRC. The top 5 most significant target networks were listed in Table 2. The kinase target networks included Polo Like Kinase 1 (PLK1), Cyclin Dependent Kinase 1 (CDK1), Cyclin Dependent Kinase 2 (CDK2), Aurora Kinase A (AURKA) and Aurora Kinase B (AURKB). The transcription factor targets network mainly correlated to E2F Transcription Factor 1 (E2F) family and Forkhead-Related Activator 2 (FREAC2). miR-493 (ATGTACA), miR-369-3P (GTATTAT), miR-18A, miR-18B (GCACCTT), miR-448 (ATATGCA) and miR-129 (GCAAAAA) were potentially related to USP5.

We constructed PPI network by BioGRID (fig. 10)

and a total of 105 interactors and 155 published interactions were found. In addition, 4 unique small molecule chemical associations were displayed in network, including vialinin A, degrasyn (WP1130), curcusone D and 2,6-Diaminopyridine-3,5-bis(thiocyanate) (PR-619).

Emerging evidence showed that UPS were characterized in promoting tumorigenesis and tumor progression^[32]. Multiple DUBs has been confirmed as novel promising biomarkers and therapeutic targets for cancer, in which USP5 is one of the potential deubiquitinated proteins^[6]. The present study aimed at analyzing the relationship between USP5 expression, prognosis and the potential functional role of USP5 in CRC.

TABLE 2: THE KINASE, TRANSCRIPTION FACTOR-TARGET AND miRNA NETWORKS OF USP5 IN CRC

Enriched category	GeneSet	LeadingEdgeNum	p value	FDR
Kinase target	Kinase_PLK1	42	0	0
	Kinase_CDK1	74	0	0
	Kinase_CDK2	96	0	0
	Kinase_AURKA	16	0	0
	Kinase_AURKB	35	0	4.84E-04
	V\$E2F_Q6	72	0	0
Transcription factor target	V\$E2F_Q4	73	0	0
	V\$E2F_Q4_01	66	0	0
	V\$E2F1_Q6	67	0	0
	V\$FREAC2_01	92	0	0
	ATGTACA, miR-493	137	0	0
miRNA target	GTATTAT, miR-369-3P	89	0	0
	GCACCTT, miR-18A, miR-18B	56	0	0
	ATATGCA, miR-448	96	0	0
	GCAAAAA, miR-129	86	0	2.83E-04

Note: LeadingEdgeNum: The number of leading edge genes and V\$: The annotation found in Molecular Signatures Database (MSigDB) for transcription factors

In this study, we identified the aberrantly highly expressed USP5 in tumor tissues compared with normal tissues based on GEO datasets. A large cohort of 821 CRC samples and 583 normal samples were involved in the comprehensive meta-analysis. TCGA and The Human Protein Atlas results drew the same conclusion. Additionally, we found diagnostic values of USP5 high expression. Considering the early detection problem, USP5 deserves further clinical validation as a potential diagnostic marker. Kaplan-Meier survival analysis showed that high level of expression of USP5 results in shorter survival rate in CRC patients and the above conclusions are

consistent with literature review^[15].

Since USP5 has been involved in several significant physiological functions, we need to figure out USP5 co-expression genes and its enrichment by GSEA. USP5 neighboring gene networks generally showed different degrees of amplification in CRC, which indicated that USP5 might involve in proteasome, ribosome, DNA replication, cell cycle, spliceosome and several metabolic pathways. As a classic deubiquitinated protein, USP5 definitely plays a role in proteasome. USP5 in regulating RNA splicing^[13] and DNA damage repair^[33] are consistent with the former studies. In pancreatic cancer, USP5

can promote tumor progression by regulating cell cycle^[11]. But whether USP5 can promote cell cycle in CRC still needs further research.

Next, we used GSEA enrichment analysis to reveal the target kinase, transcription factors and miRNA. We found that USP5 is associated with a network of kinases including PLK1, CDK1, CDK2, AURKA and AURKB in CRC. These kinases regulate tumor cell proliferation and cell cycle^[34-37]. In fact, PLK1 is an essential gene for the correct execution of cell division^[38]. AUR/PLK1 axis directly regulates mitosis and other non-canonical targets, including cellular-myelocytomatosis (c-myc)^[39-41]. Although there are some surprisingly debated topics about whether PLK1 is an oncogene or a tumor suppressor gene, this does not affect the use of PLK1 inhibitors as an anticancer drug during last decades^[42,43].

The transcription factor targets of USP5 mainly correlated to E2F family. E2F family, as an important transcriptional factors family, has been implicated in the regulation of many cell processes related to cell proliferation, differentiation, cell cycle, apoptosis and DNA repair^[44]. Our analysis suggests that E2F family might be an important target of USP5 and regulate cell proliferation and cycle through USP5. Further studies need to test this hypothesis.

Several miRNAs were found related to USP5 in our study. miR-493, miR-129 and miR-448 suppresses

proliferation and invasion in cancer^[45-47], while miR-18a, miR-18b and miR-369 has the opposite effect^[48-50]. Although these miRNAs have different degrees of relationship with tumor occurrence and development, their relevance to USP5 needs further experimental research.

According to the results of PPI network data, we found lots of genes were correlated with USP5. Some of them had already been verified, like FOXM1, Suppressor of Mothers against Decapentaplegic (SMAD) Ubiquitination Regulatory Factor 1 (SMURF1) and Tumor Necrosis Factor Receptor (TNFR)-Associated Factor 6 (TRAF6)^[51-53]. Others might be a hotspot in the future researches. Four inhibitors such as WP1130, vialinin A, curcucosone D and PR-619 might inhibit USP5, in which the first two inhibitors have been used and confirmed previously^[9,54].

Cell cycle disorder resulted in tumor cell aberrant proliferation is one of the 10 tumor hallmarks^[55]. The above evidence indicated that USP5 may be related to CRC cell cycle regulation, so we verified in our experiments. Overexpression of USP5 can change the expression of cyclin protein marker, confirming that USP5 can indeed regulate the cell cycle in CRC. The mechanism might be USP5 regulates the deubiquitination of many key proteins in cell cycle signaling pathways.

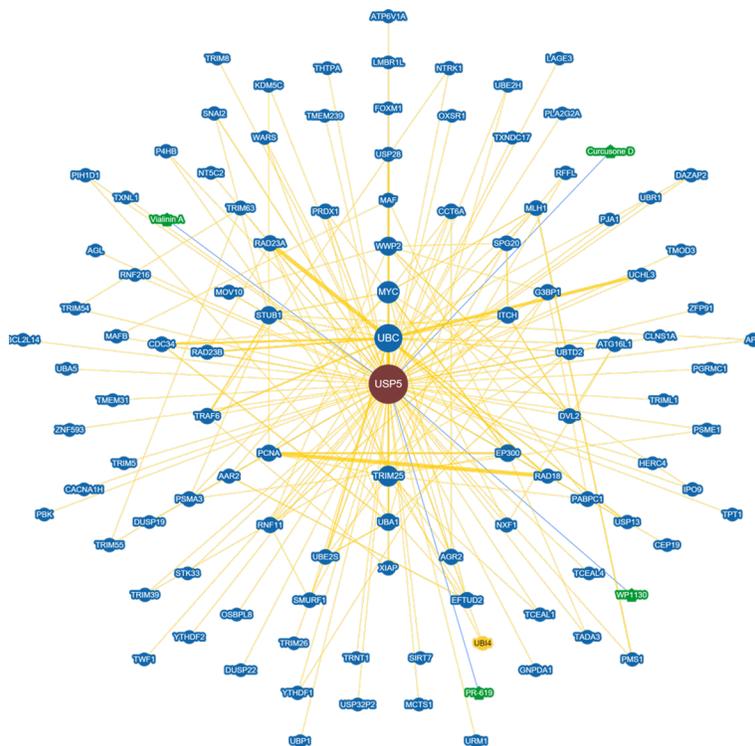


Fig. 10: PPI network analysis by BioGRID

In conclusion, this study mainly used meta-analysis and bioinformatics methods to improve the expression and functional analysis of USP5 in CRC. Our results still need to be confirmed by further experimental studies.

Conflict of interests:

The authors declared no conflict of interests.

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