Protein-Protein Interaction Analysis Reveals A Novel Cancer Stem Cell Related Target TMEM17 in Colorectal Cancer

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Abstract

Background Cancer stem cells (CSCs) have been increasingly reported for tumour recurrence and chemoresistance in colorectal cancer (CRC). However, it is still lack of effective therapy on CSCs.

Methods Here we describe a two-step strategy to generate CSC targets with high selectivity for colon stem cell markers, consisting in first step to generate specific proteins interacted CSC markers and second step to validate in survival analysis. TMEM17 was found and further investigated its biological functions in CRC cells. Finally, we utilized the Gene Set Enrichment Analysis (GSEA) to dig the potential mechanisms of TMEM17 in CRC.

Results By combining protein-protein interaction (PPI) database and high-throughput gene profiles, network analysis revealed a cluster of colon CSCs related genes. In the cluster, *TMEM17* was identified as a novel CSCs related gene. *In vitro* functional study, our results show that TMEM17 depletion can suppress cell proliferation and sensitize chemotherapy drugs to CRC cells. Enrichment analysis of differentially expressed genes was conducted using GSEA algorithm, Wnt/β-catenin pathway was found to be associated with TMEM17 expression. Further validation in clinical samples revealed TMEM17 expression was much higher in tumour than normal tissue and it was associated with poor survival in CRC patients.

Conclusion Collectively, our finding unveils the critical role of TMEM17 in CRC and targeting TMEM17 is potential effective therapeutic for CRC patients.

Introduction

Colorectal cancer is one of the most common cancer types in the world. Latest report has shown that there were over one million new cases in 2018 globally.[1] Recent years, surgery and chemotherapy have improved the outcome of the CRC patients, however, the tumour recurrence and chemoresistance are still the major causes of therapy failure.[2, 3] A current hypothesis is that the existence of a small portion of cancer cells, termed “cancer stem cells” (CSCs), is the main reason of these treatment failures. In line with this hypothesis, studies have discovered stem cell markers including LGR5, CD24, CD44, EPCAM, CD133 on the CRC population.[4-11] In addition, it has been shown that the CSCs can endure the toxicity of chemotherapy and have the capable of self-renewal and generate mature differentiated cancer cells. [12, 13] Although many features of colon cancer stem cells have been identified, its application value in clinic still need to be examined.

During the last 20 years, the high-throughput data analysis has become a new efficient and cost saving method for the cancer research, since abundant amount of patients’ genomic profiles has been taken up in public data sets which are free of access.[14] In this study, we discovered a cluster of cancer stem cell related proteins using a systematic and protein interaction network analysis. Among these proteins, TMEM17 is relative unknown and was found closely related to the colon cancer stem cell markers. TMEM17 belongs to the transmembrane (TMEM) protein family, which is involved in numerous

pathological processes of the cancer development, such as local invasion, metastasis formation and intravasation.[15, 16] Some family members of TMEM17 such as TMEM48, TMEM45A and TMEM97 were reported as potential prognostic biomarkers for cancers, while other TMEM proteins such as TMEM16A was found related to calcium regulation and TMEM173 was reported to control the immune response in carcinogenesis.[16-18] Hence, they represent new possible targets for cancer therapy.

To our knowledge, only two studies have shown that TMEM17 is associated with the cancer development. One study claimed that TMEM17 is a pro-oncogenic protein in the breast cancer, while another study declared that TMEM17 is an anti-oncogenic protein in the lung cancer.[19, 20] It is still unknown why TMEM17 demonstrated these contradict effects and what is its effect on other type of cancer including CRC. Here we describe a two-step strategy to generate CSC targets with high selectivity for colon stem cell markers, consisting in first step to generate specific proteins interacted CSC markers and second step to validate in survival analysis. TMEM17 was found and further investigated its biological functions in CRC cells. Furthermore, depletion of TMEM17 can enhance the sensitivity of chemotherapy drugs and suppress Wnt/β-catenin signaling. Our study identifies an important role of TMEM17 in colon cancer and elucidates a potential cancer stem cell target to sensitize chemotherapy.

Methods

Patients

We retrospectively analyzed the gene expression profiles of frozen colorectal cancer tumor tissue samples from one of the largest individual data sets: CIT/GSE39582 CRC cohort. The data set was obtained directly in its processed format from GEO database through Bioconductor package 'GEOquery'. Overall patients were included in this study. The batch effects were corrected using 'combat' algorithm implemented in R package 'sva' and z-scores for each gene were used for the following analyses. Both paper charts and electronic medical records were carefully reviewed when necessary.

Construction and analysis of protein-protein interaction network

To find potential therapeutic targets on colon CSCs, nine colon stem cell markers were selected from previous studies to construct a protein-protein interaction (PPI) network related to colon CSCs (**Table 1**). The protein interaction information of these markers were obtained using the BioGRID database (Version 3.5.168)[21, 22]. To investigate the association of chemotherapeutic sensitivity, 232 patients with chemotherapy and complete prognostic information in the CIT cohort (GSE39582) were used as the discovery data set.[23] To obtain genes related to prognosis of colorectal cancer and avoid the influence of sample distribution, the corresponding genes resulted from PPI analyses were further selected using the log-rank test with 1000 times randomization (80% portion of samples each time) to assess the association between each gene and patients' disease-free survival in the discovery cohort. Genes with significant frequency more than 500 in repeated log-rank tests were identified as key genes.

Validation cohort

The CIT cohort, one of the largest individual data sets of colorectal cancer is used to validate the potential therapeutic value of TMEM17. The expression of TMEM17 were analysed in 17 cancer samples and its paired normal tissue, while other 566 patients' data were used to conduct a prognostic analysis. The optimal cut-off point of TMEM17 expression was determined based on disease-free survival (DFS) information of these patients using the function 'surv_cutpoint' from R package 'survminer'.

Short interfering RNA

The used *TMEM17* siRNA sequence: si-TMEM17 #1: GCAGCATTATGATGCTTCA; si-TMEM17 #3: GGTCATGTATAGAAGAGAT. The Lipofectamine RNAiMAX kit (Invitrogen) was used for siRNA transfection following the manufacturer's instructions. Cells were transfected with 100 nM final concentration of siRNA duplexes at the optimal seeding density. After 24 hours, cells were digested and re-seeded for following experiments.

RT-quantitative PCR

Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA) and the RNeasy Mini Kit (Qiagen, Germany) and reverse transcribed into cDNA using the cDNA Synthesis Kit (Transgen Biotech, China). RT-PCR was performed using and the KAPA SYBR Fast qPCR kit (KAPA Biosystems, Wilmington, MA). For quantification of mRNA levels, *18S* level was used as an internal control. The specific primers used for TMEM17 were: 5'- GTTCAGTGATTCCAATCGGACC- 3'; 3'- ACCACAGTGGGAA ATAGTAGGT- 5'.

Immunoblotting

Cells were collected and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors for 30 mins. Equal amounts of protein extract were separated on SDS polyacrylamide gels and transferred to polyvinyl difluoride (PVDF) membranes. Membranes were blocked with 5% BSA for 2 hours at room temperature and then probed primary antibody overnight at 4 °C. The used antibodies were: anti-TMEM17 (Santa Cruz, CA; sc-514433), anti-EPCAM (Beyotime, China; AF0141), anti-LGR5 (Abcam, UK; ab75850), anti-MYC (Abcam, UK; ab32072), anti-Vimentin (Cell Signaling Technology, USA; #5741), anti-Snail (Cell Signaling Technology, USA; #3879) and anti-GAPDH (Cell Signaling Technology, USA; #5174).

Cell proliferation assay

For the cell proliferation assay, optimal cells were plated in triplicate in a 96-well format. After 24 hours, the medium was refreshed with optimal drug treatment. Cells were then lysed with CellTilter-Glo (CTG, Promega, Madison,WI), and the fluorescence signal was detected with a microplate reader on days 0, 2, 3, 4 and 5.

Colony formation assay and Tumorsphere formation assay

For the clonogenic assay, optimal cells were seeded in 6 well plates and refresh the medium every three days in 37℃. Colonies were formed after 8 to 10 days culture. The colonies were fixed with methanol and

stained with crystal violet (0.5% crystal violet, 20% methanol).

For the tumorsphere assay, single-cell suspensions were plated (5000 cells/well) in a 12 well ultra-low attachment plates with Mammocult medium (Stem cell Technologies), supplemented with fresh hydrocortisone (0.5μg/ml) and heparin (1:500) and culture in 37℃. 0.5ml fresh medium was added every three days and tumorspheres were formed after 7-10 days culture. The spheres were stained with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) (Sigma-Aldrich) and quantified.

Pathway Analysis

Enrichment analysis for differentially expressed genes between high/low TMEM17 expression using R package 'gProfileR' in the CIT data set. The cut-off point of high/low TMEM17 expression was based on the optimal cut-off point in disease-free survival (DFS) analysis in the CIT. For interested biological pathways, Gene Set Enrichment Analysis (GSEA) was further performed using Bioconductor package 'HTSanalyzeR'.[24, 25]

Tissue microarray and immunohistochemistry staining

A total of 318 CRC patients with pTNM stage □ to □ from January 2002 to June 2006 were included in this study and the pathological specimens were constructed in a tissue microarray (TMA). Tumour staging was assessed according to the criteria of the Seventh Edition of the American Joint Committee on Cancer (AJCC) stage.[26] The clinicopathological data were collected from the CRC database of the follow-up office and approved by the Institutional Review Board of the The Sixth Affiliated Hospital, Sun Yat-sen University.

Paraffin-embedded tissue samples were cut into 5μm sections and antigen retrieval was performed with citrate buffer (Beyotime, China; P0081). After blocking with 10% goat serum, samples were incubation with primary antibody overnight at 4°C, followed by diaminobenzidine staining. IHC staining was evaluated in semi-quantitative method as described before.[27] Each TMA spot was marked with an intensity score and percentage of positive tumour cells score from 1 to 4. TMA scores were determined by the intensity score multiply proportion of area score. A final score was determined as the average of the duplex. The optimal cut-off point of TMEM17 expression was conducted based on X-tile software (X-tile 3.6.1).[28]

Statistical analysis

Graphs were expressed as *mean* ± *SD* from three independent experiments. Statistical difference between two groups was evaluated by two-tailed student's *t*-test, and that of multiple groups was analysed by two-way ANOVA. Survival curves were evaluated by Log-rank (Mantel-Cox) test. *P*-values < 0.05 were considered as statistically significant.

Results

Protein-protein interaction network identified TMEM17 as a CSC related marker

To find potential therapeutic targets on colon CSCs, a PPI network was constructed based on a list of colon stem cell markers (**Figure 1A**, **Table 1**). A total of 683 records were found from PPI analysis, containing 276 different proteins. The corresponding genes of these 276 proteins were verified using log-rank test to evaluate the relationship between each gene and patients' diseases free survival in the CIT data set. Eleven genes were identified and listed base on the significant frequency in the resampling survival analyses and further filtered by the average *P* value (**Figure 1B**, **Table 2**). Among these genes, the function of *TMEM17* in the development of CSC is unknown and its effect on the development of CRC has not been reported yet. Analysing of the mRNA expression, an increased expression of *TMEM17* was found in tumour tissue as compare to adjacent normal tissue (**Figure 1C**). In addition, increased *TMEM17* expression was associated with tumour recurrence and poor survival (**Figure 1D-E**). This result indicated that it could be a novel biomarker to predict CRC prognosis.

Genetic depleting TMEM17 supressed cell proliferation in CRC

In order to investigate TMEM17 biological function in cancer cells, TMEM17 expression was depleted by using siRNA. The depletion effect was conformed using RT-qPCR and immunoblotting assay (**Figure 2A-B**). The cells were transfected with *TMEM17* siRNA. Significantly reduced colony formation and cell proliferation were found (**Figure 2C-D**). These results suggest that TMEM17 plays as a crucial role in colon cancer cell proliferation and may be used as a therapy target to CRC.

Targeting TMEM17 enhanced the sensitivity of chemotherapy drugs in CRC

Chemotherapy failure is one of crucial reasons for the tumour recurrence in stages □-□ CRC patients. Hence, enhancing the sensitivity of chemotherapy drugs is a potential therapeutic strategy. To investigate the potential pharmacology value of TMEM17, cells were treated with chemotherapy drugs after siRNA transfection. The cell proliferation rate was significantly suppressed in TMEM17 siRNA transfected cells with drug treatment as comparing to the cells receive the drug treatment alone and neither 5-Fu or oxaliplatin treatment affect this outcome (**Figure 3A-B**). In line with these findings, si-TMEM17 enhanced the sensitivity of 5-Fu and oxaliplatin treatment in a long-term cell culture (**Figure 3C**).

Targeting TMEM17 suppressed CSC characteristic in CRC cells

To test the functional importance of TMEM17 in CSC, colon cancer cells were cultured in serum free medium to examine sphere formation. Depleting of TMEM17 significantly suppressed the spheres formation (**Figure 4A**) and enhanced the sensitivity of chemotherapy drugs (**Figure 4B-C**). Notably, the CSC markers, including EPCAM and LGR5, were downregulated in si-TMEM17 transfected cells (**Figure 4D**). We also investigated the expression of TMEM17 when cells were cultured in different culture mediums. TMEM17 was increased in tumorsphere culture, accompanying with CSC markers up-expression (**Figure 4E**).

TMEM17 related CSC features was associated with Wnt/ β-catenin signaling

In order to find the underlying mechanism of TMEM17 mediated CSC development, pathway analysis was performed with patients' genomic profiles in the CIT data set. The result suggested that high *TMEM17* expression was associated with active epithelial mesenchymal transition, Wnt/β-catenin signaling and TGF β signaling (**Figure 5A**, **Supplementary Table 1**). Among these signaling pathways, the Wnt/β-catenin signaling, which is known to regulate tumour initiating cell activation and differentiation[29-32], was enrichment in high *TMEM17* patients (**Figure 5A-C**). Also, depletion of TMEM17 suppress some makers of Wnt/β-catenin signaling in immunoblotting analysis (**Figure 5D**). In addition, patients with high *TMEM17* expression have shown a significant enrichment of stem cell gene sets (**Figure 5E**). These data suggest that *TMEM17* may be a positive regulator of the development of CSC.

TMEM17 is upregulated in CRC and is related to poor CRC survival

Based on the functional studies of TMEM17, we hypothesized that TMEM17 activity may affect the clinical outcome of CRC patients. We constructed a tissue microarray containing a large cohort of CRC patients and tested the expression of TMEM17 (**Figure 6A**, **Supplementary Table 2**). An increased expression of TMEM17 was found in tumour tissue as compared to the normal tissue. In addition, TMEM17-high group was associated with a significant lower survival rate as compared to the TMEM17-low group (**Figure 6B and C**). The survival analysis revealed a 10-year survival rate of 64% in TMEM17-low group, while 48% in TMEM17-high group (P= 0.037).

Discussion

CSCs are believed to be highly chemoresistant and play a key role as tumour initiating cells in cancer recurrence following chemotherapy.[33] However, there is no effective CSCs-targeting strategy in the clinical use.[34, 35] One main reason is there are no valid target on CSCs. This study is aimed to find a potential therapeutic target on CSCs using a big data analysis. A cluster of potential target protein was found combining the protein-protein interaction (PPI) database and high-throughput gene profiles. Among these proteins, a novel gene *TMEM17* was selected and further investigated for its biological functions in CRC cells.

The existence of CSCs was proposed decades ago. Although increasing studies find evidence support the CSCs theory, their effort still face a fundamental scepticism that many still doubt if CSCs is a distinct tumour cell population and whether CSCs are involved in the recurrence of every cancer types.[35] In CRC, a series of lineage-tracing studies confirmed a $LGR5^{+}$ cell population had the ability to undergo differentiation in different models.[36-38] These studies supporting that CRC is composed of heterogeneous cell populations including a small fraction of CSC. Currently, most of anti-CSCs strategies were based on targeting the stem cell markers or inhibiting the relevant pathway signalling, such as anti-CSCs antigens and Wnt inhibitors.[39] The identification of novel therapies to target CSCs is the goal of many cancer researches. In this study, we performed a PPI analysis using selected CSC markers and discovered that TMEM17 may as a potential target on CSCs.

Genome-scale human PPI networks are helping to find a cluster of genes from the same features and provide more potential targets for the cancer therapy.[40] PPI network analysis was shown to be a reliable tool to interpret the function of abundant genes associated with the development of cancer and autism.[40] Increasing studies using PPI analysis in cancer research indicate that it is a potential method to discover new therapeutic targets.[41, 42] Here, we constructed a PPI network using nine colon CSC markers and discovered a cluster of significant targets. Most of these genes were related to CSCs, such as *CD63*[43], *MCM2*[44], *CAV1*[45], *GLIS2*[46], *CDC20*[47, 48], *LATS2*[49] and *FGF2*[50, 51]. Interestingly, *TMEM17*, being the second highest resampling frequency in the survival analysis, has no report on CSCs and its pathological function is confuse in other tumours.[19, 20] Hence, it's deserved to be investigated.

PPI analysis is highly promising method for therapeutic target discovery depends on its high efficiency. However, it also subjects to the limitations of bioinformatics analysis. Therefore, a series of *in vitro* experiments were performed using CRC cell lines to validate the pathological function of TMEM17. But there are still some limitations in this study. Firstly, although we analysis the prognostic information of public database and used a large cohort of CRC patient samples, *in vitro* experiment was lacking. Secondly, our results reveal that depletion of TMEM17 inhibits CSC by suppressing Wnt/β-catenin signaling. However, how TMEM17 affects β-catenin is still unclear, and the detailed mechanism should be clarified in the future.

In conclusion, we performed a PPI analysis based on colon CSC makers and discovered a novel CSC related gene *TMEM17*. Identifying the critical role of TMEM17 in CRC cells, indicating that TMEM17 is a potential effective therapeutic for CRC patients.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board (IRB) of The Sixth Affiliated Hospital of Sun Yat-sen University.

Consent for publication

All authors read and approved the final manuscript. The authors declared no financial conflict of interest.

Availability of data and materials

The data sets generated and analyzed during the current study are available in the GEO database (CIT/GSE39582, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39582) and Genomic Data Commons Data Portal (TCGA CRC, https://portal.gdc.cancer.gov/).

Competing interests

The authors declared no financial conflict of interest.

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Author's contributions

XJW supervised the project and contributed to the design and interpretation of all experiments. ZLY, YFC and FG contributed to the design, conduct, and interpretation of all experiments. ZLY and ZRC performed tumour sample preparation, gene knockdown, and tumorsphere assays. ZLY and YFC performed western blot analyses. FG performed bioinformatics and statistical analyses. YFC, JK, YFZ and PL contributed to collection of patient samples and clinical information and performed immunohistochemistry staining. ZLY, BZ, FG and XJW wrote the manuscript with input from all co-authors.

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Tables

Table 1. List of Colon Cancer Stem Cell Markers.

Gene	Other name	Function	References
Lgr5	GPR49	Wnt signaling gene	[4, 8, 36-38]
ALDH1A1	ALDC, ALDH1	Enzyme	[7]
CD24	CD24A	Cell adhesion molecule	[8]
CD29	Integrin b1	Cell adhesion molecule	[8]
CD44	CDW44	Cell adhesion molecule, Hyaluronic acid receptor	[5, 8, 9]
CD133	Prominin 1	Self-renewal, Tumour angiogenesis	[6, 8]
CD166	ALCAM	Cell adhesion molecule	[8]
EPCAM	ESA, MK-1	Cell adhesion molecule	[9]
MSI1	Musashi-1	RNA-binding protein	[10, 11]

Table 2. Stem cell marker related proteins.

Gene	Full name	Frequency in resampling
CD63	CD63 molecule	979
TMEM17	transmembrane protein 17	958
MCM2	minichromosomal maintenance complex component 2	952
CAV1	caveolin 1	891
GLIS2	GLIS family zinc finger 2	849
CDC20	cell division cycle20	810
LATS2	Large tumour suppressor kinase 2	732
ANGPT2	angiopoietin 2	727
ECSIT	ECSIT signalling integrator	711
NOB1	NIN1 (RPN12) binding protein 1 homolog	704
FGF2	Fibroblast growth factor 2	699

Figures

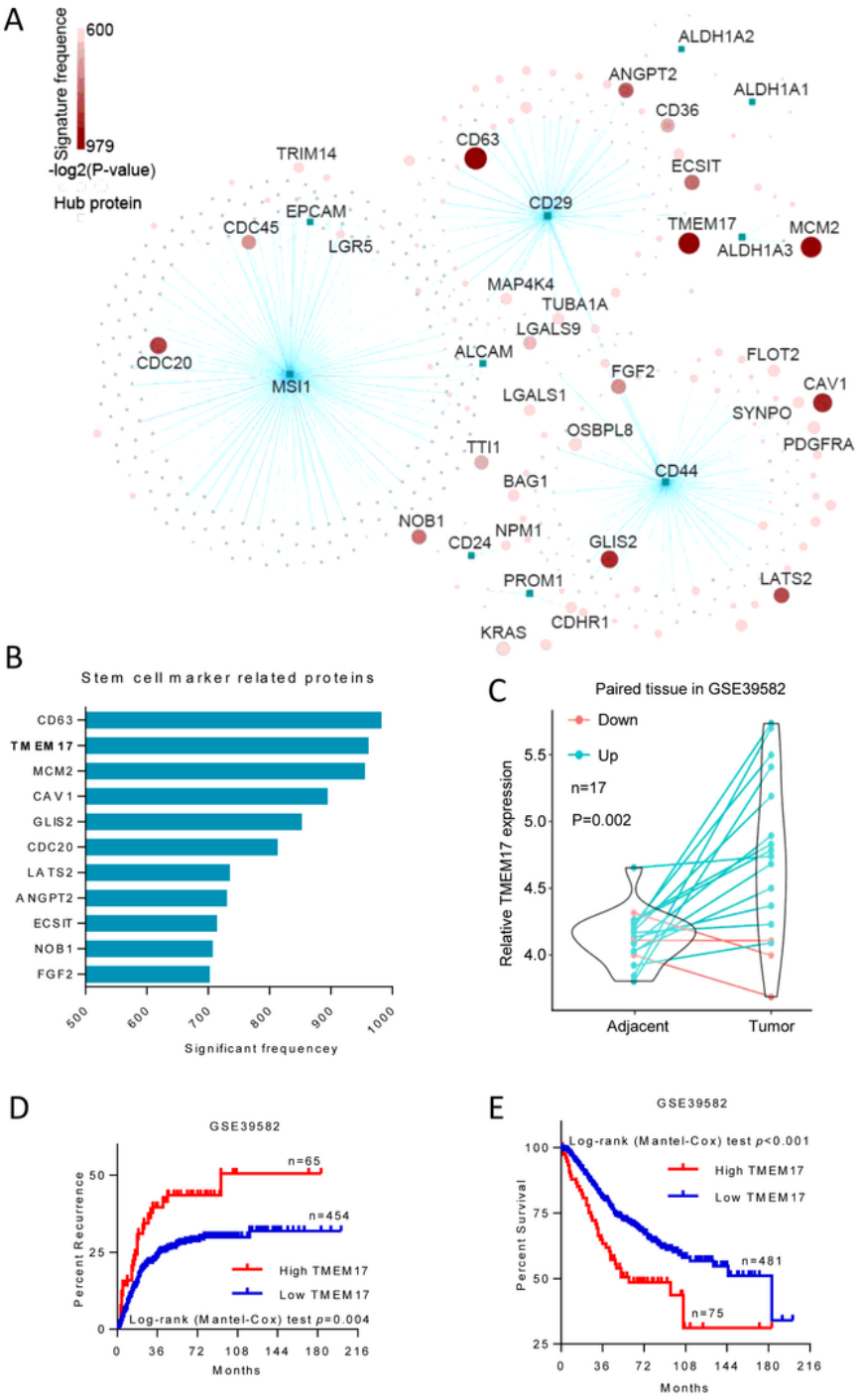

Figure 1

PPI network and survival analysis identified TMEM17 as a CSC related gene. (A) PPI network of the nine colon stem cell markers. Node size is -log2 transformed averaged P-values in 1000 randomization log-rank tests. Node color represents the frequency calculated by the number of times that the corresponding gene significantly associated with survival in the same analysis. Nodes with labels represent key genes related to CSC (frequency > 500). Edges represent physical PPIs between proteins obtained from BioGRID

database. (B) The significant frequency of 11 CSC related genes in survival analysis (log-rank test, P < 0.05; frequency > 500). (C) TMEM17 expression is significantly higher in CRC samples than that in paired normal colon tissue (P = 0.002). (D and E) Kaplan-Meier survival analysis revealed that high TMEM17 expression was significantly correlated with tumour recurrence (D) and short overall survival (E).

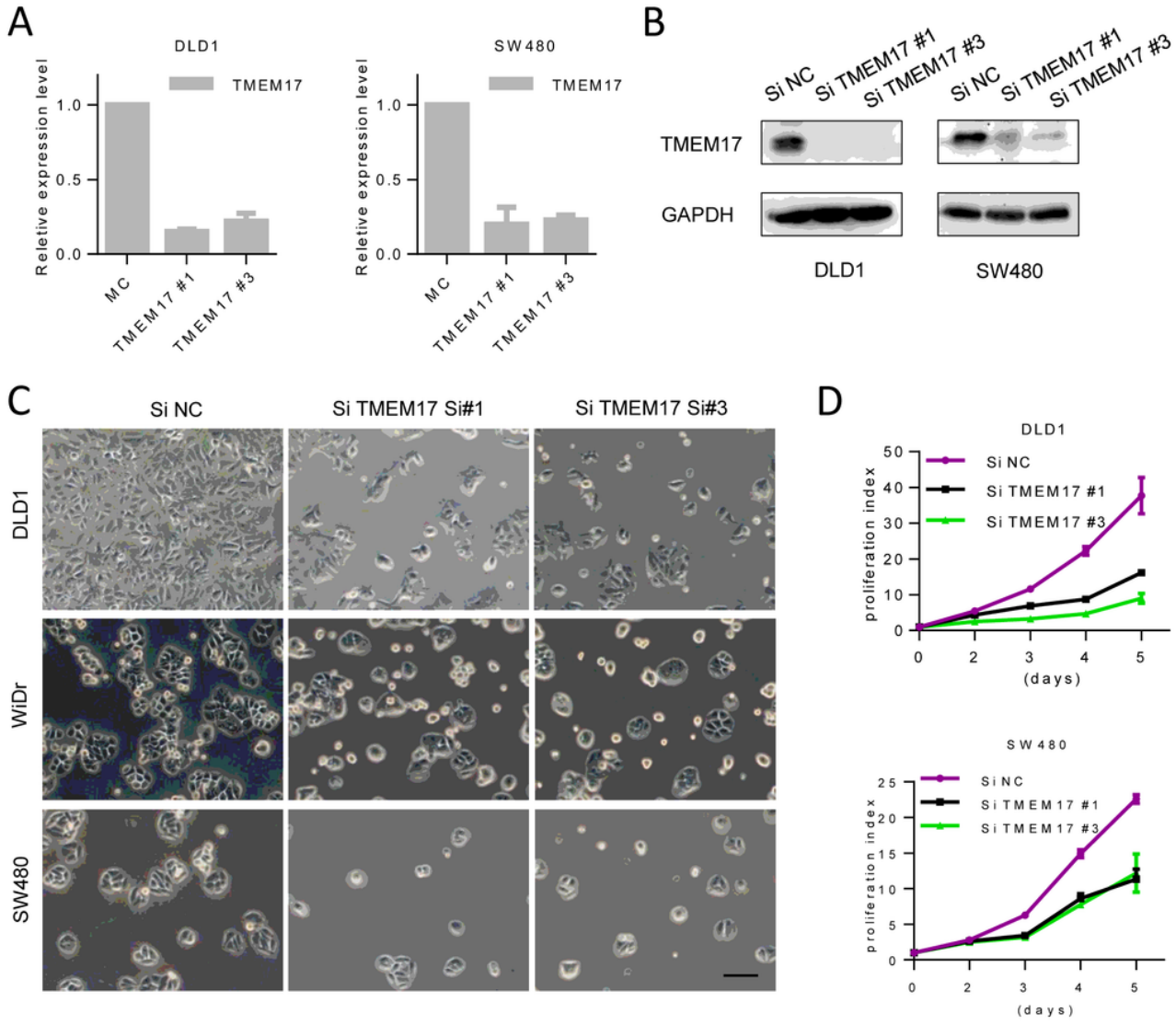

Figure 2

Depletion of TMEM17 suppressed CRC cells proliferation. (A and B) The transfection efficiency after depleting TMEM17 by siRNA in CRC cells were tested by RT-PCR (A) and immunoblotting (B). (C) Representative images of 48 hours after CRC cells treated with si-TMEM17 in adherent culture. The bar = 200um. (D) Growth curves of CRC cells with depleting TMEM17 in a period of five days culture.

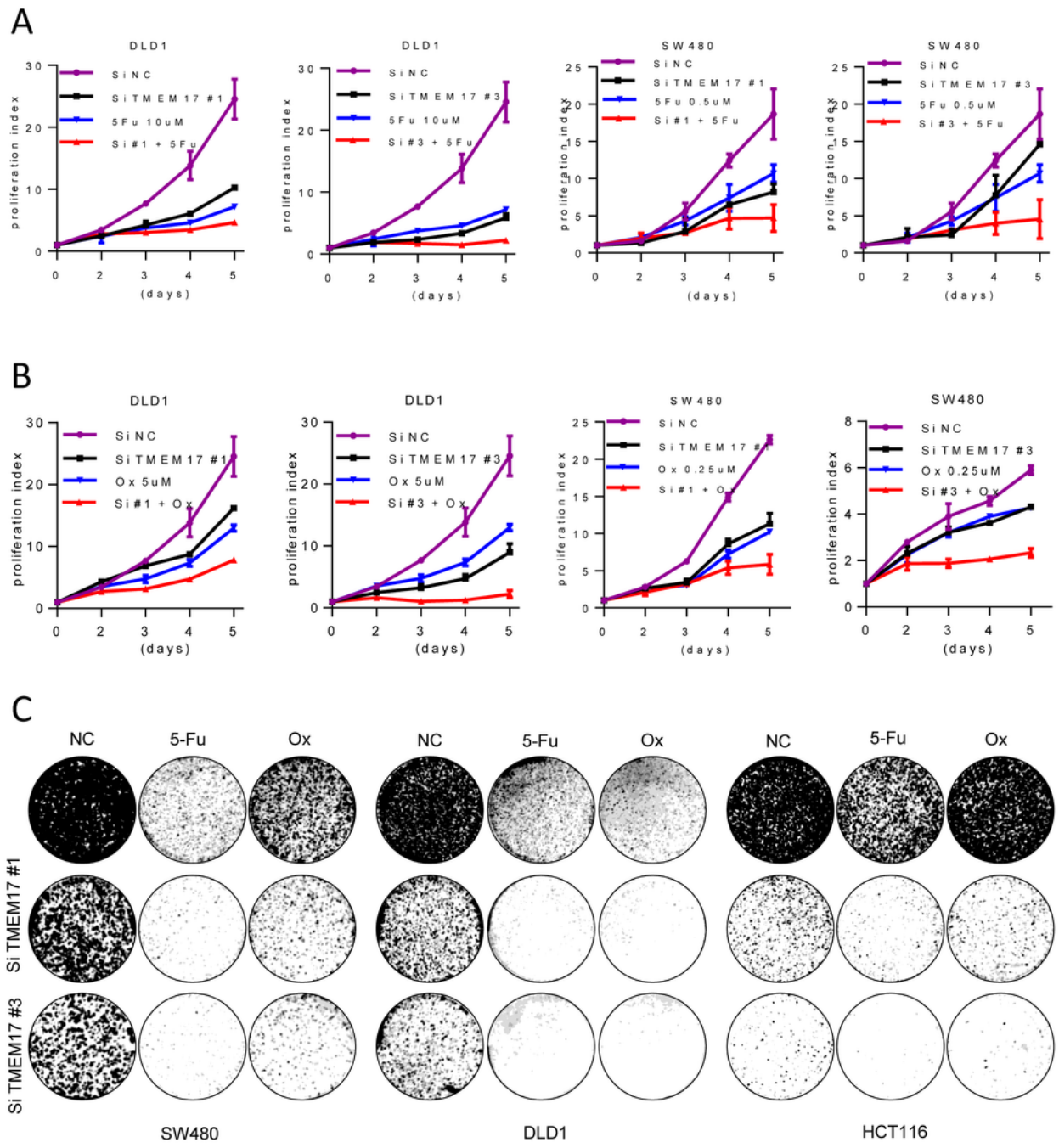

Figure 3

Targeting TMEM17 enhanced the sensitivity of chemotherapy drugs in CRC. (A) Growth curves of CRC cells with depleting TMEM17 and 5-Fu treatment in a period of five days culture. (B) Growth curves of CRC cells with depleting TMEM17 and oxaliplatin treatment in a period of five days culture. (C) Clonogenic assay of CRC cells with depleting TMEM17 and 5-Fu/oxaliplatin treatment in a period of eight to ten days culture.

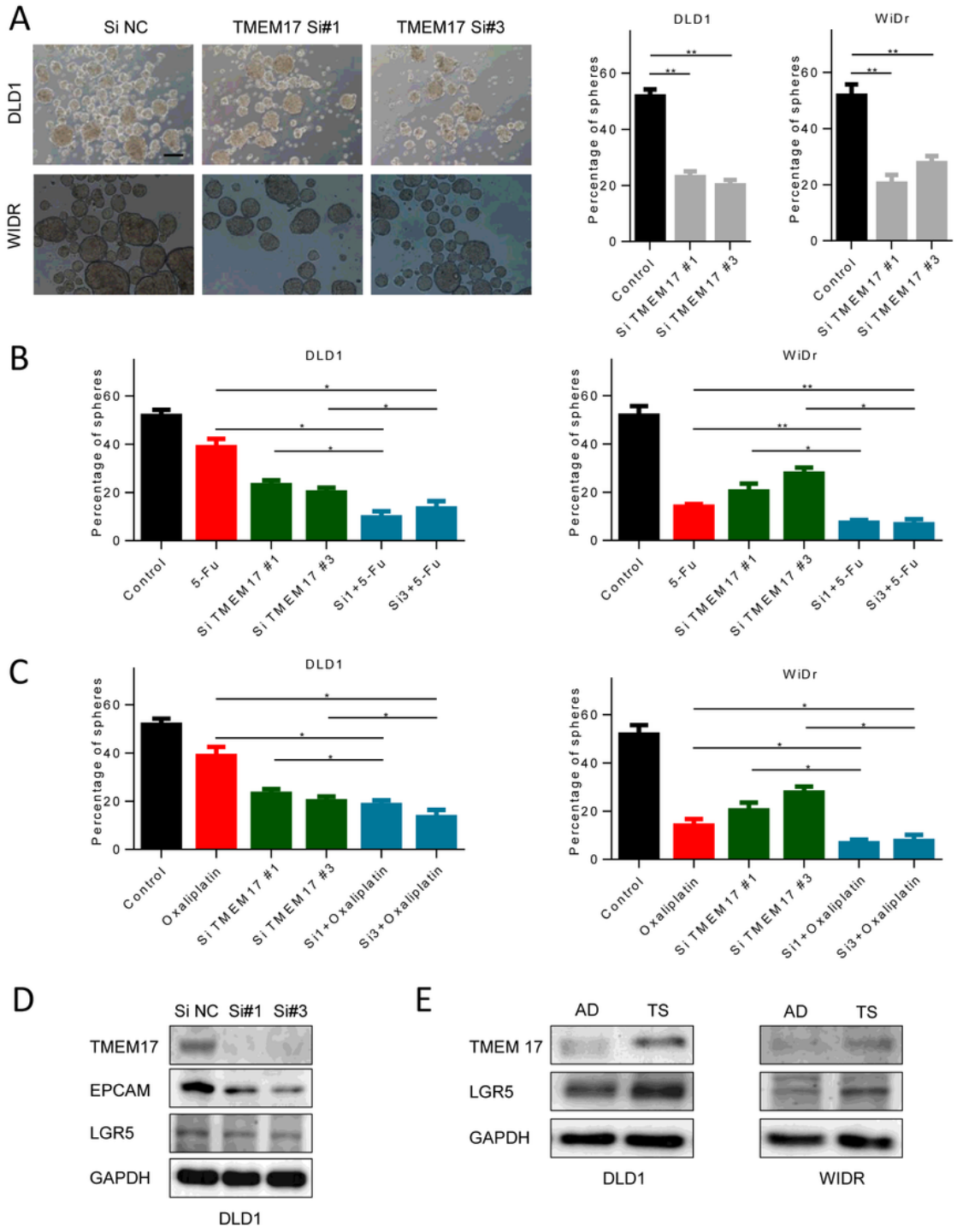

Figure 4

Targeting TMEM17 suppressed CSC characteristic in CRC cells. (A) Genic depleting TMEM17 reduced cancer cell sphere formation, n=3. The bar = 400um. (B) Tumorsphere formation assay of CRC cells with depleting TMEM17 and 5-Fu treatment, n=3. (C) Tumorsphere formation assay of CRC cells with depleting TMEM17 and Oxaliplation treatment, n=3. (D) Immunoblotting assay of the expression of TMEM17, EPCAM and LGR5 proteins from CRC cells with scramble or TMEM siRNA. (E) Immunoblotting

assay of the expression of TMEM17, EPCAM and LGR5 proteins from CRC cells with adherent culture or tumorsphere culture. (F) Clonogenic assay of DLD1 and DLD1 oxaliplatin resistance cells. (G) Immunoblotting assay of DLD1 and DLD1 oxaliplatin resistance cells. Error bars represent ± SD. **P<0.01, *P<0.05, paired sample T test (A-C).

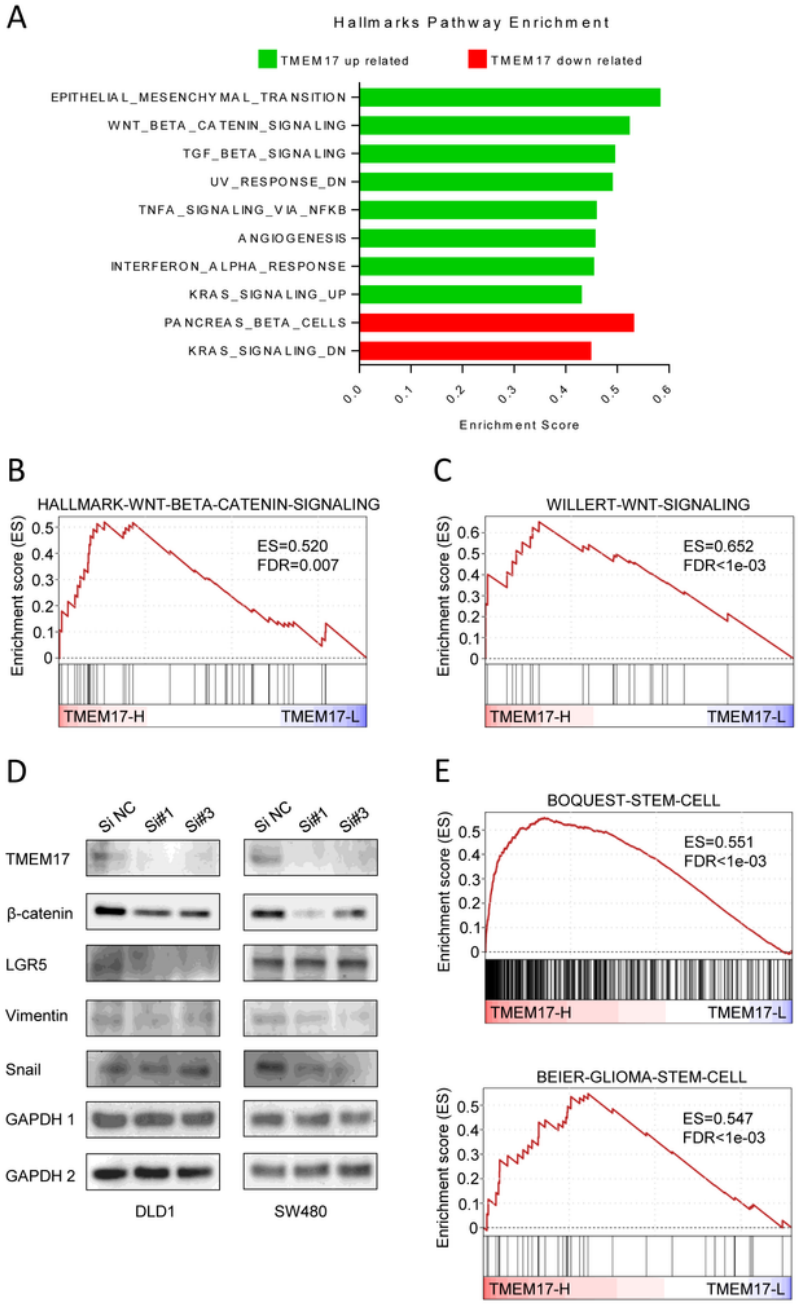

Figure 5

Pathway enrichment analysis between differential expression groups of TMEM17. (A) Significantly dysregulated pathways were identified by GSEA in the CIT cohort for cancer hallmark pathways. Top ten pathways were selected for presentation by absolute enrichment score (positive score is green and negative score is red). (B) GSEA plot of the Hallmarks Wnt/ β-catenin signaling in the CIT cohort. (C) GSEA plot of Willert Wnt signalling in the CIT cohort. (D) Immunoblotting assay of the expression of TMEM17 and several Wnt signaling markers proteins from CRC cells with scramble or TMEM siRNA. (E) GSEA plot of Boquest Stem Cell signalling and Beier Glioma Stem Cell signalling in the CIT cohort.

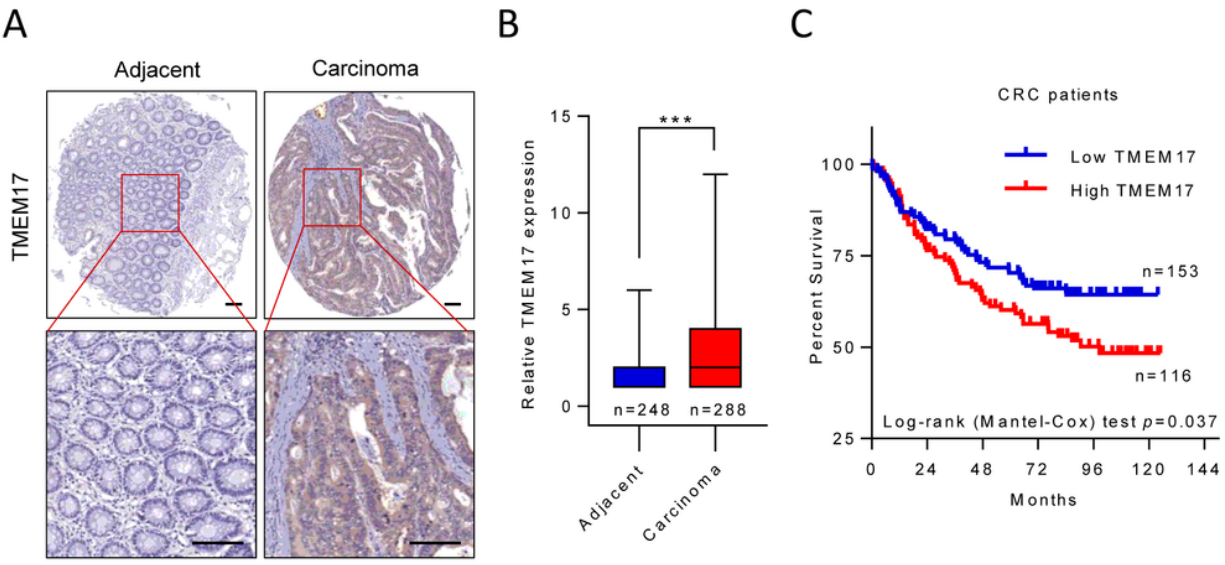

Figure 6

TMEM17 is upregulated in CRC and is related to poor CRC survival. (A) Representative TMEM17 immunochemistry staining in CRC and normal colon tissue. The scale bar represents 100μm. (B) Tissue microarray assay of TMEM17 expression in CRC and normal colon tissue. Error bars represent ± SD. ***P<0.001, 2-tailed unpaired Student's t test. (C) High TMEM17 expression was associated with poor overall survival in CRC patients.