SMC4 Promotes Proliferation and Migration of Colon Adenocarcinoma Cells

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Research

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Abstract

Background: Structural maintenance of chromosomes 4 (SMC4) is one of the SMC family members, which was located in 3q25.33 and involved in chromosome enrichment and separation, DNA recombination and repair, has been implicated in the promotion of cancers proliferation and metastasis. However, the possible role of SMC4 in colon adenocarcinoma (COAD) metastasis and EMT progression remained unclear.

Methods: The expression of SMC4 in COAD tissues was screened by bioinformatics analysis and immunohistochemical assay. si-RNA-mediated transfection was performed in COAD cells. The proliferation viability was measured using MTT, colony formation and EdU assays. SMC4-induced autophagy occurred was explored by AO staining, autophagy-related markers were detected by Western blots and Immunofluorescence staining. The migration ability was determined using wound healing and Transwell assay. The expression of EMT markers and transcriptional factors were detected using western blot assays.

Results: We found that SMC4 was upregulated in COAD tissues, and its overexpression was linked with COAD patients' aggressive tumor characteristics. Furthermore, prognostic analysis shown that COAD patients with high SMC4 expression had a remarkably shorter survival and acted as an important factor for predicting poor overall survival in GC patients. Moreover, SMC4 depletion inhibited COAD cells proliferation and migration through epithelial-to-mesenchymal transition (EMT) progression. Taken together, we proposed that SMC4 involved in COAD progression and might be a valid prognostic marker.

Conclusions: We proposed that SMC4 involved in COAD progression and might be a valid prognostic marker.

1. Introduction

COAD is a relatively common malignancy of the digestive tract and one of the leading types of cancer that cause death worldwide [1]. In 2018, the global incidence rate is 10.2% and the mortality rate is 9.2% [2, 3]. Due to the lack of effective biomarkers in the early diagnosis and treatment of COAD, patients with COAD are diagnosed in advanced stage with poor prognosis [4].Therefore, the further study of the abnormally expressed proteins associated with COAD is of great significance for the early detection of COAD, the effective evaluation of prognosis and the development of effective biomarkers.

Structural maintenance of chromosomes 4 (SMC4) is one of the SMC family members, which located in 3q25.33. This ATPase family maintains the stability of chromosomal structure and participates in mitosis of eukaryotic cells. SMC4 is mainly involved in chromosome enrichment and separation, DNA recombination and repair [5, 6]. In recent years, studies have shown that SMC4 was highly expressed in a variety of cancers and related to the malignant progression of tumors, such as lung, liver and breast cancer [7–9]. Ma *et al*. found that SMC4 was upregulated in breast cancerous tissues and in T2-3N0 or ER/PR-positive patients, higher mRNA expression level of SMC4 was associated with worse survival rates

[9]. In hepatocellular carcinoma, SMC4 was overexpressed and correlated with tumor dedifferentiation, advanced stage and vascular invasion [10]. These already discovered functions of SMC4 indicated that it played an indispensable role in cancer tumorigenesis and progression.

Cancer cell proliferation and migration underlay development and metastatic dissemination, which are major problems in cancers [11]. The main cause of death in patients with COAD is invasion and metastasis, therefore, explore and block the mechanism of COAD patients' recurrence and metastasis is the key to improve the survival of patients. SMC4 was reported that corrected with glioma cells cycle and proliferation [12]. The similar function of SMC4 was found in liver cancer that SMC4 could affect the cancer cells proliferation and migration through JAK2/STAT3 pathway [10]. Whereas, the role of SMC4 in proliferation and metastasis of COAD rarely reported.

Herein, we sought to exhibit that the SMC4 overexpression indicated high invasion and exacerbated prognosis in COAD. Furthermore, functional experiments validated the expression of SMC4 significantly correlates with COAD cells proliferation, migration and EMT progression. Our findings might provide novel evidence for the function of SMC4 in COAD patients and its clinical treatment.

2. Materials And Methods

2.2 Clinical specimens

Shanghai Outdo Biotech Co. Ltd. selected 94 patients at random who underwent routine treatment in 2009 and paraffin-embedded COAD tissues meeting rigorous follow-up criteria. Clinical pathological parameters containing age, gender, tumor size, clinical stage, TNM stage and 5-year survival, were seriously inspected. Ages of patients ranged from 27 to 90, with an average age of 58.5 years. 48:46 was the ratio of male-to-female. In conformity with the seventh edition of the American Joint Committee on Cancer, 21 COAD specimens were staged as early stage (I-II) and 36 as late stage (III-IV). The median survival time was 48 months. In addition, 78 cases from adjoining non-tumor tissues were obtained from the margin of the cancer resection in these patients.

2.3 Cell culture and transfection

The COAD cell lines (SW620, SW480, HCT-116, HT-29 and Caco2) purchased from the ATCC were cultured in DMEM medium (Gibco, Gaithersburg, MD, USA) which supplemented with 10% FBS and appropriate antibiotics, and maintained in humidifies 5% CO_2 at 37°C. Three kinds of SMC4 siRNAs (si-SMC4#1, si-SMC4#2 and si-SMC4#3) (RIBOBIO, China) were purchased. In the light of the knockdown effect, control siRNA, si-SMC4#1 and si-SMC4#3 were applied in this research. The sequence of si-SMC4#1 and si-SMC4#3 were 5'-CCACAAGAGTAGCATATCA-3' and 5'-GAAAGTCCTTGATGCAATA-3'. COAD cells (SW480 and HCT-116) were transfected with 30nM of siRNA by adding Lipofectamine 3000 (Invitrogen, USA) as stated by the manufacturer's protocol.

2.4 Western blots

Total protein was extracted with RIPA buffer containing a mixture of protease inhibitor and phosphatase inhibitor. Protein samples were separated on SDS-PAGE and transferred to 0.45 um PVDF membranes (Millipore). After blocking with 5% fat-free milk, incubated at 4°C with the primary antibody overnight and then with horseradish peroxydase-coupled secondary antibody (Millipore). Enhanced chemiluminescence (Millipore) was used to detect signal.

2.5 Reagents

Antibodies against β-Actin, E-cadherin, Vimentin, Snail and were purchased from Cell Signaling Technology (Boston, USA). Antibody against SMC4 was purchased from Proteintech (Wuhan, China), MMP-2, MMP-9, Beclin-1 and LC3-II were purchased from Cell Signaling Technology (Boston, USA).

2.6 Immunohistochemistry (IHC)

For immunohistochemical studies, tissues were deparaffinized using xylene, while rehydrated in graded alcohol washes. Antigen retrieval was performed by placing the slides in sodium citrate buffer at 80°C for 40 min. After rinsing with PBS three times, the endogenous peroxidase was blocked by 3% H_2O_2 for 15 min at room temperature (RT). The slides were subsequently incubated with the indicated antibody (1:200 dilution) at RT 4°C overnight followed by incubating with the secondary antibody at for 1 h, DAB was immunostained and hematoxylin was counterstained.

2.7 Evaluation of IHC staining

The SMC4 staining of COAD tissue sections was judged by a couple-scoring system, combined staining intensity and area extent. The intensity of staining was graded as below: 0 meant no obvious staining; 1 meant weak staining; 2 meant moderate staining; 3 meant strong staining. The area extent was graded as below (proportion of positive cells): none or < 5% positive cells: 0; 5%-25% positive cells: 1; 26%-50% positive cells: 2; >50% positive cells: 3. The total scores were divided into low or high expression groups via multiplying the intensity score and the ratio of positive cells score. 0 ~ 1 was negative (-), 2 ~ 4 was weak positive (+), 5 ~ 7 was moderate positive (++), and ≥ 8 was strong positive (+++). Tissue sections scored as '++' and '+++' were considered as strong positives (high-level expression) of SMC4. Two pathologists scored all the specimens in a blinded manner. Final score established for cases with discrepancies was determined by reassessment of both pathologists with a double-headed microscope.

2.9 Colony formation assay

Cells (2000 cells) were seeded in 6-well plates in triplicate and incubated. After 2 weeks, cells were fixed by 4% paraformaldehyde and stained with hematoxylin for 20 min. Then, stained cells were washed with PBS for 30 min. Colonies (containing > 50 cells) were counted directly and images were captured.

2.10 MTT assay

Approximately 2000 cells per well were seeded into 96-well plates, then added the MTT (100 μL per well) at 0, 24, 48 and 72 h respectively. Removed the MTT from the wells after 4 h and added 100 μL DMSO into each well. The relative number of viable cells was assessed by measuring absorbance.

2.11 Wound healing assay

Cells were seeded in a 6-well plate and cultured overnight. A sterile 200 μL pipette tips were used to create wounds through the monolayer, then used pre-warmed PBS washed plates to remove cellular debris. Cell migration was monitored at 0 h, 48 h respectively and images were captured using microscope.

2.12 Acridine 0range (AO) staining

Cells were stained with 1 μg/mL^{-1} of AO (Macklin, Shanghai, China) for 15 min and washed with PBS at RT in the dark. The formation of acidic vesicular organelles (AVOs) was determined under a fluorescence microscope (AO, bright red fluorescence in acidic vesicles).

2.13 Immunofluorescence staining

Adherent cells in 6-well plates culture slides fixed with 4% paraformaldehyde for 15 min, 0.5% Triton X-100 (CWBIO, China) was used to permeabilize and blocked with 3% BSA for 2 h. Cells were incubated with primary antibody (Beclin-1 and LC3-II) in 3% BSA at 4°C overnight, PBS washed for three times, incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG secondary antibody (Invitrogen) for 1 h, nuclei were stained with DAPI solution and then captured by fluorescence microscope (Leica SP5II).

2.14 Statistical analysis

The data analysis was performed by SPSS 25.0 software and GraphPad Prism 8.0 software. T-test for independent means were used for group comparisons. Kaplan-Meier analysis calculated the survival curves. Univariate and multivariate hazard ratios of study variables were examined by Cox proportional hazards regression model. Group comparisons for continuous data were done by Mann-Whitney U test or one-way ANOVA. Biochemical experiments were performed in triplicate and at least three independent experiments were evaluated. The value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 SMC4 was highly expressed in COAD tumor tissues

We analyzed the Timer (https://cistrome.shinyapps.io/timer/) and Human Protein Atlas (HPA) databases (https://www.proteinatlas.org) and found that SMC4 expression levels were elevated in a variety of tumor subsets, especially in COAD (Fig. 1A and 1B). To investigate the role of SMC4 in the development of COAD, we interrogated publicly available data and discovered that the mRNA expression of SMC4 was markedly increased in COAD tissues from GEO DataSet (GDS4515/201664_at) and the Cancer Genome Atlas (TCGA) database ($P < 0.001$) (Fig. 1C and 1D). Moreover, SMC4 expression in patients with late

stage and lymph node (LN) metastasis was higher than early stage and no nodal metastasis in database (Fig. 1E and 1F). We then detected SMC4 in 87 adjacent non-tumor tissues and 94 COAD tissues by IHC (Fig. 1G). IHC staining showed that the positive and strongly positive rates of adjacent non-tumor colonic mucosa tissues were only 29.89% (16/87) and 12.64% (11/87), but significantly increased in COAD tissues (88.30%, 83/94; 53.19%, 49/94) ($P < 0.01$) (Fig. 1H). Subsequently, we observed the association between expression of SMC4 and certain clinical parameters. As summarized in forest plot (Fig. 1I), SMC4 overexpression was significantly correlated to Grade 3 ($P = 0.000$), late stage (III + IV) ($P = 0.001$) and LN metastasis ($P = 0.043$), but no significance with age, gender, tumor size and tumor invasion depth. These observations suggested that SMC4 might play a critical role in initiation and progression of COAD.

3.2 SMC4 was strongly associated with poor prognosis in COAD

Next, we further investigated the prognostic value of SMC4 expression in COAD. We analyzed the data of 94 patients by Kaplan-Meier, the result revealed that OS (Fig. 2A) was correlated with expression of SMC4 ($P = 0.000$), the higher level of SMC4 expression meant poorer prognosis than lower level of SMC4 expression. Especially, compared with negative HSDL2 expression patients, high HSDL2 expression patients with differentiation status ($P = 0.020$, $P = 0.001$), early clinical stage ($P = 0.000$), TNM (T3 + T4) ($P = 0.000$), and LN (-) ($P = 0.000$) had significantly curtailed OS (Fig. 2B-F). Forest plots showed the results of univariate logistic regression analysis (Fig. 2G), we observed that the high-level expression of SMC4 related to COAD patients' stage and LN metastasis. Moreover, the analysis of Multivariate Cox confirmed that SMC4 expression could be a significant independent prognostic marker in COAD (Fig. 2H). Therefore, we could arrive at the result that SMC4 may become the considerable prognosis indicator.

3.3 Depletion of SMC4 reduced the proliferation, colonization and induced autophagy of COAD cells

To elucidate the biological function of SMC4 in COAD, we firstly detected the protein expression of SMC4 in COAD cell lines (SW480, SW620, HCT-116, HT-29 and Caco2) and found that SMC4 was upregulated in SW480 and HCT-116 cells (Fig. 3A). According above result, SW480 and HCT-116 were transfected with non-targeting siRNA as si-Control (si-Con) or three different SMC4-specific lentiviral si-RNAs, SMC4 expression was distinctly suppressed in si-SMC4#1 and si-SMC4#3 groups compared to si-Con groups by Western blots (Fig. 3B). Subsequently, we analyzed the effect of SMC4 on the proliferation of cells by MTT assay. The results showed that silencing of SMC4 significantly inhibited the growth of COAD cells (Fig. 3C). In the colony forming experiment, it was found that the SMC4 depletion decreased the colonization in COAD cells (Fig. 3D). In addition, EDU assay showed that the percentage of EDU positive cells reduced in COAD cells with down-regulation of SMC4 expression (Fig. 3E). Therefore, these *in vitro* studies showed that SMC4 depletion impaired the proliferation and colonization of COAD cells.

Under different conditions, autophagy could enhance cell survival or increase cell death. To confirm the affection of SMC4 on autophagy in COAD cells, AO staining was performed and showed orange-colored autophagosomes were observed following the depletion of SMC4 (Fig. 3F), indicated that SMC4 could

induced COAD cells generated an autophagic response. We further evaluated the effect of SMC4 on the expression of LC3-II and Beclin-1 which related to autophagic process (Fig. 3G). The expression of LC3-II and Beclin-1 was activated and upregulated in SMC4 depletion cells. Immunofluorescence staining further verified our findings (Fig. 3H). These results revealed that SMC4 could induce COAD cells autophagy.

3.4 Suppression of SMC4 inhibits the migration of COAD through EMT

According to the results of IHC analysis in Fig. 1E, a positive correlation between SMC4 levels and LN metastasis was shown obviously (Fig. 1E), so we began to investigate whether SMC4 affects COAD cell metastasis. Through the wound healing and Transwell experiments, we found that the down-regulation of SMC4 expression level led to the inhibition of wound healing and the significantly decrease of migration ability *in vitro* (Fig. 4A, 4B). Studies have shown that EMT promotes the metastatic potential of cancer cells. Therefore, we examined the potential effect of SMC4 on EMT related markers. Western blots results showed that the expression of E-cadherin increased, but the Vimentin, Snail and MMP2, MMP9 expression decreased in the SMC4 silenced cells. These results indicate that the high level of SMC4 reduces the COAD metastatic process by activating EMT.

3.5 Bioinformatic analysis of SMC4 related genes and pathways

To gain the insight of SMC4 biological meaning in COAD, the function module of LinkedOmics was used to examine SMC4 co-expression mode in COAD cohort. As Volcano plot shown in Fig. 5A, SMC4 connected with 6422 genes, 3,293 genes (dark red dots) were shown significant positive correlations with SMC4, whereas 3,129 genes (dark green dots) were shown significant negative correlations (false discovery rate, FDR < 0.01). The top 10 significant genes positively and negatively correlated with SMC4 were shown in the sequence map (Fig. 5B). The results of KEGG pathway enrichment and GO analysis (biological process, cellular component and molecular function) were shown in Fig. 5C-5G, SMC4 was connected with kinetochore organization, cell cycle, metabolic process and other process. These results provide the basis for our follow-up studies.

4. Discussion

The SMCs complex is mainly composed of a dimer of ring SMC protein and each SMC molecule is composed of 1000–1500 amino acids [13]. There is a hinge domain in the middle, two double helix domains at both ends and spherical domains of Walker A and Walker B motifs at the ends. SMC1 binds to SMC3 to form a dimer, which simultaneously binds to SCC/SCC3 to form a chromatin agglutinin complex (Conhesion). The Conhesion mainly consists of SMC2 and SMC4 dimer, and leads to genomic instability and abnormal gene regulation by interrupting the near chromatin binding [14].

As the core subunit of Conhesion, SMC4 was highly expressed in tumor tissues and the overexpression of SMC4 is closely related to tumor proliferation, metastasis and poor prognosis of patients with cancer [9]. In the p53 network, researches confirmed SMC4 expression might affect chromosomal stability through p53 pathway [15]. A previous study suggested that downregulation of SMC4 could reduce cell proliferation and resulted in severe defects in chromosome assembly in HeLa cells [16]. Zhou *et al*. reported that the SMC4 expression was correlated with liver cancer progression including tumor differentiation late stage and vascular invasion [10]. In prostate cancer, higher expression of SMC4 is significantly associated with the metastatic cascade and poor prognosis [17]. In present study, analysis by database and IHC scores showed that SMC4 was overexpression in COAD tissues and related to tumor differentiation, clinical stage, lymph node metastasis and shortened OS. Similar trend was either found in glioma and lung cancer to support our findings. Moreover, patients with poor differentiation of SMC4 overexpression had a shortened OS. Therefore, SMC4 expression level can predict patients' prognosis with COAD, and has the potential to be an efficient tool for the appropriate management of personalized therapy.

Cancer cell proliferation and migration underlay development and metastatic dissemination, which were major problems in cancers [18]. SMC4 was related to the tumorigenesis and development of cancers by regulating cancer cell growth and metastasis. Zheng *et al*. found that low microRNA-124-5p expression correlated with poor prognosis and promoted colorectal cancer cell proliferation via targeting SMC4 [19]. In glioma cells, SMC4 overexpression markedly promoted the proliferation rate, migration and invasive capability of cells *in vitro* and *in vivo*. Therefore, we considered that SMC4 may involves in the cell growth and migration in COAD. To confirm this hypothesis, we firstly performed colony formation, MTT, EdU and wound healing assays, the results verified that SMC4 depletion could reduce the cancer cell proliferation, colonization and migration. Moreover, with a high propensity to metastasize cancer, the EMT process was critical in cancers [20]. We observed the change of EMT markers in COAD cells transfected with or without SMC4. Western blots presented that SMC4 significantly down-regulated the expression level of epithelial markers, while upregulated mesenchymal markers, accelerating COAD metastasis. These results revealed that SMC4 promoted COAD migration by EMT progression and provide the preliminary molecular mechanisms underlying the role of SMC4 in COAD metastasis.

In all, we identified that overexpression of SMC4 is significantly related to COAD patients' stage, LN metastasis and poor prognosis. We showed that upregulation of SMC4 facilitates proliferation, migration and EMT of COAD cells. Hence, these results provided a novel biomarker for the prediction of COAD prognosis and the establishment of targeted therapies.

Abbreviations

SMC4: structural maintenance of chromosomes 4; COAD: colon adenocarcinoma; GC: gastric carcinoma; EMT: epithelial-to-mesenchymal transition; LN: lymph node; OS: overall survival

Declarations

Ethic Statement: our study adhered with the Helsinki Declaration and was ratified by the Human Ethics Committee. Patients were acquainted that the excised samples stored by the hospital were possibly utilized to identify information/images (if applicable) for scientific researches and publication, and their privacies were guaranteed to be protected. Retrospectively collection of subsequent survival data through medical record analysis.

Consent for publication: All the included patients signed informed consent for publication.

Availability of data and materials: the datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: all authors conceived, planned and carried out the experiments that led to the paper. All authors wrote the paper, and reviewed successive versions. All authors read and approved the final manuscript.

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Figures

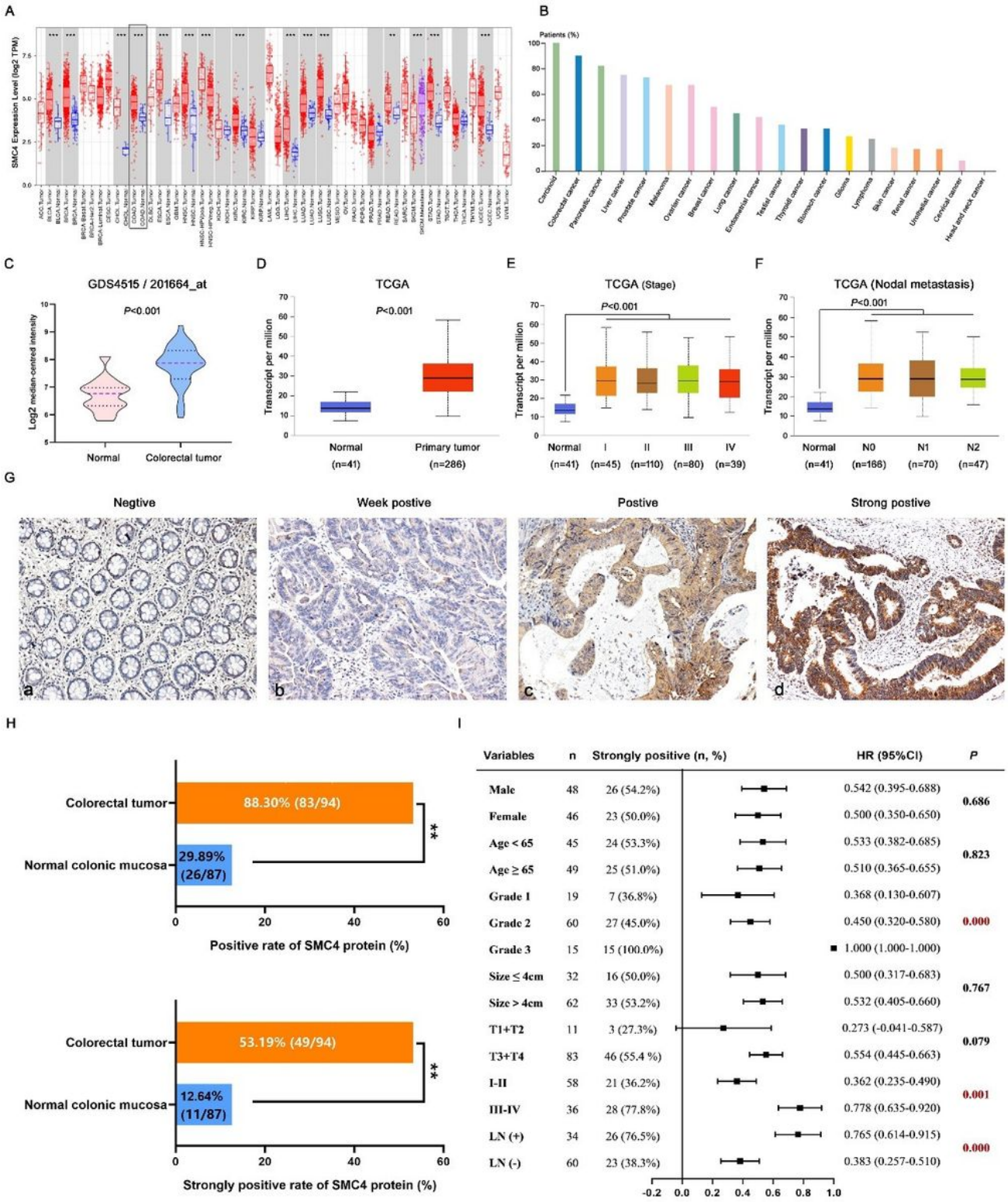

Figure 1

SMC4 expression was up-regulated in COAD. (A, B) Expression of SMC4 in pan-cancers from Timer and HPA database. (C, D) Box plots derived from gene expression data in GEO DataSet (GDS4515/201664_at) (C) and TCGA (D) comparing the mRNA expression of SMC4 in normal and COAD tissue (P<0.001). (E, F) SMC4 expression in normal, different stages (I, II, III, IV) (E) and with or without nodal metastasis (F) patients' tissues in TCGA database. (G) SMC4 expression in adjacent non-tumour tissues (a) and COAD

tissues (b, c, d) as examined by IHC (×200). Representative examples of SMC4 staining were shown. (H) Statistical results of IHC that SMC4 protein expression positive and strongly positive staining rates in adjacent normal colonic mucosa tissues and COAD tissues. (I) Correction between SMC4 expression and clinicopathological significance of COAD.

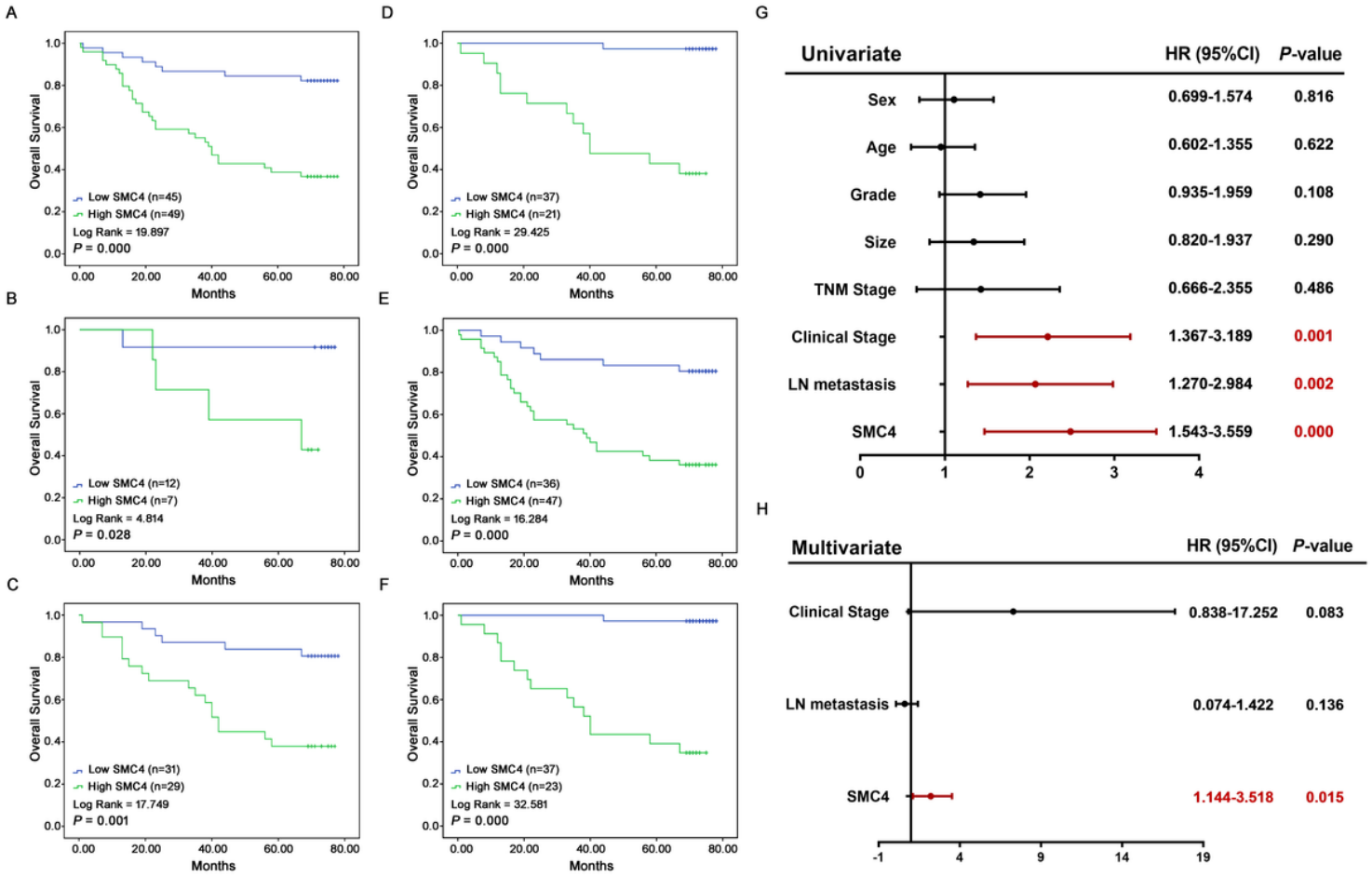

Figure 2

The correlation between SMC4 expression and prognosis of COAD patients. (A) Overall survival rates of COAD patients in different expression level of SMC4 analyzed by Kaplan-Meier. (B) Overall survival rates of COAD patients with Grade 1 with different expression level of SMC4. (C) Overall survival rates of COAD patients with Grade 2 in relation to SMC4 expression. (D) Overall survival rates of COAD patients with Stage I-II in relation to SMC4 expression. (E) Overall survival rates of COAD patients with TNM (T3+T4) in relation to SMC4 expression. (F) Overall survival rates of COAD patients with LN (-) in relation to SMC4 expression. (G, H) Forest plots showed the results of univariate (G) and multivariable (H) logistic regression analysis.

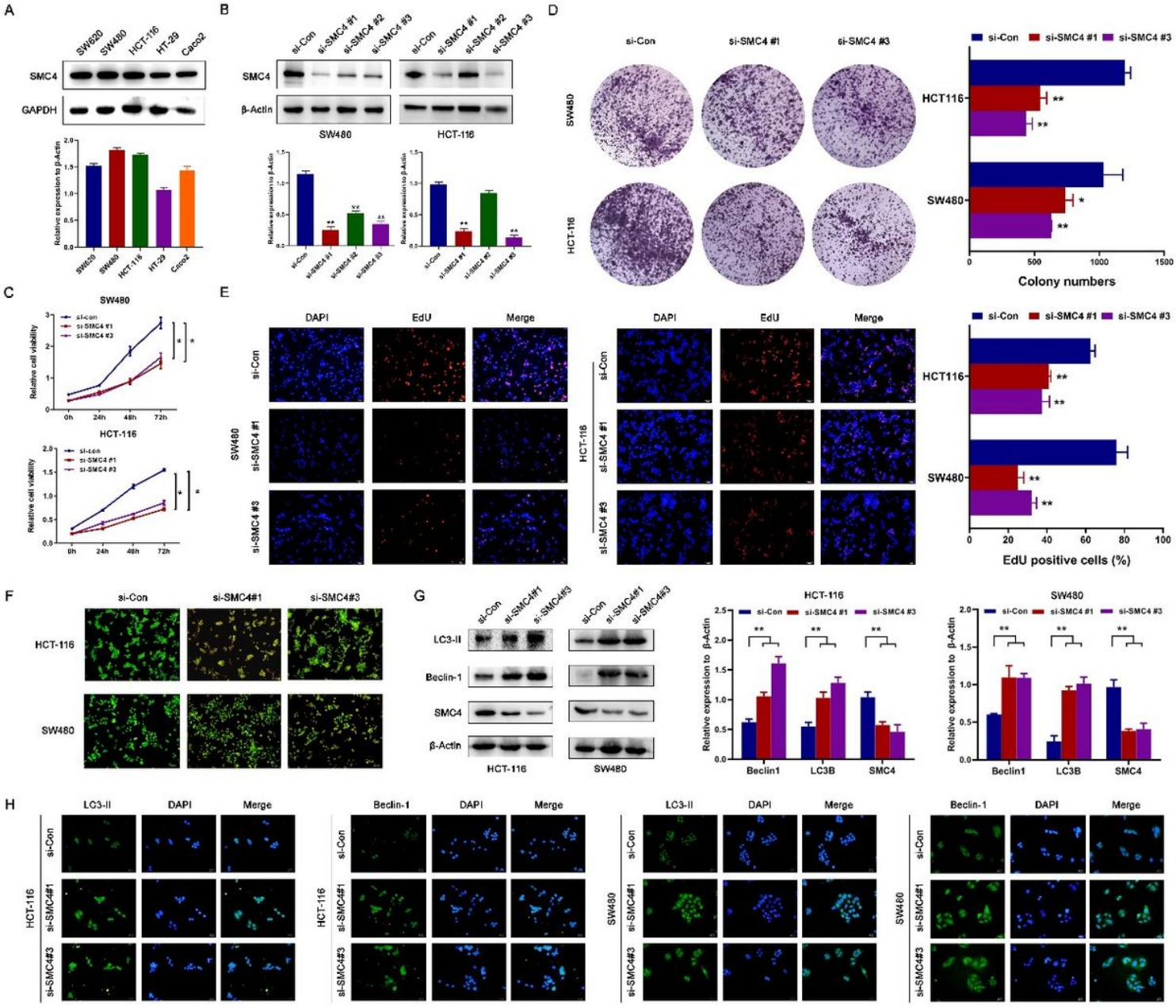

Figure 3

SMC4 was related to proliferation, colonization and autophagy of COAD cells. (A) Western blots confirmed the protein expression of SMC4 in COAD cells. β-Actin was used as a loading control. (B). Different si-SMC4 sequences transfection efficiency was detected by Western blots. β-Actin was used as a loading control. (C-E) Cell proliferation was examined by MTT (C), colony formation (D) and EdU (×100) (E) in the constructed cells. (F) The formation of autophagosomes was evaluated by AO staining. Magnification: 200×. (G) Autophagic process markers LC3-II and Beclin-1 were confirmed by Western blot in the constructed COAD cells. (H) Immunofluorescence staining of LC3-II and Beclin-1. Magnification: 400×. Data were represented as mean ± standard error of mean of at least three independent experiments, *P<0.05; **P<0.01.

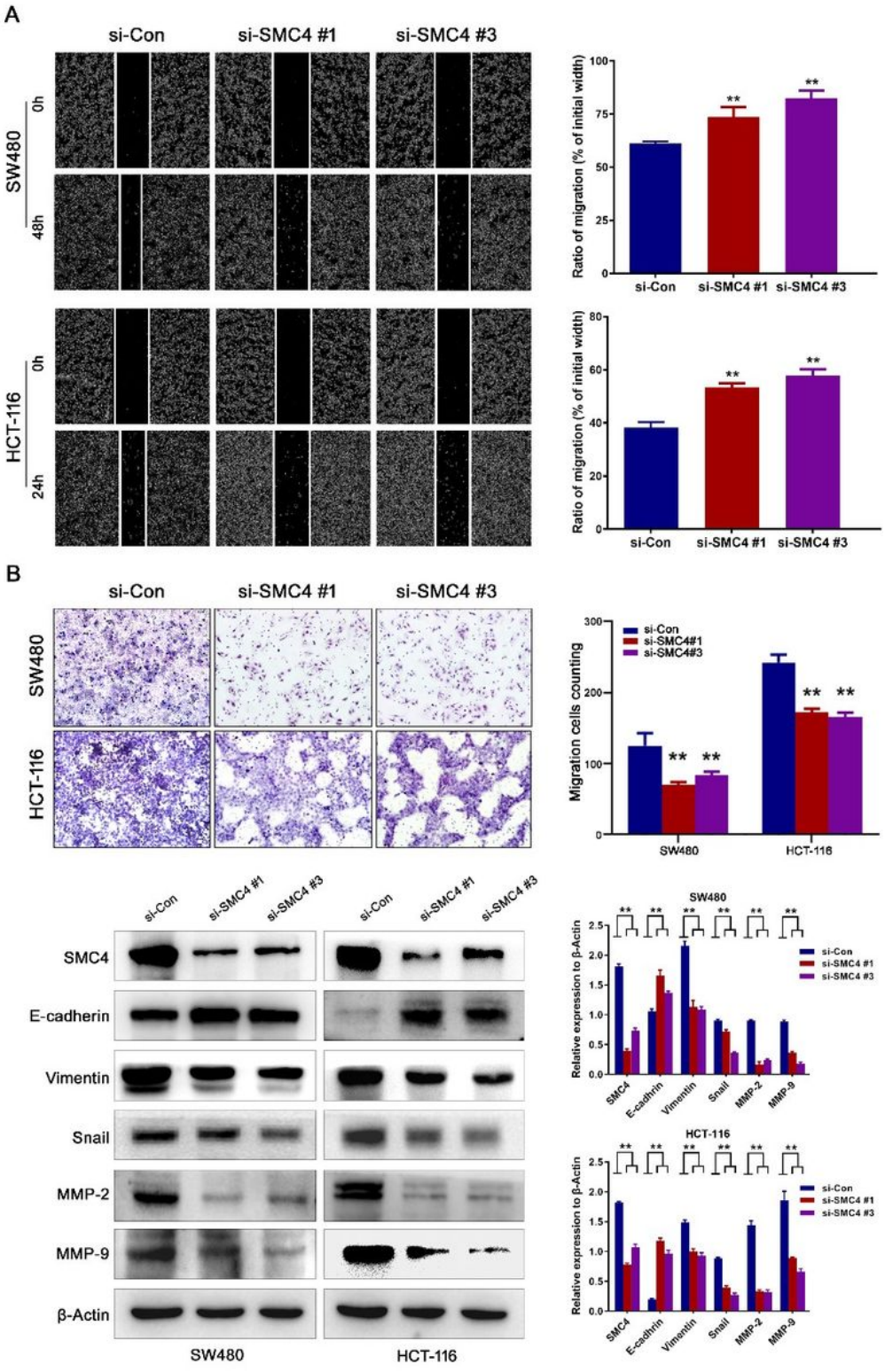

Figure 4

SMC4 boosted the process of COAD cells metastasis. (A) The rate of migration into scratched area in the constructed cells detected by wound healing. (B) Migration assay showed the effect of SMC4 expression on migratory ability of COAD cells. (C) Analysis the effect of SMC4 differential expression by Western blots on EMT markers (E-cadherin, Vimentin, Snail, MMP-2, MMP9) in COAD cells. β-Actin was used as a loading control.

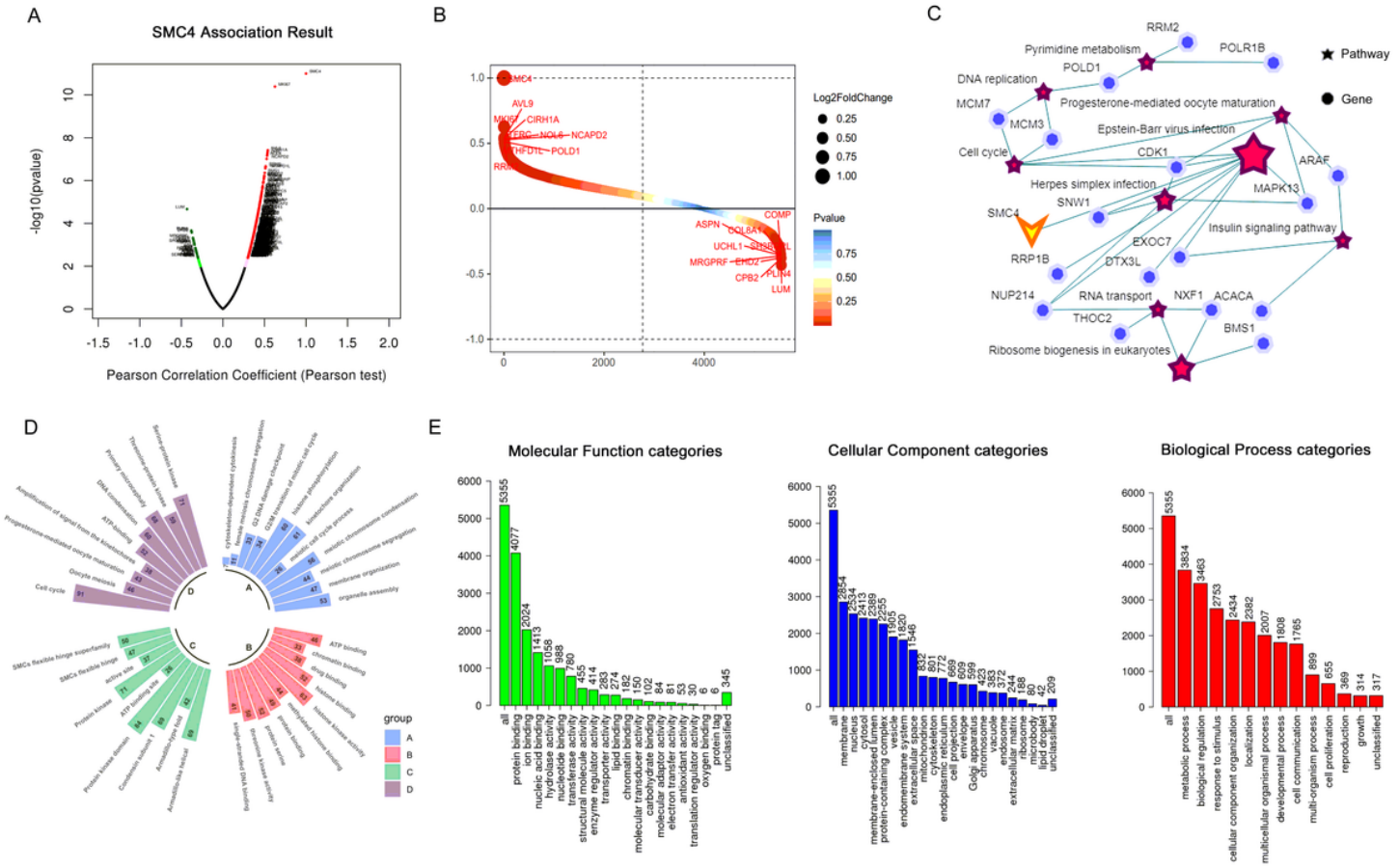

Figure 5

Bioinformatic analysis of SMC4 related genes and pathways. (A) The corrected genes of SMC4. Dark red dots were on behalf of positive genes and dark green dots were on behalf of negative genes (FDR<0.01). (B) Sequence map to show the top 10 of positive and negative related genes of SMC4. (C) Analysis of KEGG pathway showed the genes and pathways related to SMC4. (D) Classification for SMC4 related process and functions. A: biological process; B: molecular function; C: protein domains; D: pathways. (E) KEGG pathway enrichment and GO analysis (biological process, cellular component and molecular function).