Prognostic Value and Biological Function of LRRN4 in Colorectal Cancer

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Abstract

Background: Several nervous and nerve-related biomarkers have been detected in colorectal cancer (CRC) and can contribute to the progression of CRC. However, the role of leucine-rich repeat neuronal 4 (LRRN4), a recently identified neurogenic marker, in CRC remains unclear.

Methods: We examined the expression and the clinical outcomes of LRRN4 in CRC from TCGA-COREAD mRNA-sequencing datasets and immunohistochemistry on the Chinese cohort. Furthermore, the colony formation, flow cytometry, wound healing assay and mouse xenograft models were used to investigate the biological significance of LRRN4 in CRC cell lines with LRRN4 knockdown or overexpression in vitro and in vivo. In addition, weighted co-expression network analysis and DAVID were used to explore the potential molecular mechanism.

Results: We provide the first evidence that LRRN4 expression, at both the protein and mRNA level, was remarkable high in CRC compared to controls and positively correlated with the clinical outcome of CRC patients. Specifically, LRRN4 was an independent prognostic factor for progression-free survival and overall survival in CRC patients. Further functional experiments showed that LRRN4 promoted cell proliferation, cell DNA synthesis and cell migration and inhibited apoptosis. Knockdown of LRRN4 can correspondingly decrease these effects in vitro and can significantly suppress the growth of xenografts. Several biological functions and signaling pathways were regulated by LRRN4, including proteoglycans in cancer, glutamatergic synapse, Ras, MAPK and PI3K.

Conclusions: Our results suggest that LRRN4 could be a biological and molecular determinant to stratify CRC patients into distinct risk categories, and mechanistically, this is likely attributable to LRRN4 in regulating several malignant phenotypes of neoplastic cells via cancer-related pathways.

Background

Tumor progression is a complex and dynamic process of interaction between cancer cells and tumor microenvironment[1]. Nevertheless, in the last decade, the role of the nervous system, as a crucial component of the tumor microenvironment, has gained much attention in different tumor types including colorectal cancer (CRC)[2]. The intestine is highly innervated, both from outside the intestines and the enteric nervous system and increasing evidence has suggested the role of nervous system as a contributor in CRC[3].

Nowadays, detecting perineural invasion has been applied to assess risk stratification in CRC patients and is thought to be a sign of tumor metastasis and invasion and a portent of poor prognosis of patients[4, 5]. Although no direct evidence supported the specific bi-directional communication between neurons and cancer cells of CRC, it should exist in theory[6]. Recent studies have indicated that not only cancer cells can stimulate the growth of nerve fibers by secreting neurotrophic factors, but nerve fibers can also infiltrate the tumor microenvironment, stimulating tumor growth and cancer cell dissemination[7]. Meanwhile, several neurogenic biomarkers were significantly associated with

clinicopathological features of CRC patients and even take part in carcinogenesis and progression of CRC, such as NDRG4, ADRB2, NPY, GABA and et al[8–11]. In our previous study, we had demonstrated that CRC stem cells can generate sympathetic and parasympathetic neurons to comprise the nervous system of CRC tissues. When the expression of neural marker MAP2 was silenced in human CRC cells, the growth of xenograft tumors was correspondingly inhibited in mouse models, indicating the importance of neurogenic markers in CRC[7]. Although previous studies indicated that the expression of neurogenic molecules in cancer cells is indeed important in CRC, exploring more neurogenic factors is still needed to further understand the crosstalk between cancer cells and neural cells in CRC.

Leucine-rich repeat neuronal 4 (LRRN4), a novel member of the LRRN protein family, was first identified in 2005[12]. It is expressed in various regions of the central neural system, especially hippocampus. LRRN4 plays an important role in hippocampus-dependent long-lasting memory[12]. Besides, LRRN4 has been detected in dorsal root ganglion neurons of adult mice and is closely related to the development of dorsal root ganglion[13, 14]. Previous studies also demonstrated that LRRN4 is expressed in various non-neuronal tissues including lung, ovary and heart[12, 15]. Apart from the functions of LRRN4 in normal tissue and benign disease, it is also involved in cancers. Recently, a structural alteration of LRRN4 was found in high hyperdiploid acute lymphoblastic leukemia with relapse[16]. LRRN4 has been identified as a marker of primary mesothelial cells, while it was found as either non-detectable or downregulated in mesothelioma[17]. Although LRRN4 was not highly expressed in normal colon tissue, the level of LRRN4 was much lower in CRC tissue, as detected by the coupling methods of hydroxyapatite chromatography and SDS-PAGE followed by mass spectrometry analysis[18]. However, the clinical significance of LRRN4 remains to be clarified by investigation in a larger sample. What's more, the functions of LRRN4 are largely unknown.

In this study, we investigated the clinical impact of LRRN4 expression in CRC samples from The Cancer Genome Atlas (TCGA)-COREAD cohort and Chinese CRC cohort. Then, the effects of LRRN4 on cell proliferation, cell cycle, apoptosis and migration were investigated in CRC cells. Furthermore, the xenograft model was utilized to explore whether LRRN4 impacts the xenograft tumor growth in vivo. Finally, we explored the potential molecular mechanism by weighted co-expression network analysis (WGCNA) and Database for Annotation, Visualization and Integrated Discovery (DAVID).

Methods

Patients

Expression of LRRN4 and clinicopathological features of TCGA-COREAD patients were obtained from UCSC Xena Browser (https://xenabrowser.net/), 376 primary colorectal cancer tissues and 51 normal mucosal tissues were included. The clinicopathological features including age, gender, overall survival (OS) status, progression-free survival (PFS), microsatellite instability (MSI) status, tumor location, histological types, pathologic stages, lymphatic invasion, perineural invasion, venous invasions and adjuvant chemoradiotherapy.

Expression of LRRN4 and clinicopathological features of 81 primary colorectal cancer tissues and corresponding distant normal mucosal tissues were obtained from West China Hospital, Sichuan University. Informed consent was obtained from all patients. The protocol conformed to the Declaration of Helsinki and sll tests were approved by the Institutional Review Board of West China Hospital, Sichuan University. The clinicopathological features were obtained from patient medical records, including age, gender, tumor location, histological types, pathologic stages, grade, adjuvant chemoradiotherapy. Patients were followed up to obtain the information of OS and PFS status.

Immunohistochemistry

Immunostaining analysis of LRRN4 was performed as described in our previous study[19]. The primary anti-LRRN4-antibody (Abcam, UK, ab133372) was used at a 1:200 dilution and the DAB kit (GeneTech, China) was used following the protocol. All slides were scored by two independent investigators. X-tile was used to generate the optimal cut-off score with low LRRN4 expression and high LRRN4 expression.

Cell culture and transduction

The CRC cell lines Caco2 (SCSP-5027) were obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Science, SW480 (CCL-228^{TM}), HCT-116 (CCL-247^{TM}) and LoVo (CCL-229^{TM}) were obtained from the American type culture collection. These cells were grown in Dulbecco's Modified Eagle's medium (DMEM; HyClone, USA) supplemented with 10% fetal bovine serum (GEMINI, USA) and 1% penicillin-streptomycin mixture (HyClone, USA). All cells were cultured at 37 ℃ and in a humidified atmosphere of air containing 5% CO_2. STR profling and mycoplasma contamination were performed to keep the authenticity of cell line on regular basis.

Knockdown and overexpression of LRRN4 were achieved by lentiviral transduction. The sequence of LRRN4 was obtained from National Center for Biotechnology Information. The lentiviruses containing LRRN4 small hairpin RNA (shRNA) for knockdown were constructed by GeneChem (China). The plasmid for overexpression of LRRN4 was constructed by Sangon Biotech (China) and lentiviruses for overexpression of LRRN4 were produced and tittered as described elsewhere.

RNA extraction and real-time quantitative PCR

Total RNA of CRC cells was extracted with Trizol reagent (Molecular Research Center, USA), according to the manufacturer’s instructions. After confirming the RNA quality, cDNA was synthesized using PrimeScript RT Reagent Kit (Takara Bio, Japan). Gene expression differences were detected by real-time quantitative PCR, using SYBR Green Master Mixture (Roche, Switzerland). The primers are 5’-CTTGCTTCTGTCGCCACACAC-3’ (forward) and 5’-AGGAGCCAAGACAAGTCACA-3’ (reverse). The data were normalized to the expression of the housekeeping gene GAPDH. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Colony formation assay

For colony formation assay, cells (500 cells per well) were incubated in 6-well plates and allowed to grow until the colony can be recognized. Cells were washed with PBS three times and fixed using 4% paraformaldehyde for 30 min followed by staining with crystal violet at room temperature for 30 min. The plates were then rinsed with distilled water and dried before the count. Colonies containing more than 50 cells in each well were counted.

Flow cytometry

EdU (5-ethynyl-20-deoxyuridine) incorporation assay was performed using EdU assay kit (YF®647A Click-iT EdU Imaging Kits; US Everbright, China) following the manufacturer's guidelines. Briefly, cells were incubated with 10 μM EdU and subsequently fixed in 4% paraformaldehyde. After EdU staining, cell nuclei were stained with DAPI the cell proliferation was detected by BD FACS Canto^{TM} System (BD Biosciences, USA).

DNA content analysis was detected using cell cycle analysis kit (Sangon Biotech, China) following the manufacturer's guidelines. Briefly, cells were dissociated by trypsin and fixed with chilled 70% ethanol overnight. The staining working fluid of propidium iodide (PI) and RNase A was used to stain DNA for 30 min. Cells were washed and filtered through a 40 μm cell strainer before flow cytometry. Cell cycle distributions were then analyzed by BD Accuri^{TM} C6 Plus flow cytometer (BD Biosciences, USA).

Cell apoptosis was detected using YF®647A-Annexin V and PI Apoptosis Kit (US Everbright, China) following the manufacturer’s instruction was followed except that the cell nuclei staining dye was changed from PI (supplied with the kit) to DAPI (Beyotime, China). After staining, the activity of Annexin V/DAPI was then examined using BD FACS Canto^{TM} System (BD Biosciences, USA).

Wound healing assay

Cells were seeded into six-well plate for adherent culture. When cells reached 80% confluence, 200 μl pipette tips were used to make a thin wound. Then the detached cells were washed off twice and incubated with DMEM basic medium. After that, the images were acquired at 0, 24 and 48h after wounding using a phase-contrast microscope. The relative wound healing closure was calculated by measuring the area of the gap at 0, 24 and 48 h.

Xenograft model

Male severe combined immune deficiency (SCID) mice, 4-6 weeks old, were purchased from the Beijing Vital River Laboratory Animal Technology (China) and housed under pathogen-free conditions. Animals studies was approved by the Institutional Review Board of West China Hospital, Sichuan University. 3×10^6 Caco2 cells were suspended with PBS and mixed with Matrigel (Corning, USA) at a 5:2 ratio. Then, the cells were injected subcutaneously into the right flank of each mouse. The large diameter and small diameter of tumors were monitored twice a week. The tumor volumes were calculated by the formula $(\pi)/6 \times (\text{large diameter}) \times (\text{small diameter})^2$. When the established criteria for end-point were reached, mice were anesthetized according to the 2020 AVMA Guidelines on Euthanasia state. In short, the mice

were anesthetized by intraperitoneal injection of 0.1 mL of 1% phenobarbital sodium, and then the tumors were dissected and weighted.

WGCNA and DAVID

The R package WGNCA was performed for the enrichment of co-expression network of LRRN4 as previously described[20]. In short, genes with log2 (RSEM>1) from TCGA-COREAD were included in the network. A power of 3 was selected to compute into a Topological Overlap Matrix, with over 50 genes for a dendrogram, using the function Topological Overlap Matrix similarity. Similar modules were merged at a cutoff (0.25) to obtain moderately large and distinct modules. Then, the correlations among gene expression modules and clinical traits were calculated using the module-trait relationships of WGCNA. The tumor location, MSI status, histological type, pathological stage, lymphatic invasion, perineural invasion, venous invasion, preoperative CEA were chosen as clinical traits. In addition, the association of gene significance (GS) and module membership (MM) was also assessed how close the significance of gene expression is to the magenta module. Finally, to further explore the biofunction of LRRN4 by bioinformatic, Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were employed using DAVID database. GO annotation included cellular component (CC), biological process (BP) and molecular function (MF) terms. A p value cutoff of 0.05 was used for significant enrichment.

Statistical analysis

All statistical analyses were performed using R statistical software (version 4.0.5), SPSS software (version 27.0) and GraphPad Prism software (version 7.00). LRRN4 expression values were RSEM-normalized and shown as log2 values, analyzed in UCSC Xena Browser. The χ^2 tests method was used to determine the difference in expression of LRRN4 among normal mucosal and tumor, as well as the relationship of the LRRN4 expression in CRC with clinicopathological features. Kaplan-Meier analyses and log-rank tests were used to estimate the survival curves. Univariate and multivariate analyses were employed to establish a Cox proportional hazard regression model alone or after adjusting for clinical variables. In the analyses of results obtained from experiments in cells, the data were presented as the mean ± SD of at least three independent assays. Statistical comparisons between groups were performed using the unpaired Students t-test or the Mann-Whitney U test. A *p*-value less than 0.05 was considered statistically significant.

Results

LRRN4 is highly expressed in CRC and correlates with some clinicopathological features

To investigate the potential significance of LRRN4 in CRC patients, the relationship between LRRN4 expression and clinicopathological characteristics was analyzed. As shown in Fig. 1, the expression level of LRRN4 is much higher in CRC compared to normal colorectal tissue ($p < 0.001$). Moreover, the LRRN4

expression was statistically different in different pathological stage (p = 0.037), lymphatic invasion (p = 0.029), patients' progression-free survival (PFS) status (p = 0.009), and overall survival (OS) status (p = 0.002). No statistical difference was observed in the rest of the clinicopathological parameters, including age, gender, location, microsatellite MSI status, histological types, perineural invasion and venous invasion.

High LRRN4 mRNA expression is an independent prognostic factor for poor PFS and OS in CRC patients

To investigate the potential survival significance of the expression LRRN4, we performed Kaplan-Meier survival analysis. The patients first were classified into low- and high-LRRN4 expression groups using X-tile plots to generate the optimal cut-off score. The survival analysis showed that high LRRN4 expression was associated with poor PFS (Fig. 2A, p = 0.001) and OS (Fig. 2B, p = 0.030).

To further assess whether the LRRN4 expression could independently predict PFS and OS in CRC patients, both univariate and multivariate Cox regression analyses were performed by adjusting for gender, age, stage, MSI status, lymphatic invasion, venous invasion and adjuvant chemoradiotherapy as covariates. Univariate analyses showed that high LRRN4 expression was significantly associated with poor PFS (Table 1, p = 0.015) and OS (Table 2, p = 0.005). In multivariate analyses, high expression of LRRN4 remained a strong prognostic value for PFS (Table 1, HR = 1.797, 95%CI = 1.009-3.200, p = 0.047) and OS (Table 2, HR = 1.733, 95%CI = 1.014-2.961, p = 0.044), even after adjusting for other covariates, indicating its potential prognostic values for PFS and OS in CRC patients.

Table 1
Univariate and multivariate Cox proportional hazards regression analyses for PFS of LRRN4 and clinical features

Variables	Uni-variable Cox			Multi-variable Cox		
	HR	95%CI	*p* value	HR	95%CI	*p* value
LRRN4 (High *vs.* Low)	1.992	1.143-3.469	**0.015**	1.797	1.009-3.200	**0.047**
MSI(High *vs.* Low)	0.677	0.316-1.448	0.314	1.209	0.524-2.792	0.656
Age	1.346	0.753-2.407	0.316	1.020	0.997-1.044	0.092
Gender (Male *vs.* Female)	1.348	0.772-2.354	0.294	1.118	0.619-2.020	0.712
Histological type (Non-mucinous *vs.* Mucinous)	0.394	0.121-1.278	0.121	0.425	0.128-1.410	0.162
Pathologic stage (III/IV *vs.* I/II)	2.900	1.620-5.192	**0.000**	1.707	0.814-3.578	0.157
Lymphatic invasion (Yes *vs.* No)	2.348	1.346-4.095	**0.003**	1.067	0.423-2.690	0.890
Perineural invasion (Yes *vs.* No)	1.932	1.093-3.418	**0.024**	1.268	0.637-2.522	0.499
Venius invasion (Yes *vs.* No)	2.045	1.170-3.575	**0.012**	1.215	0.523-2.824	0.650
Adjuvant chemoradiotherapy (No *vs.*Yes)	3.540	1.184-6.652	**0.000**	2.781	1.289-6.000	**0.009**

HR, hazard ratio, *CI*, confdence interval
p value below 0.05 was considered signifcant and highlighted (bold)

Table 2
Univariate and multivariate Cox proportional hazards regression analyses for OS of LRRN4 and clinical features

Variables	Uni-variable Cox			Multi-variable Cox		
	HR	95%CI	*p* value	HR	95%CI	*p* value
LRRN4 (High *vs.* Low)	2.063	1.247-3.413	**0.005**	1.733	1.014-2.961	**0.044**
MSI (High *vs.* Low)	0.913	0.463-1.801	0.792	0.957	0.449-2.039	0.909
Age	1.032	1.011-1.053	**0.002**	1.035	1.012-1.059	**0.003**
Gender (Male *vs.* Female)	1.667	0.993-2.798	0.053	1.541	0.898-2.644	0.116
Histological type (Mucinous *vs.* Non-mucinous)	1.239	0.588-2.611	0.573	1.254	0.570-2.760	0.574
Pathologic stage (III/IV *vs.* I/II)	2.914	1.723-4.927	**0.000**	4.691	2.333-9.431	**0.000**
Lymphatic invasion (Yes *vs.* No)	2.025	1.214-3.377	**0.007**	0.633	0.268-1.496	0.298
Venius invasion (No *vs.* Yes)	0.990	0.597-1.640	0.968	2.180	0.949-5.005	0.066
Adjuvant chemoradiotherapy (No *vs.* Yes)	2.481	1.487-4.138	**0.001**	0.540	0.291-1.001	0.051

HR, hazard ratio, *CI*, confdence interval
p value below 0.05 was considered signifcant and highlighted (bold)

The prognostic significance of LRRN4 for CRC patients is validated in protein level

Having found the significance of the expression of LRRN4 mRNA in CRC prognosis, we further investigated the expression of LRRN4 protein in samples from 81 CRC patients by immunohistochemistry (Fig. 3A, 3B). We first analyzed the expression levels of LRRN4 on colorectal cancer and normal mucosal tissues. Consistent with the results of the TCGA data analysis, there were more patients (83%) with high-LRRN4 expression in CRC compared to normal colorectal tissue (39%) (Fig. 3C, p 0.0001). Then the correlation of LRRN4 expression and clinical characteristics in colorectal cancer patients was analyzed. The results showed that high LRRN4 expression was statistically related to pathological stage (Table 3, p = 0.001), lymph nodes metastasis (Table 3, p = 0.006) and OS status (Table 3, p = 0.001). Next, we performed survival analysis and found high LRRN4 expression was correlated with poor PFS (Fig. S1, p =

0.129) and OS (Fig. 3D, $p = 0.0003$), although no statistical significance was found in PFS. Univariate and multivariate Cox regression analyses were further performed by adjusting for gender, age, location, histological type, pathologic stage, grade and adjuvant chemoradiotherapy as covariates. In univariate analysis, high LRRN4 expression was significantly associated with poor OS (Table 4, $p = 0.001$). In multivariate analysis, LRRN4 remained a strong prognostic value for OS (Table 4, HR = 3.999, 95%CI = 1.564-10.224, $p = 0.004$), indicating the significance of LRRN4 expression for outcomes in CRC patients.

Table 3
The Correlation of LRRN4 expression and Clinical Characteristics in CRC Patients

Characteristics	LRRN4-Low (%)	LRRN4-High (%)	*p* value
Gender			
Male	17(53.1)	27(55.1)	1.000
Female	15(46.9)	22(44.9)	
Age			
≤65	15(46.9)	32(65.3)	0.113
>65	17(53.1)	17(34.7)	
Overall survival status			
Alive	24(75.0)	17(34.7)	**0.001**
Death	8(25.0)	32(65.3)	
Progression-free survival status			
Progression-free	20(62.5)	15(45.5)	0.216
Progression	12(37.5)	18(54.5)	
Location			
Colon	20(62.5)	37(75.5)	0.225
Rectum	12(37.5)	12(24.5)	
Histological type			
Mucinous	9(29.0)	16(32.7)	0.808
Non-mucinous	22(71.0)	33(67.3)	
Pathologic stage-T			
T 1/2	6(18.8)	5(10.2)	0.328
T 3/4	26(81.3)	44(89.8)	
Pathologic stage-N			
N0	21(65.6)	16(32.7)	**0.006**
N1/2	11(34.4)	33(67.3)	
Pathologic stage-M			

HR, hazard ratio, *CI*, confdence interval
p value below 0.05 was considered signifcant and highlighted (bold)

Characteristics	LRRN4-Low (%)	LRRN4-High (%)	*p* value
M0	31(96.9)	42(85.7)	0.100
M1	1(3.1)	7(14.3)	
Pathologic stage			
Stage I	5(15.6)	3(6.1)	**0.006**
Stage II	16(50.0)	10(20.4)	
Stage III	10(31.3)	29(59.2)	
Stage IV	1(3.1)	7(14.3)	
Adjuvant chemoradiotherapy			
No	9(28.1)	19(38.8)	0.351
Yes	23(71.9)	30(61.2)	
Differentiation			
Low/Middle	29(93.5)	42(89.4)	0.527
High	2(6.5)	5(10.6)	

HR, hazard ratio, *CI*, confdence interval
p value below 0.05 was considered signifcant and highlighted (bold)

Table 4
Univariate and multivariate Cox proportional hazards regression analyses for OS of LRRN4 and clinical features

Variables	Uni-variable Cox			Multi-variable Cox		
	HR	95%CI	p value	HR	95%CI	p value
LRRN4 (High *vs.* Low)	3.773	1.735-8.206	**0.001**	3.999	1.564-10.224	**0.004**
Age	1.012	0.987-1.037	0.351	1.018	0.990-1.047	0.219
Gender (Female *vs.* Male)	0.892	0.478-1.664	0.720	0.867	0.433-1.738	0.687
Location(Rectum *vs.* Colon)	0.749	0.366-1.532	0.428	1.189	0.527-2.679	0.677
Histological type (Non-mucinous *vs.* Mucinous)	0.528	0.280-0.995	**0.048**	0.496	0.229-1.075	0.076
Pathologic stage (III/IV *vs.* I/II)	2.099	1.066-4.132	**0.032**	1.357	0.596-3.093	0.467
Adjuvant chemoradiotherapy(Yes *vs.* No)	0.705	0.374-1.328	0.279	0.865	0.422-1.777	0.694
Differentiation(Low/Middle *vs.*High)	0.883	0.271-2.876	0.837	0.559	0.156-2.000	0.371

HR, hazard ratio, *CI*, confdence interval
p value below 0.05 was considered signifcant and highlighted (bold)

LRRN4 promotes cell proliferation in colorectal cancer cell lines

In light of our above results in CRC patients suggesting that LRRN4 was closely correlated with stage and prognosis of CRC, thus we further conduct cell experimental to explore the potential biological function of LRRN4 in CRC cells. We initially investigated the LRRN4 expression in CRC cell lines (Caco2, SW480, HCT-116, and LoVo, Fig. 4A). LRRN4 was highly expressed in Caco2 and SW480 compared to in HCT-116 and LoVo cell lines. Therefore, we generated sublines of Caco2 and SW480 silencing LRRN4, which were infected with lentivirus expressing two different LRRN4-shRNA (Caco2-kd1 and Caco2-kd2; SW480-kd1 and SW480-kd2) and corresponding control (Caco2-scr, SW480-scr), respectively. Meanwhile, HCT-116 and LoVo were infected with lentivirus overexpressing LRRN4 (HCT-116-OE and LoVo-OE) and corresponding control (HCT-116-EV and LoVo-EV) respectively. The knockdown of LRRN4 in Caco2, SW480 and overexpression of LRRN4 in HCT-116 and LoVo cells were validated by real-time quantitative PCR (Fig. 4B-C).

To determine the role of LRRN4 in cell proliferation, we carried out EdU assay. As shown in Fig. 4D-E, knockdown of LRRN4 significantly suppressed the cell proliferation of Caco2 and SW480 cells (Caco2-kd1, Caco2-kd2, SW480-kd1 and SW480-kd2 vs. Caco2-scr, SW480-scr), while the LRRN4 overexpression promoted the proliferation of HCT-116 and LoVo cells (HCT-116-OE, LoVo-OE vs. HCT-116-EV, LoVo-EV). Moreover, we investigated the capacity of colony formation in the cell lines with different LRRN4 expression. The results showed that knockdown of LRNN4 significantly decreased colony formation rate in CRC cells (Caco2-kd1, Caco2-kd2, SW480-kd1 and SW480-kd2 cells vs. Caco2-scr, SW480-scr) (Fig. 4F). However, an increase in the size and number of colonies was observed in HCT-116 and LoVo cells with LRRN4 overexpression compared to corresponding control cells (HCT-116-EV and LoVo-EV) (Fig. 4G), which further verified the promotive effect of LRRN4 on cell proliferation.

LRRN4 promotes cell DNA synthesis and inhibits apoptosis in CRC cells

The role of LRRN4 in CRC cell proliferation could be regulating the cell cycle or cell apoptosis. Therefore, the cell cycle and apoptosis were analyzed using flow cytometry. The cell cycle distribution analysis showed significantly decreased cell populations in the S phase in both Caco2 and SW480 with knockdown of LRRN4 (Caco2-kd1, Caco2-kd2, SW480-kd1 and SW480-kd2) compared to their corresponding control cells (Caco2-scr, SW480-scr) (Fig. 5A). On the contrary, overexpression of LRRN4 induced a higher percentage of S phase distribution in HCT-116 and LoVo cells compared to their control (Fig. 5B). We next analyzed whether LRRN4 exerted any impacts on cell apoptosis. When compared to their corresponding control, knockdown of LRNN4 exhibited a higher percentage of apoptotic cells in Caco2 and SW480 cells (Fig. 5C). On the contrary, the rates of apoptotic cells were significantly decreased in CRC cells with overexpression of LRRN4 (HCT-116-OE and LoVo-OE) compared to the control cells (HCT-116-EV and LoVo-EV) (Fig. 5D). Taken together, these data indicated that LRRN4 plays a functional role in promoting DNA synthesis and inhibiting cell apoptosis.

LRRN4 accelerates cell migration

To further explain the correlation between LRRN4 and pathological stage in CRC patients from cell level, wound healing assay was employed to evaluate the effect of LRRN4 on cell migration. Knockdown of LRRN4 significantly impaired the migratory capacity of Caco2 and SW480, resulting in impaired wound closure (Fig. 6A) at two different time points (24h and 48h). To confirm the above findings, cell migration was determined in CRC cells with overexpression of LRNN4. The results showed that overexpression of LRRN4 significantly accelerates wound closure compared to control, indicating a promotive effect of LRRN4 on cell migration in HCT-116 and LoVo cells (Fig. 6B).

LRRN4 accelerates xenograft tumor growth in vivo

After having demonstrated the positive impact of LRRN4 on CRC cells in vitro, we sought to confirm its role in a more physiologically relevant in vivo model. A xenograft model was utilized to investigate whether LRRN4 impacts tumor growth in vivo. Cells were injected subcutaneously into SCID mice. As shown in Fig. 7A, the sizes of xenograft tumors of cells with knockdown of LRRN4 (Caco-kd1 and Caco2-

kd2) were much smaller than that of control (Caco2-scr). In the growth curve analysis (Fig. 7B, C), the tumor growth was significantly inhibited by knockdown of LRRN4, indicating the promotion role of LRRN4 in CRC xenograft tumors.

LRRN4 affects several cancer-related biological functions and pathways

To investigate the potential molecular mechanisms of LRRN4 involved in CRC, we first used WGCNA to identify gene co-expression modules and link them to LRRN4 expression. We found significant correlations between module eigengenes and the following traits, including LRRN4 expression, histological type, lymphatic invasion and perineural invasion. The magenta module was positively associated with LRRN4 expression and several clinicopathological phenotypes, which would be further analyzed (Fig. 8A). Next, we calculated the relationship between module membership of magenta module and gene significance. For most traits, strong correlations were observed (LRRN4: cor = 0.27, p = 9.3e−128; histological type: cor = 0.3, p = 1.1e−158; lymphatic invasion: cor = 0.45, p < 1e−200; perineural invasion: cor = 0.43, p < 1e−200), indicating that the genes most representative of the magenta module's overall expression profile were those most strongly related to LRRN4 expression and clinicopathological traits (Fig. 8B).

To attribute biological meaning to the magenta module, we enriched a module significance for the gene ontology and pathway annotations returned from DAVID database. In total, 8 cell components, 13 biological processes, and 5 molecular functions were enriched (Fig. 8C-8E). The enriched biological processes included cell adhesion, response to hypoxia, cell migration and negative regulation of cysteine-type endopeptidase activity involved in apoptotic process, which was linked to malignant features of CRC cells. Microtubule binding, ion channel binding, laminin binding, fibroblast growth factor binding and potassium ion transmembrane transporter activity were highly enriched molecular functions. Meanwhile, the LRRN4-related genes in the magenta module were enriched in several cancer-related pathways, including Ras signaling pathway, Proteoglycans in cancer and Glutamatergic synapse, which are reported to be implicated in tumorigenesis and tumor progression including CRC (Fig. 8F).

Discussion

Increasing evidence has shown that the expression of neurogenic biomarkers in cancer cells plays a functional role in crosstalk between cancer cells and neuronal cells to promote carcinogenesis and progression[4–7]. In this study, we first revealed the correlation of LRRN4 expression, a novel neurogenic marker, with CRC in both mRNA level and protein level. The expression of LRRN4 was high in CRC and was correlated with pathological stage and OS status in the TCGA cohort and our cohort. However, a previous study found that LRRN4 was of low abundance in normal colon tissue and the level of LRRN4 was much lower in CRC tissue, which was detected by coupling methods of hydroxyapatite chromatography and SDS-PAGE followed by mass spectrometry analysis[18]. In fact, in a few cases, we also found that the expression of LRRN4 is higher in normal tissues than in tumors. This heterogeneous

result may be caused by variations in method or sample size. In addition, a high expression level of LRRN4 was associated with poor OS in the TCGA cohort and our cohort. High expression of LRRN4 was statistically associated with poor PFS in the TCGA cohort, while only a trend was found in our cohort, which might be caused by the limited number of patients who developed progression in our cohort. Although the clinical significance of LRRN4 expression in CRC has never been explored before this study, a similar prognostic significance of other members of the LRRN protein family was found in gastric cancer patients[21]. Evidence has shown that members of the LRRN protein family are specifically expressed in nerve tissues and are mainly involved in neuronal development and regeneration[13–15, 21–23]. But, their abnormal expression plays an important role in neurological and non-neurological malignancies, such as neuroblastoma and gastric cancer[21, 24, 25]. Overall, our results suggested the potential functional role of LRRN4 in the progression of CRC and that LRRN4 is of prognostic value, the same as other members of the LRRN family.

Although a few reports demonstrated the differential expression of LRRN4 in primary mesothelioma and CRC patients, the functions of LRRN4 in malignant cells are unclear[17]. To elucidate the role of LRRN4 in the tumorigenesis and progression of CRC, we knocked down or overexpressed LRRN4 in CRC cells. Our results showed that LRRN4 plays a functional role in promoting cell proliferation, DNA synthesis and in inhibition of apoptosis, indicating a function in cancer cells similar to other members of the LRRN family. LRRN1 is involved in the regulation of proliferation and can protect cells from FBS deprivation-induced apoptosis in neuroblastoma cells[24]. Meanwhile, LRRN1 suppresses the apoptosis of gastric cancer cells[21]. Moreover, based on the structural feature of LRRN4, it contains not only leucine-rich repeat domains, but also a fibronectin type III repeat domain, which has been recognized as a cell adhesion molecule in cell migration[12, 21, 26]. Present results further confirm the promoting role of LRRN4 in cell migration and provide experimental evidence to explain the malignant biological behavior that we observed in CRC patients.

Our results in both CRC patients and cells indicated that LRRN4 plays an oncogene role in CRC. We further analyzed the potential mechanism of LRRN4 in CRC malignant phenotype by using the WGCNA and DAVID. Several signaling pathways were found to be regulated by LRRN4, including Ras signaling pathway, the mitogen-activated protein kinases (MAPK), and phosphoinositide-3 kinase (PI3K) pathways, which are known to correlate with the malignant phenotype of cancer cells[27–31]. However, there was no evidence suggesting that LRRN directly regulates these signaling pathways. LRRN3, a LRRN family member, potentiates Ras-MAPK signaling by facilitating internalization of EGF in clathrin-coated vesicles and LRRN4 also is regulated mainly through the Ras-MAPK signaling pathway[23]. Interestingly, crosstalk among these LRRN4-related signaling pathways (Ras, MAPK, and PI3K pathway) has been reported in cancer, indicating a crucial regulatory role of LRRN4 in carcinogenesis and progression[28]. Although we did not further verify the LRRN4-activated cancer pathways by an experimental approach in this study, we provided bioinformatic evidence. In particular, we enriched the LRRN4-related signal network by using WGCNA, which has been thought to be a powerful tool to enrich the gene regulation network[32].

Conclusion

In conclusion, our findings have firstly revealed that LRRN4 was intricately associated with the survival of patients with CRC and was an independent prognostic factor for PFS and OS. The further experiment demonstrated that LRRN4 promotes cell proliferation, DNA synthesis, migration and suppresses apoptosis in CRC cells, which is mainly due to being activated by several crucial cancer-related pathways, including RAS, MAPK and PI3K signaling pathway. Our study highlights the potential of an oncogenic feature of LRRN4, which could be used as promising prognostic and therapeutic targets for CRC.

Abbreviations

CRC
colorectal cancer
LRRN4
leucine-rich repeat neuronal 4
DAVID
Database for Annotation, Visualization and Integrated Discovery
WGCNA
weighted co-expression network analysis
OS
overall survival
PFS
progression-free survival
MSI
microsatellite instability
shRNA
small hairpin RNA
EdU
5-ethynyl-20-deoxyuridine
DMEM
Dulbecco's Modified Eagle's medium
SCID
severe combined immune deficiency
MAPK
mitogen-activated protein kinases
PI3K
phosphoinositide-3 kinase.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Review Board of West China Hospital, Sichuan University, approval No. 2018156A, from 18 September 2018.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

LR, FCW and ZT designed and managed the entire study; XC, CYL, LFW, YJM, LX, HQR, WXL and ZJ performed the experiments, XC and CYL analyzed the data and wrote the main manuscript text; YDJ, LR, FCW and ZT wrote and revised the manuscript; MWT and MXM provided professional advice about the research. All authors read and approved the final manuscript.

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Not applicable.

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Figures

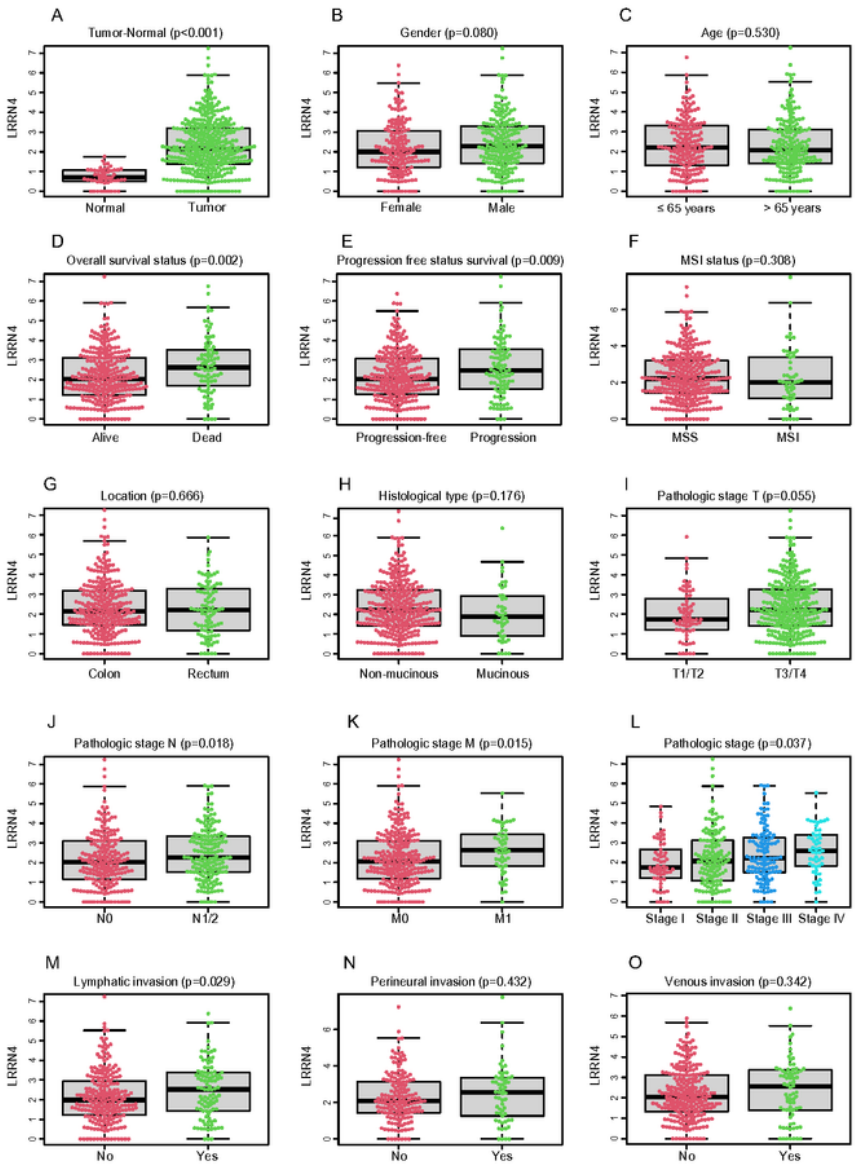

Figure 1

Expression of LRRN in CRC tissue with different clinicopathological characteristics from TCGA-COREAD cohort. (A) Expression of LRRN4 in CRC and normal colorectal tissues. The LRRN4 expression in CRC tissue with different gender (B), age (C), overall survival (OS) status (D), progression-free survival (PFS) status (E), microsatellite instability (MSI) status (F), tumor location (G), histological types (H), pathological T stage (I), pathological N stage (J), pathological M stage (K), pathological stage (L), lymphatic invasion (M), perineural invasion (N) and venous invasion (O)

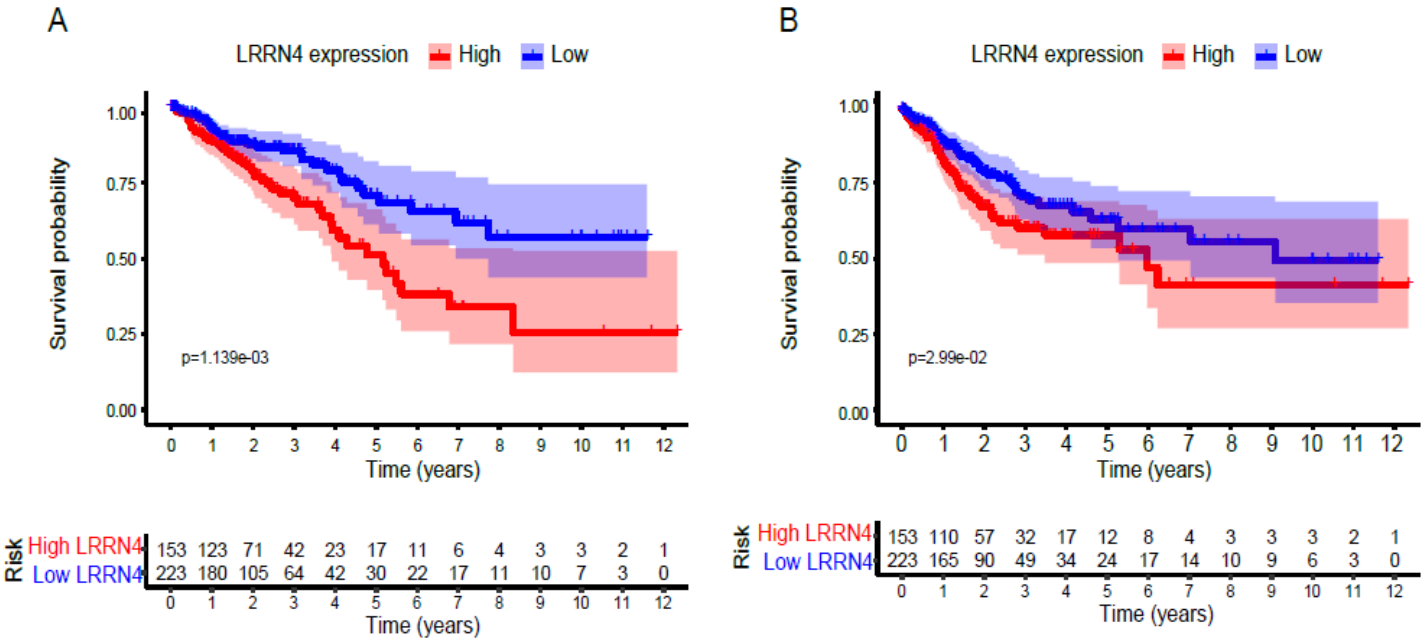

Figure 2

Kaplan-Meier curves of PFS, OS of CRC from TCGA based on the expression of LRRN4. (A) Kaplan-Meier curves of PFS, (B) Kaplan-Meier curves of OS

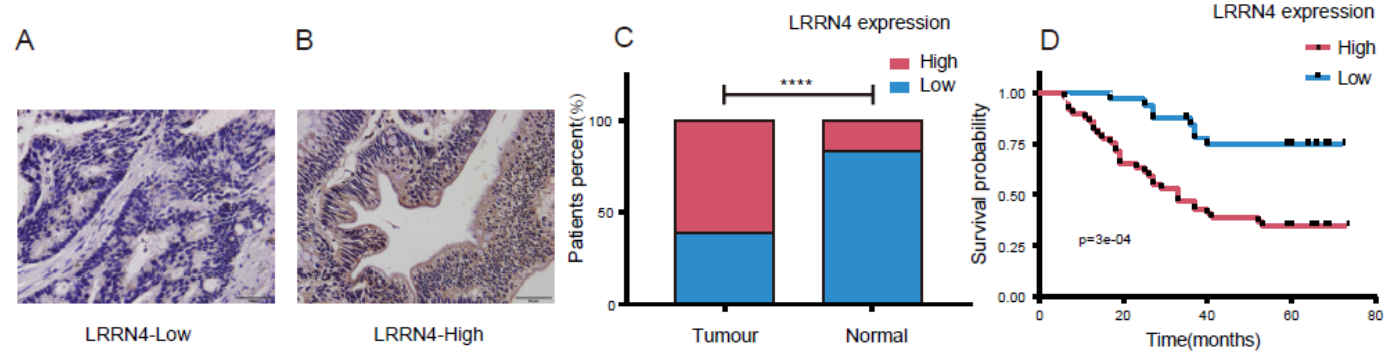

Figure 3

Expression of LRRN4 protein and its clinical significance in CRC patients. The representative IHC images with low expression (A) and high expression (B) of LRRN4 protein. Magnification 400×, scale bars correspond to 50 μm. (C) Expression of LRRN4 in CRC and normal colorectal tissues. (D) Kaplan-Meier curves of OS of CRC based on the expression of LRRN4. **** , $p < 0.0001$

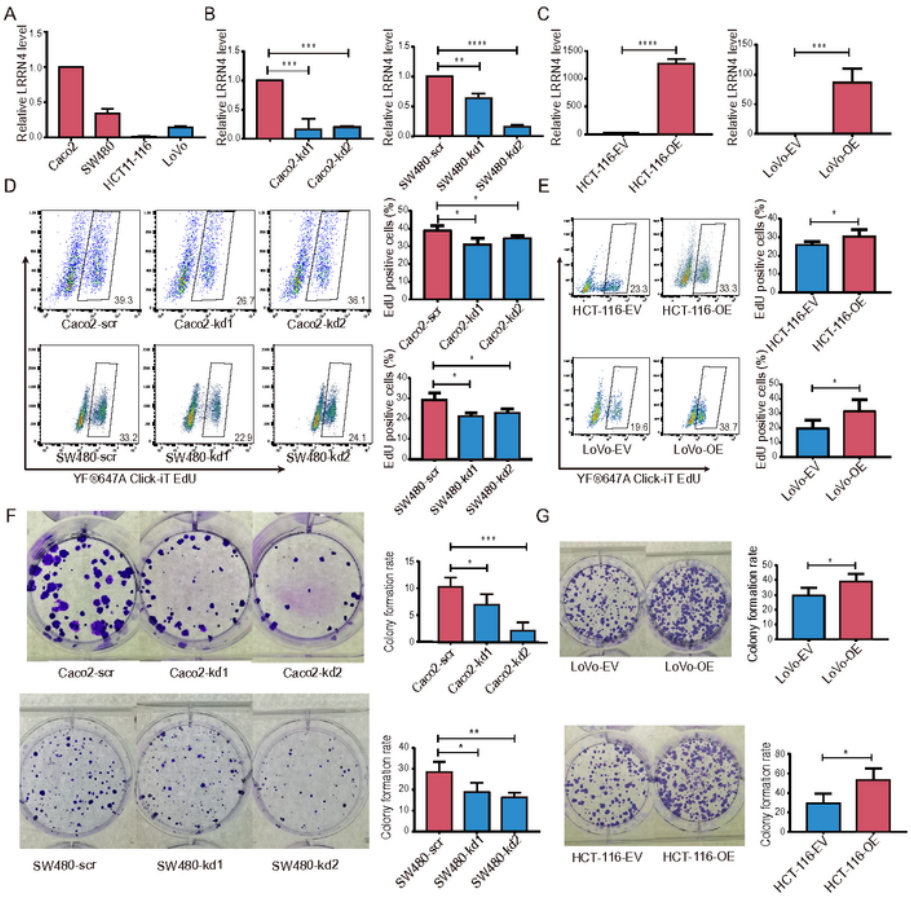

Figure 4

Expression of LRRN4 in CRC cell lines and LRRN4 promotes cell proliferation. (A) Expression of LRRN4 in Caco2, SW480, HCT-116, and LoVo cells. (B) LRRN4 expression in Caco2, SW480 cells with constitutive expression of two different LRRN4 shRNA (kd1 or kd2). (C) LRRN4 expression in HCT-116 and LoVo cells with constitutive express LRRN4 open reading frame (OE). (D) The EdU proliferation assay and quantification in Caco2 and SW480 with knockdown of LRRN4. (E) The EdU proliferation assay in HCT-

116 and LoVo with overexpression of LRRN4. (F) Colony formation assay of cell clonal proliferation ability in Caco2 and SW480 cells with knockdown of LRRN4. (G) Colony formation assay of cell clonal proliferation ability in HCT-116 and LoVo cells with overexpression of LRRN4. Bars represent means with SEM from at least three independent experiments. * , p < 0.05; **, p < 0.01; *** , p < 0.001; **** , p < 0.0001

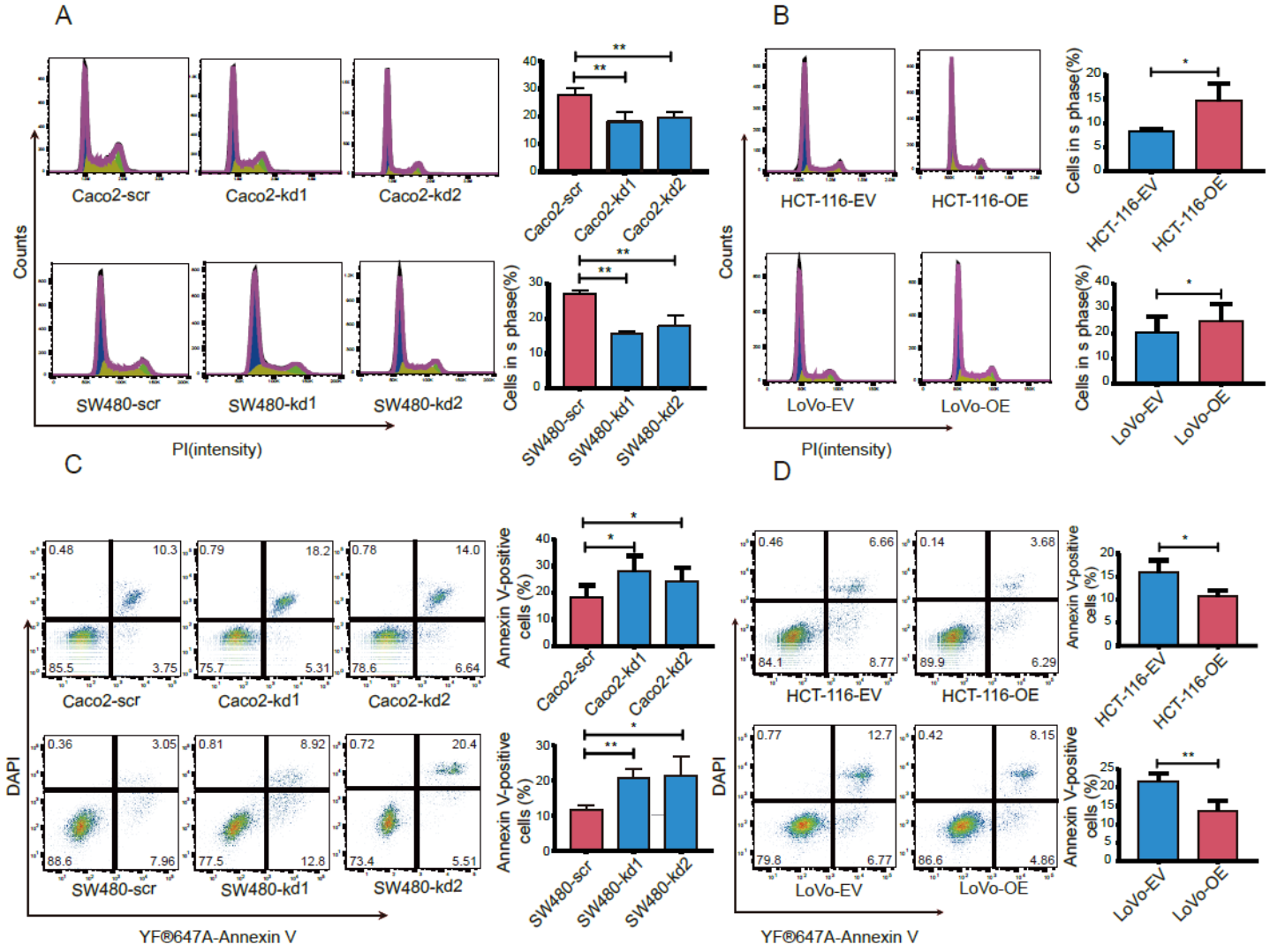

Figure 5

LRRN4 promotes cell DNA synthesis and inhibits apoptosis in CRC cells. (A) Representative histogram of the gated cells in the G0/G1, S and G2/M phases and quantitative analysis of S phase proportion in Caco2 and SW480 cells with knockdown of LRRN4. (B) Representative histogram of the gated cells in the G0/G1, S and G2/M phases and quantitative analysis of S phase proportion in HCT-116 and LoVo cells with overexpression of LRRN4. (C) Representative plots of YF®647A-Annexin V flow cytometry and DAPI staining experiments and quantitative analysis of Annexin V-positive cells in Caco2 and SW480 cells with knockdown of LRRN4. (D) Representative plots of YF®647A-Annexin V flow cytometry and DAPI staining experiments and quantitative analysis of Annexin V-positive cells in HCT-116 and LoVo cells with overexpression of LRRN4. Bars represent means with SEM from at least three independent experiments. * , p < 0.05; ** , p < 0.01

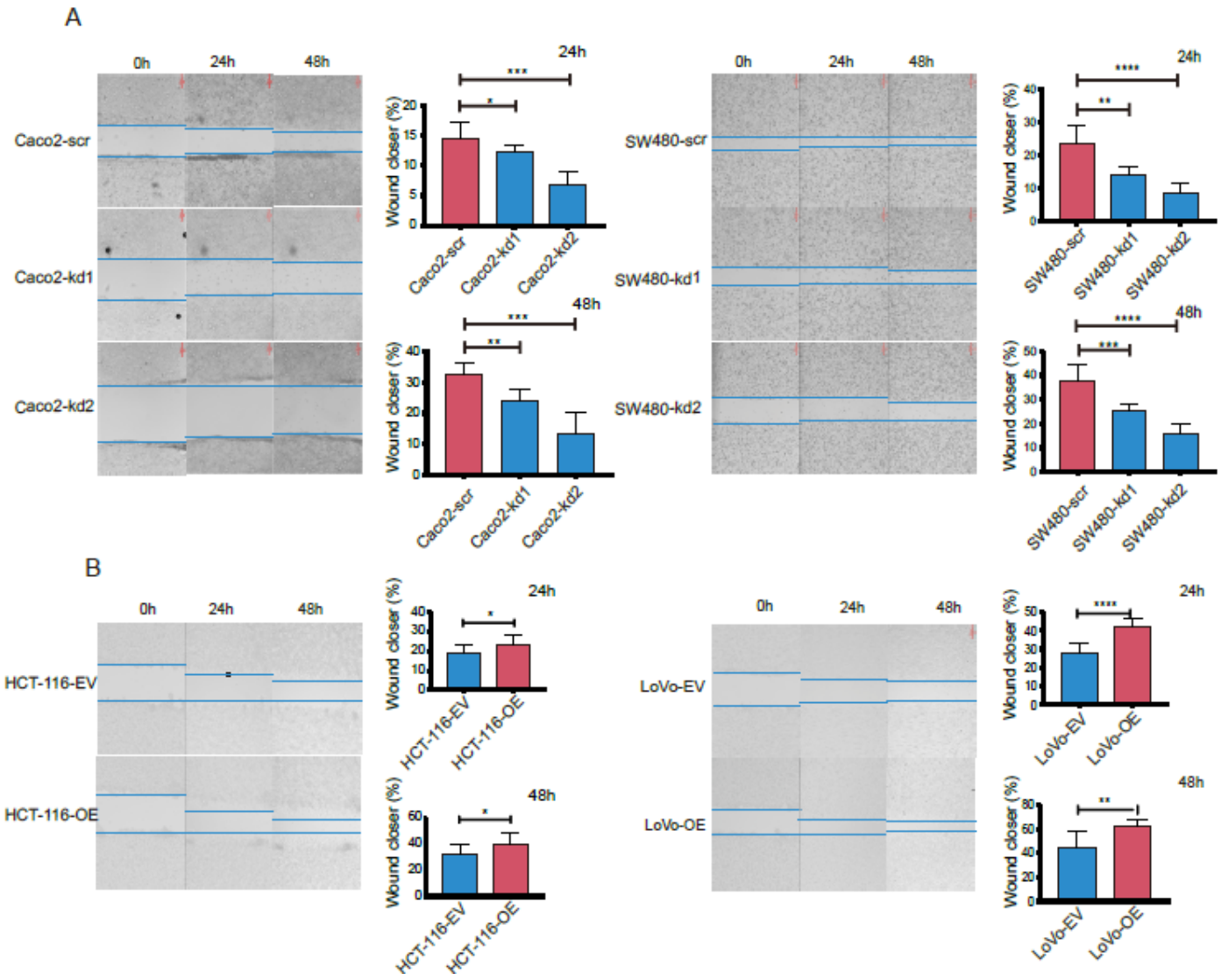

Figure 6

LRRN4 accelerates cell migration. (A) Representative images of scratched monolayer re-epithelialization and quantitative analysis in Caco2 and SW480 cells with knockdown of LRRN4. (B) Representative images of scratched monolayer re-epithelialization and quantitative analysis in HCT-116 and LoVo cells with overexpression of LRRN4. Bars represent means with SEM from at least three independent experiments. * , $p < 0.05$; ** , $p < 0.01$; *** , $p < 0.001$; ****, $p < 0.0001$

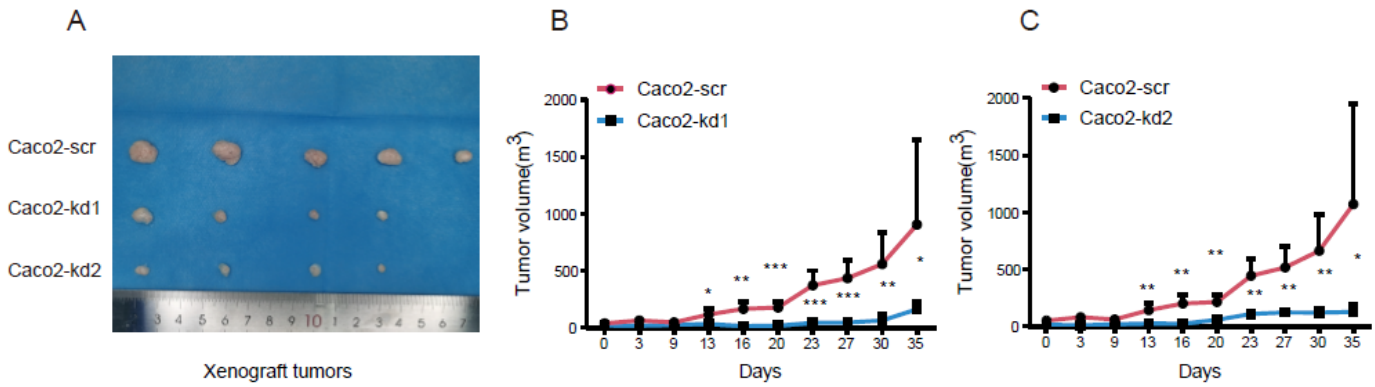

Figure 7

Effects of LRRN4 on Caco2 xenograft tumor growth in SCID mice. (A) Image of harvested xenograft tumors from SCID mice in each group (Caco2-scr, Caco2-kd1 and Caco2-kd2) at necropsy. (B) Tumor volume (mm3) of xenografts with Caco2-scr and Caco-kd1 as measured twice a week. (C) Tumor volume (mm3) of xenografts with Caco2-scr and Caco2-kd2 as measured twice a week. * , $p < 0.05$; ** , $p < 0.01$; *** , $p < 0.001$

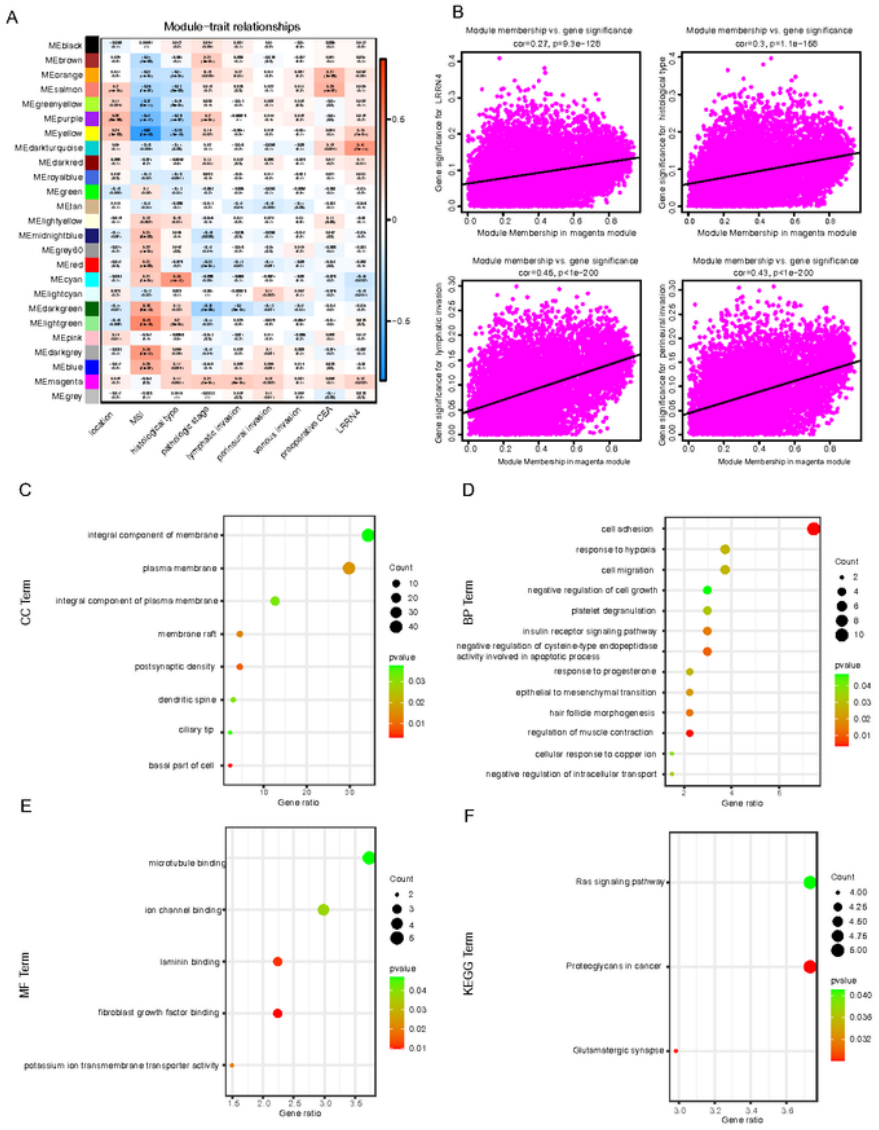

Figure 8

The GO and KEGG pathways of LRRN4-related genes. (A-B) Association between gene expression modules and clinicopathologic characteristics and LRRN4 expression. Pearson's correlation analysis was performed to obtain Pearson (p-value). (C) The cellular component (CC), (D) biological process (BP), (E) molecular function (MF), and (F) the KEGG pathway of LRRN4-related genes in the magenta module, where $p < 0.05$ are displayed

Supplementary Files

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- Fig.S1.pdf