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ORIGINAL RESEARCH

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Suppressing the *MLK3* promotes glutamine metabolism: mechanism and implications in progression of colon cancer

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Abstract

This study was designed to explore the potential role of mixed-lineage protein kinase 3 (*MLK3*) in colorectal cancer (CRC) progression and its relationship with glutamine metabolism. The immunohistochemical staining results of *MLK3* were primarily collected through 100 CRC patients. Wound healing and transwell assays were used to detect migration ability of CRC cells by transfecting cells with *siMlk3*. Gene set variation analysis (GSVA) and Spearman's rank correlation coefficient were used as bioinformatics tools to explore the signaling pathways related to *MLK3*. Western blotting was performed to analyze the downstream of glutamine metabolism. The results suggested an increased expression of *MLK3* in CRC tissues, which was related to adverse clinicopathological characteristics in those CRC patients. Knockdown of *MLK3* inhibited the proliferative and migratory potential of CRCs. Bioinformatics analysis confirmed the relationship between *MLK3* expression and cancer malignancy related signaling pathways. CRC cell lines transfected with *siMlk3* suppressed glutamine metabolism by downregulating the glutamine transporter alanine-serine-cysteine transporter 2 (ASCT2). These results suggested the vital role of *MLK3* in CRC progression, which may be related to the suppression of glutamine transporter, namely alanine, serine, cysteine transporter 2 (ASCT2).

Keywords

Colorectal cancer; *MLK3*; Glutamine metabolism; ASCT2

1. Introduction

Colorectal cancer (CRC) is characterized by high migration capacity and poor prognosis, and is one of the most common cancer types globally [1]. The 5-year relative survival rate for CRC is relatively poor, *i.e.*, approximately 64% from 2009 to 2015 [2]. Therefore, more effective biomarkers are needed for CRC prognosis.

Mixed lineage kinase 3 (*MLK3*), one of the mitogen-activated protein kinases (MAPK) members, has recently attracted attention as a chief adaptor molecule due to its role in inflammation and neurodegenerative diseases [3, 4]. It activates the MAPKs family, including c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), extracellular-signaling regulated kinase (ERK), and p38 MAPK signaling pathways. Also, it can negatively regulate Ras homolog gene family member A (RhoA), and the guanosine triphosphate phosphohydrolase (GTPase), during disease progression [5]. Increased *MLK3* expression has been detected in various human cancers, including breast cancer, pancreatic cancer, as well as ovarian cancer tissues with undesirable outcomes [6–8]. *MLK3* expression was significantly upregulated in breast cancer cell lines as compared to adjacent normal tissues [9]. Schroyer *et al.* [10] suggested a promoting role of *MLK3* in CRC progression under oxidative stress. Notably, *MLK3* is upregulated in various types of cancers and is closely associated with poor prognosis. Based on an immune-competent mouse model, Kumar *et al.* [11] demonstrated that *MLK3* could be a potential therapeutic target in breast cancer by increasing T cell cytotoxicity. However, the role of *MLK3* in CRC progression has not been fully elucidated.

It is widely acknowledged that glutamine metabolism is required for progression of cancer cells based on various biological processes [12], including biosynthesis, antioxidative defense, epigenetic and posttranslational modifications as well as regulation of cell signaling pathways [13]. The intestinal mucosal cells are dependent on glutamine metabolism and rapidly undergo necrosis after glutamine depletion [14]. Interfering the glutamine metabolism is a promising anti-cancer approach [15]. Alanine-serine-cysteine amino acid transporter (ASCT2), encoded by solute carrier family 1, member 5 (SLC1A5) gene, is important for transferring glutamine into the cellular microenvironment [16]. Van *et al.* [17] suggested that knockdown of ASCT2 alone led to the death of breast cancer cells *in vitro* besides

reducing the growth of xenografted cells *in vivo*. In kirsten rat sarcoma viral oncogene (*KRAS)*-mutant CRC, inhibiting the glutamine uptake by blocking ASCT2 suppresses the migration of malignant cells, suggesting ASCT2 as a potential therapeutic target [18]. Therefore, it is important to explore the relationship between *MLK3* and glutamine transporter ASCT2, as targeting *MLK3* could suppress CRC progression partly by reducing glutamine intake.

Current study reports that *MLK3* is highly expressed in CRC and its knockdown could decrease the malignancy of CRC cells by inhibiting the glutamine transporter ASCT2.

2. Materials and methods

2.1 Bioinformatics analysis

Spearman's rank correlation coefficient analysis was used to detect *MLK3* related signaling pathways. RNA-sequencing information was acquired from the cancer genome atlas (TCGA) dataset (https://portal.gdc.cancer.gov/). The enrichment analysis of *MLK3* was performed based on the Kyoto encyclopedia of genes and genomes (KEGG) database (https://www.kegg.jp) and Gene Ontology (GO) database (http://geneontology.org/).

2.2 Collection of clinical samples

Subsequent collecting of fresh or after paraffin-embedding blocks of colorectal tissue was performed. A total of 100 patients enrolled in this study for colorectal cancer (CRC) surgical treatment from April 2008 to December 2008, and with a follow up of 6.6 to 7.2 years until July 2015 (for detailed information is provided in **Supplementary Table 1**).

2.3 Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were prepared for dewaxing, hydration and antigen extraction. After 3,3′-Diaminobenzidine (DAB) staining, tissues were counterstaining with hematoxylin, and sealed with neutral silicone resin. The slides were observed under microscope.

2.4 Cell culturing and transfection procedure

Cell lines (RKO and SW480) were obtained from Chinese Academy of Science and cultured in dulbecco's modified eagle medium (DMEM)/roswell park memorial institute (RPMI)-1640 containing 10% fetal bovine serum (FBS) under humidified conditions with 5% carbon dioxide (CO_2) at 37 °C. *MLK3*-specific siRNA (Zorin, Shanghai, China) was inserted into cells using lipofectamine 3000 (L3000015, Invitrogen, Carlsbad, CA, USA). The *MLK3*-1/-2/-3 siRNA sequences are shown in **Supplementary Table 2**.

2.5 Quantitative real-time PCR

Total RNA was extracted and quantified by the Qiagen RNeasy kit (74106, Qiagen Bioinformatics, Dusseldorf, Germany). Then, cDNA was obtained by the RT Reagent kit (RR037A, Takara Bio Lnc., Shiga, Japan). The primers used are shown in **Supplementary Table 3**. The relative amount of *MLK3* mRNA was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.6 Western blot

Sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) gel (10%), and polyvinylidene difluoride (PVDF) membranes (0.2 μm) were used for electrophoretic separation and transferring samples respectively. After blocking with non-fat milk for 1 h, the membranes were incubated with ASCT2 (1:1000, PA5-88700; Invitrogen) and ACTIN (1:1000, ab181602; Abcam) antibodies overnight. The membranes were then washed and incubated in goat anti-rabbit IgG H&L (HRP) (1:2000, ab7090; Abcam). Samples were detected by enhanced chemiluminescence method (Thermo Fisher Scientific).

2.7 Wound healing assay and transwell assay

When cells grew to 100% confluency, they were scratched by a 20 μL pipette tip, and photographed at 0 and 24 h, respectively. For the transwell migration assay (6.5 mm diameter, 8.0 mm pore size; Corning Inc.), the upper chamber was cultured with serum-free cultured cells, while lower chamber was loaded with 20% FBS medium for 48 h. Cells were enriched with methanol (30 min) and crystal violet for staining. The migrated cells were finally photographed and counted.

2.8 Statistical analysis

Statistical analysis was analyzed by GraphPad Prism 9 software (v9.0.0.121, GraphPad Software, San Diego, CA, USA) and R package. Kaplan-Meier survival curve and log-rank test was used to represent the overall survival rate with CRC patients. Data were analyzed by one-way analysis of variance (ANOVA) and presented as means ± standard deviations (SEM). It was considered statistically significant when $p < 0.05$.

3. Results

3.1 *MLK3* is upregulated in human CRC tissues

Primarily an immunohistochemical study was performed with *MLK3* in collected samples. As compared to the normal subjects, *MLK3* expression is significantly upregulated in colorectal cancer tissues (Fig. 1A), and positive staining of *MLK3* was mostly observed in the nucleus of colorectal cancer. Furthermore, *MLK3* protein level in CRCs was analyzed with various pathological markers. As shown in Fig. 1B, higher grade cancer samples expressed with higher expression of *MLK3*. The Kaplan-Meier survival result (n = 100) suggested that an increased *MLK3* expression indicated an undesirable outcome as well ($p = 0.0015$, Fig. 1C).

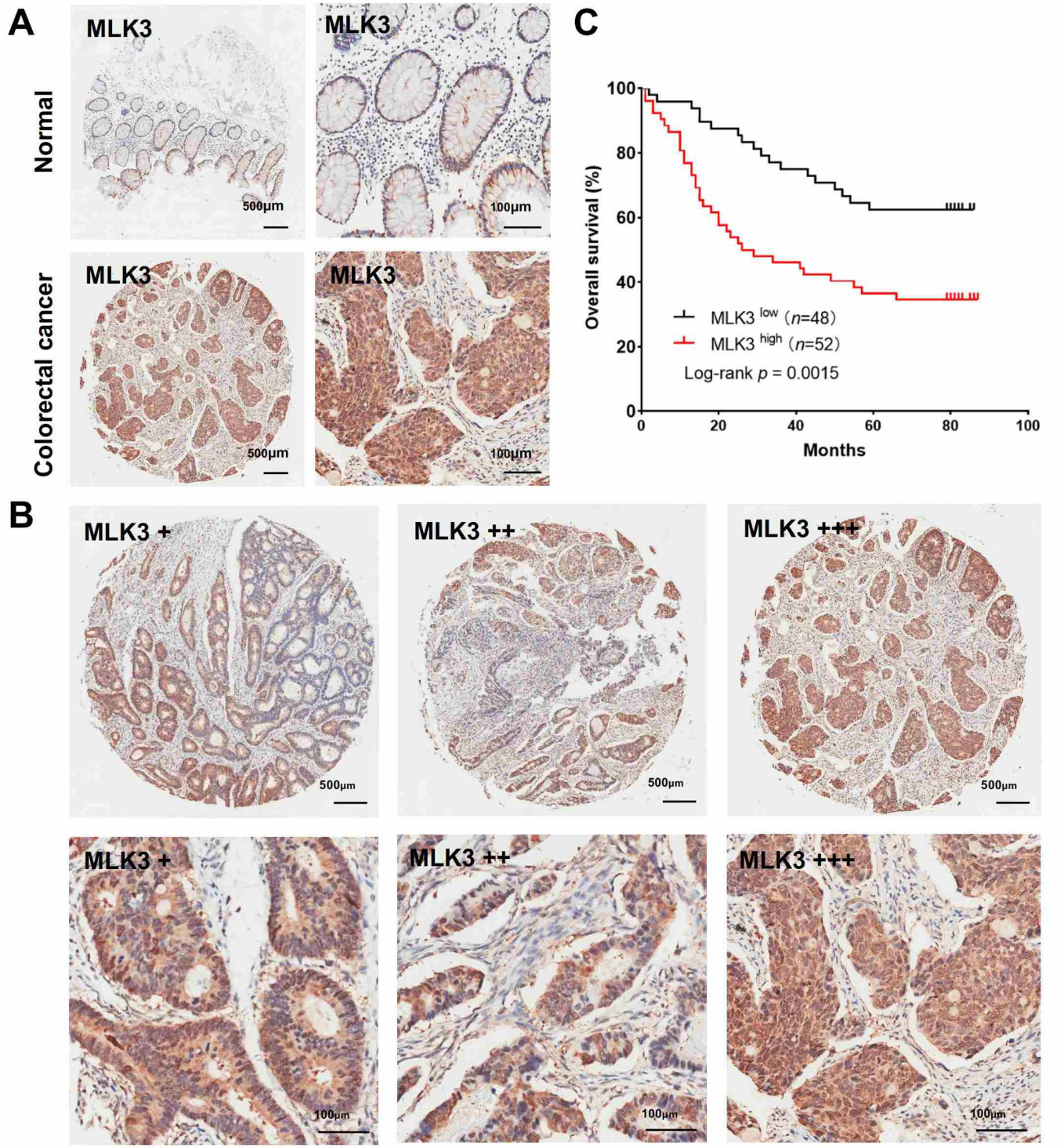


FIGURE 1. Immunohistochemical staining of *MLK3* in CRC specimens. (A) Representative images of *MLK3* expression in CRC samples and normal tissues. (B) Representative images of *MLK3* expression with various CRC status (scale bar, 500 μm and 100 μm). (C) *MLK3* expression and overall survival rate. *MLK3*, mixed-lineage protein kinase 3.

3.2 *MLK3* knockdown suppresses malignancy of CRC

Since elevated *MLK3* expression is associated with poorer clinical outcomes, we examined functional effect of *MLK3* in CRC progression by transfecting human RKO and SW480 cells with *siMlk3*. Transfection with *siMlk3* suppressed the migration ability of CRC ($p < 0.05$) (Fig. 2A,B). To further explore the promoting role of *MLK3* in CRCs stemness or epithelial-mesenchymal transition (EMT) program, their related markers, including β-catenin, Oct4, Notch-1 and Nanog were measured. As shown in Fig. 2C, the expression of these markers decreased substantially after *MLK3* knockdown.

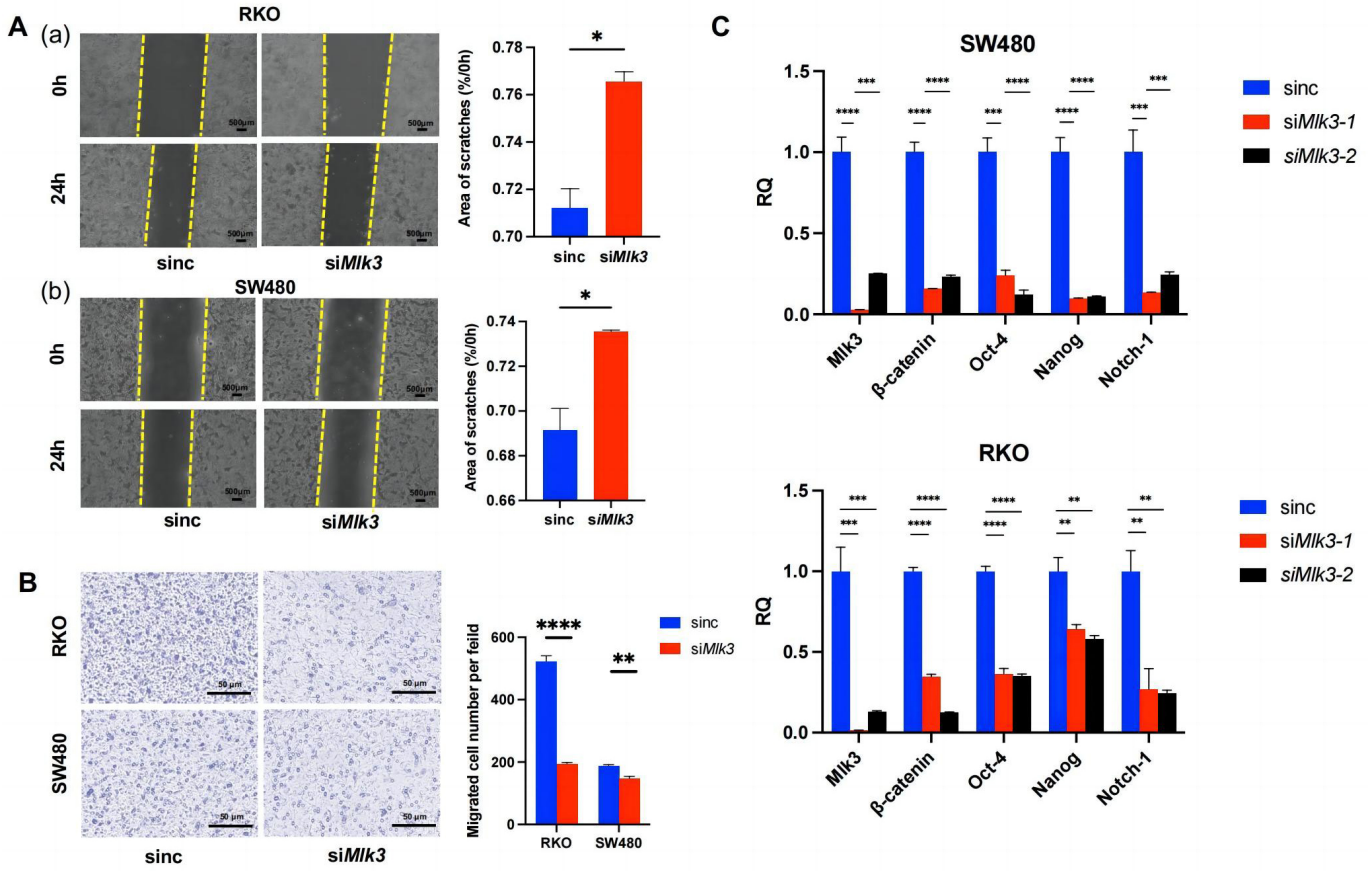


FIGURE 2. ***MLK3* knockdown suppresses malignancy potential of CRC cells.** (A) Wound healing assay with cell lines (RKO and SW480) transfected with control or *siMLK3*. Cells were photographed at 0 and 24 h at 50× magnification and wound closure (%) was evaluated. (B) Transwell assay with cell lines (RKO and SW480) transfected with control or *siMLK3*. Cells in 48 h under 50× microscope photographs. Error bars represent mean ± SEM, representing three experiments. (C) mRNA level of CRC stemness and EMT related markers in RKO and SW480 cell lines (Student's *t*-test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0005$); RQ, relative quantification; *MLK3*, mixed-lineage protein kinase 3.

3.3 *MLK3* gene-mediated signaling pathways

To comprehend the role of *MLK3* in CRC activity, a GSVA-based bioinformatic analysis was performed. The results revealed significant correlations between reactive oxygen species (ROS), EMT program, p53 pathway, tumor proliferation signature, MYC targets, degradation of extracellular matrix (ECM), and ferroptosis in group having elevated level of *MLK3* ($p < 0.05$). Similarly, KEGG analysis suggested that *MLK3* was highly enriched in the cyclic adenosine monophosphate (cAMP) signaling pathway, cell cycle and chemokine signaling pathway (Fig. 3).

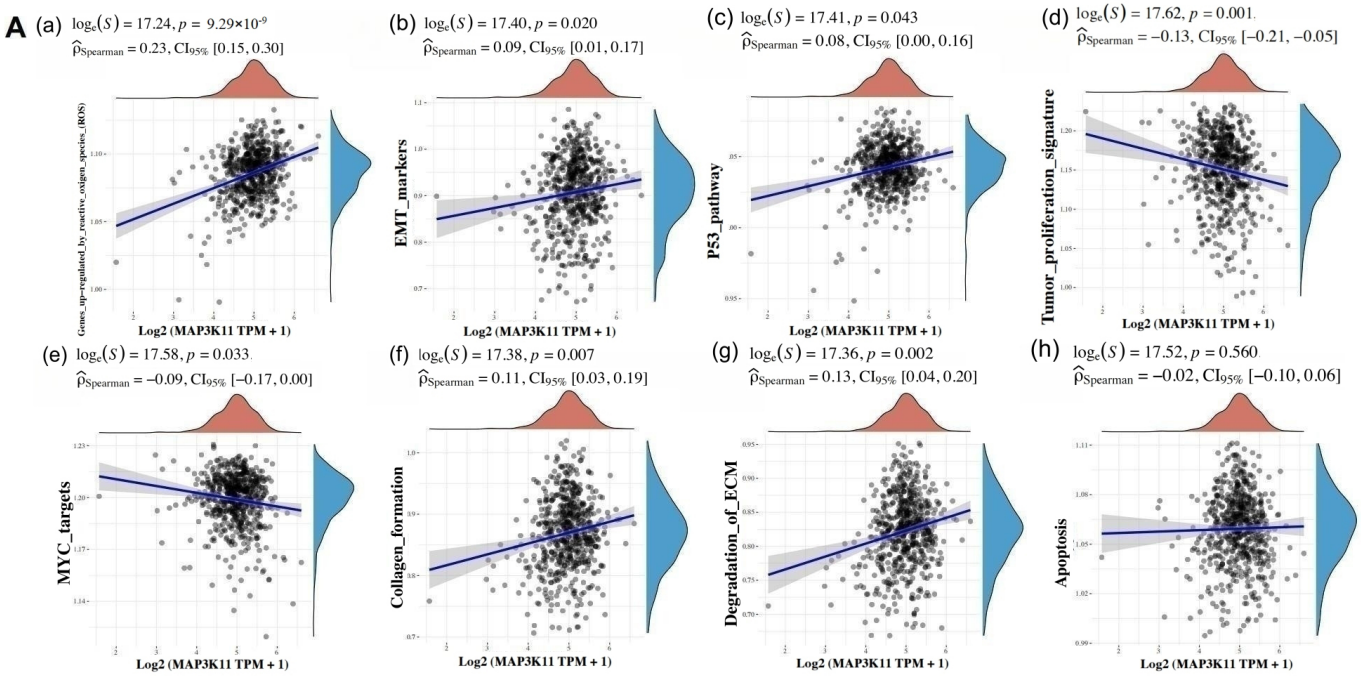
3.4 The effect of *MLK3* on ASCT2 expression

As shown in Fig. 4, knockdown of *MLK3* in cancer cell lines suppressed expression of ASCT2 proteins. It indicates that *MLK3* can target ASCT2 to exhibit a pro-tumor role in CRC progression.

4. Discussion

It is well recognized that mitogen-activated protein kinase (MAPKs) family plays a vital role in progression of cancer, *e.g.*, cancer invasion and migration. Current study focused on the biological significance of *MLK3*, which is one of the mitogen-activated protein kinase (MAP3K) subfamily [19]. Previous studies confirmed that *MLK3* can regulate a broad range of cancer cell characteristics, including cellular invasion, EMT program and migration ability [5]. However, few studies have addressed the effect of *MLK3* with glutamine metabolism on biological behavior of CRC.

The immunohistochemical results in current study suggested a significant upregulation of *MLK3* level in CRC tissues, indicating a direct relationship between increased expression of *MLK3* and adverse prognosis. The potential correlation between age, sex, and the expression of *MLK3* in colorectal cancer was not confirmed. *In vitro* experiments confirmed that *MLK3* could stimulate stemness of cancer cells, EMT and migration capacity. To explore the role of *MLK3* in CRC, Spearman's rank correlation coefficient was applied, which revealed its positive correlation with ROS, EMT-related markers, the p53 pathway, the tumor proliferation signature, and ferroptosis activity. Through illumina whole genome arrays, Velho *et al*. [20] revealed that *MLK3* played a critical role in the progression of CRC related signaling pathways including MAPK, Wnt, TGF-β, p53 and Notch. Additionally, *MLK3* exhibits ERK1/2-dependent phosphorylation under oxidative



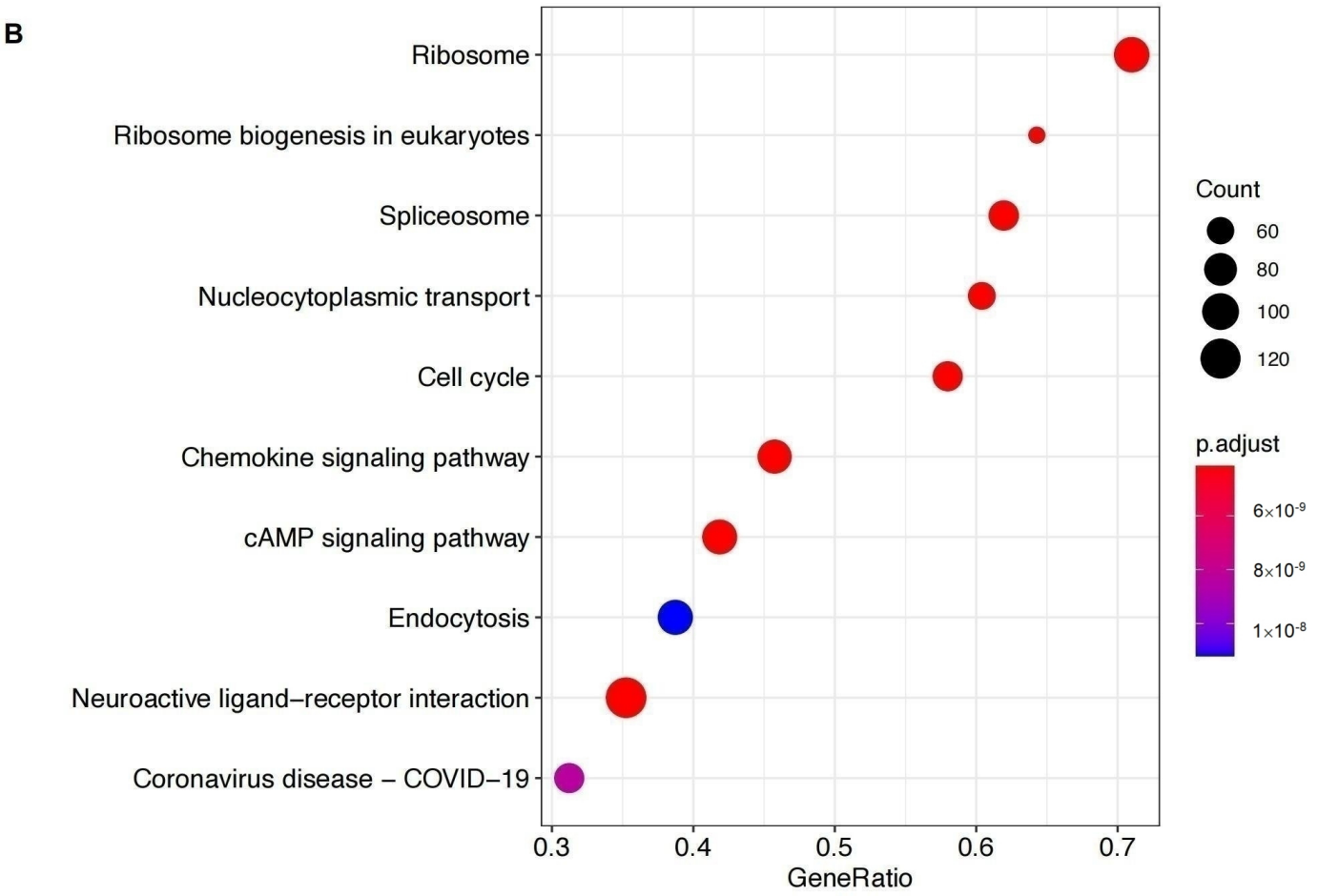
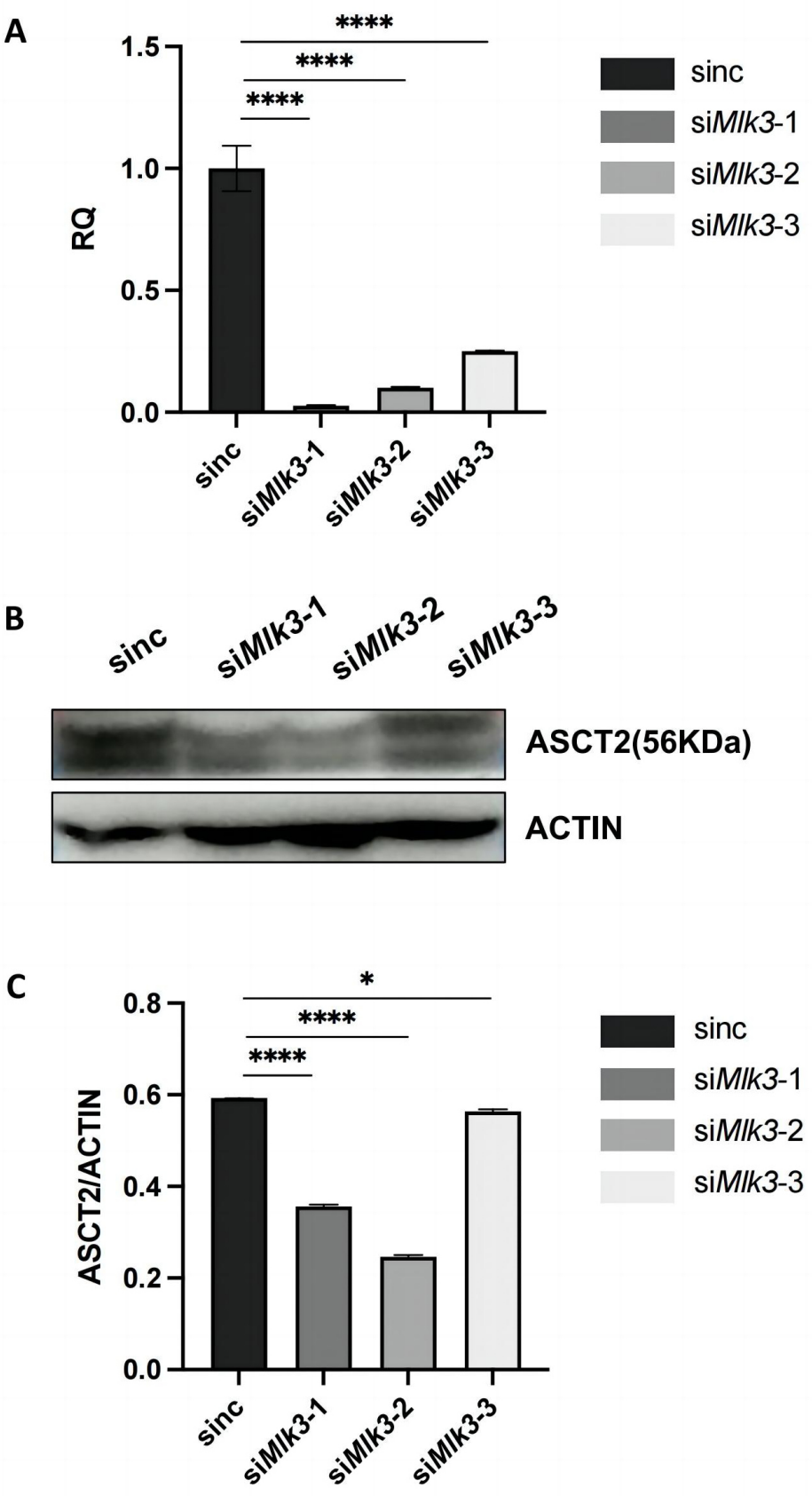


FIGURE 3. *MLK3* associated signaling pathways in CRC progression. (A) Increased expression of *MLK3* was correlated with ROS, EMT markers, p53 pathway, cancer proliferation signature, MYC targets, collagen formation, degradation of EMC and ferroptosis. (B) KEGG analysis suggested that *MLK3* was highly enriched in cAMP, cell cycle and chemokine signaling pathway.



F I G U R E 4. Western blot analysis indicating effect of *MLK3* on ASCT2 expression. (A) Relative mRNA levels of *MLK3* in SW480 cells transfected with *siMlk3*. (B) Expression of ASCT2 proteins in SW480 cells transfected with *siMLK3*. β-actin was used as loading control. (C) Statistical analysis of western blot. Error bars represent mean $\pm$ SEM. *$p < 0.05$, ****$p < 0.0005$; RQ, relative quantification; ASCT2: alanine-serine-cysteine transporter 2; *MLK3*, mixed-lineage protein kinase 3. RQ: relative quantification; ASCT2: alanine-serine-cysteine transporter 2.

stress, accelerating the invasion of CRC by activating the B-Raf and ERK1/2 in a positive loop [10].

Previous studies have uncovered the function of *MLK3* in fatty acid metabolism, suggesting a positive regulatory role in saturated fatty acid metabolism by JNK activation *in vivo*. Meanwhile, *MLK3*-deficient mice showed improved insulin resistance and decreased hepatic steatosis [21]. Current study concentrated on *MLK3* and glutamine metabolism, which is an indispensable regulator in cellular metabolism. It can biosynthesize metabolites such as α-ketoglutarate (α-KG) and generate nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide, reduced ($FADH_2$) for adenosine triphosphate (ATP) needed for cell growth [22]. In cancer cells, increased glutamic acid uptake and glutamine concentration have been widely observed, and glutamine deprivation leads to decreased survival rate [23], and apoptosis in glutamine-addicted cancers [24]. Glutamine is imported from the microenvironment by the SLC family, and ASCT2 is considered as the primary one [25]. A previous study suggested that ASCT2 can be a prognostic marker in *KRAS*-mutant CRC clinical specimens, and its overexpression is positively related with cancer invasion and migration [18]. Besides, ASCT2 knockdown is involved in the apoptosis of cancer cells manifested by decreased BCL-2 levels or increased BAX levels [26]. Current study showed that *MLK3* knockdown led to the suppression of ASCT2 expression, indicating a pro-tumor role of *MLK3* in glutamine metabolism (Fig. 5). Current research has some limitations. Firstly, the clinical subjects and cancer cell lines used in this study are less, which makes the results less convincing. Secondly, the detailed relationship between *MLK3* and glutamine metabolism or ASCT2 needs further demonstration. Hence, further studies focused on more detailed mechanisms are necessary in the future to comprehend the mechanism of action in detail.

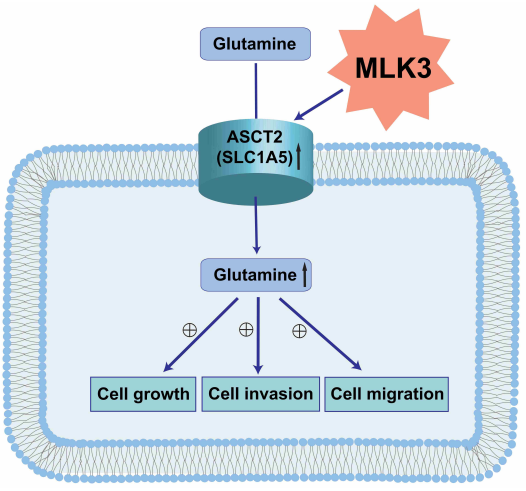


FIGURE 5. Schematic diagram of the role of *MLK3* in CRC progression. *MLK3* can promote growth, invasion and migration ability of colorectal cancer cells (CRC). The potential mechanism may be due to its suppression with glutamine metabolism by directly targeting ASCT2. *MLK3*, mixed-lineage protein kinase 3; ASCT2, alanine-serine-cysteine transporter 2.

5. Conclusions

The current study suggests an oncogenic role of *MLK3*, as increased expression of this gene signifies an independently undesirable prognosis in CRCs. Suppression of glutamine transporter ASCT2 can be the potential mechanism.

AVAILABILITY OF DATA AND MATERIALS

Data will be available on request.

AUTHOR CONTRIBUTIONS

SQW and ZQS—designed the research study and methodology, wrote the manuscript. ZQS, KJW and RPS—contributed to formal analysis, contributed to data curation, revised the manuscript, supervised the study. All authors contributed to editorial changes in the manuscript, read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the First People's Hospital of LinPing District (No. 20080102002). Informed consent was obtained from all individual participants included in the study.

ACKNOWLEDGMENT

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at https://oss.jomh.org/files/article/1674597873308581888/attachment/Supplementary%20material.xlsx.

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