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KRT19 promotes colorectal cancer progression through mTOR pathway *via* SIRT4

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Abstract. Keratin19 (KRT19) is the smallest type I KRT protein which is a member of the keratin family. KRT19 has been reported as a diagnostic marker in several cancers, such as lung, breast and pancreatic cancer. However, the diagnostic value and potential role of KRT19 in colorectal cancer (CRC) remain to be explored. Our study confirmed the KRT19 expression pattern in CRC through publicly database and in CRC patient tissues with immunohistochemistry (IHC) and Western blot. Then, we carried out a series of malignant phenotype assays like colony formation assay, transwell migration and invasion experiments to definite the role of KRT19 on CRC cells proliferation, migration and invasion. Co-immunoprecipitation (co-IP) and Western blot were utilized to clarify the underlying molecular mechanisms of KRT19 in CRC. The data from GEPIA and CRC specimens displayed that KRT19 expression was increased and correlated with a poor clinical outcome. Our *in vitro* experiments confirmed that KRT19 promoted the proliferation, migration and invasion of CRC cells. Moreover, co-IP verified the interaction between KRT19 and SIRT4. KRT19 also activated the mTOR pathway which was illustrated by Western blot. Our research provides that KRT19 may be served as a diagnostic marker for CRC, and KRT19 promotes CRC progression through mTOR pathway by interacting with SIRT4.

Key words: KRT19 — Colorectal cancer — Carcinogenesis — mTOR — SIRT4

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in humans, which ranks third morbidity and second mortality in the world (Siegel 2020; Huang 2021). As a country with a high incidence of CRC, the morbidity in China (*per* 100,000) had increased from 12.28% (1990) to 20.29% (2022) (Analysis of the prevalence of malignant tumors in China in 2022). According to the latest GLOBOCAN 2022 data, CRC remains the third most commonly diagnosed malignancy and the second leading cause of cancer-related deaths worldwide, with an estimated 1.9 million new cases and 935,000 deaths annually (Sung et al. 2023). In China, the age-standardized incidence rate of CRC continues to rise, especially among younger populations, which underscores the need for effective early detection and intervention strategies (Yin et al. 2023).

Surgical resection, radiotherapy and chemotherapy, immunotherapy and targeted therapy are the conventional treatments for CRC at present (Guend et al. 2017). Early CRC can be treated surgically, and the survival rate is more than 90%. However, for metastatic CRC, the present treatment effect is not ideal, and the 5-year survival rate is only 12.5% (Favoriti et al. 2016). Therefore, improving the successful early diagnosis is an urgent clinical challenge in CRC, and the study on the pathogenesis of CRC is also helpful to solve the problem for early diagnosis.

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Keratin 19 (KRT19) is known to be the smallest (40 kDa) type I KRT protein which is a member of the keratin family (Asfaha et al. 2015). Keratins are cytoskeletal proteins which belong to an intermediate filament family (Alsharif et al. 2021). Recent studies have revealed that keratins are involved in the processes of cell death, epithelial cell polarity and mechanical integrity (Alsharif et al. 2021; Evans and Corfe 2021). Over the past years, KRT19 is recognized as the diagnostic marker in various kinds of malignant tumors. In a Caucasian series of hepatocellular carcinomas (HCCs), KRT19 was deemed to be a prognostic marker as early as in 2006 (Durnez et al. 2006). And the team further explored the functional role for KRT19 in invasion and chemoresistance (Govaere et al. 2014). In lung cancer, researchers validated the value of KRT19 in protein level using tissue microarray and proved its predictive value (Yuan et al. 2021). Additionally, Yao et al. (2016) demonstrated KRT19 overexpression is correlated with malignant progression and poor prognosis in patients with Pancreatic ductal adenocarcinoma (PDAC). Recently, Masoumeh Mehrpouya et al. (2019) probed the metastatic potential of KRT19 among 30 cancers and indicated its application as a tumor marker. However, the prognostic value and underlying mechanism of KRT19 in CRC has not been explored in depth. KRT19 encodes a type I cytokeratin, a key component of the intermediate filament cytoskeleton in epithelial cells. It plays an important role in maintaining cellular structural integrity and is implicated in regulating cell proliferation, apoptosis, and epithelial-mesenchymal transition (EMT), all of which are relevant to cancer progression.

Thereby, present research confirmed the expression levels of KRT19 in CRC through publicly available database and corroborated with CRC patient tissue biopsies using immunohistochemistry (IHC) and Western blot. Then, a serious of *in vitro* malignant phenotype assays like colony formation assay, Transwell migration and invasion experiments were assessed to definite the role of KRT19 on CRC cells proliferation, migration and invasion. Based on previous study of our group, we found that KRT19 was just one of SIRT4 associated Differentially expressed proteins (DEPs) which were identified by LC-MS/MS. However, fewer studies have explored the association between KRT19 and SIRT4. So, we utilized co-immunoprecipitation (co-IP) and Western blot to clarify the underlying molecular mechanisms of KRT19 in CRC. The diagnosis and therapy of tumor is an ever-advancing field which depends on the premise of a deeper exploration of the molecular mechanisms of biomarkers and targets. Effective screening offers a hope for improving survival and quality of life for CRC patients. Thereby, our data implicated that KRT19 can be deemed as an essential prognostic biomarker for CRC, and KRT19/SIRT4/mTOR axis may provide a promising choice for further CRC therapeutic strategy.

Materials and Methods

Tissue microarrays

The CRC tissue microarrays (TMAs) (HColA180Su12) were commercially obtained from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China) which consisted of 87 pairs of primary CRC and matched adjacent normal tissues containing complete clinicopathological information and long-term follow-up data.

Clinical tissue specimens

Eight pairs of snap-frozen CRC samples and peritumoral specimens used for Western blot analysis were procured from the Department of Colorectal Cancer Surgery, the Second Affiliated Hospital of Harbin Medical University between October 2018 to December 2018. Selected patients had only undergone colectomy and received any neoadjuvant therapy. Pre-approval for the study was sought from the Institutional Review Board of Harbin Medical University.

Cell lines and cell culture

The human CRC cell lines HT29, RKO, SW620, LOVO, DLD-1, HCT116, SW480 and normal colorectal cells NCM460 were commercially procured from the ATCC (USA). As *per* the repositories protocol, SW620 and SW480 were maintained in Leibovitz's L-15 medium (Gibco, USA), and all other cell lines were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Sciencell, USA) at 37°C with 5% CO_2.

Plasmids, small interfering RNAs (siRNAs) production

To overexpress SIRT4/KRT19 in HT29/HCT116 cell lines, cells were transiently transfected with 2 μg plasmids pcDNA3.1 vector, SIRT4-Flag (Addgene, #151245) and full-length KRT19-HA (Umibio, Shanghai, China) with Lipofectamine 2000 (Invitrogen, USA) as *per* the manufacturer's instructions. To knockdown KRT19 expression, the cells were transfected with KRT19 specific siRNAs and control siRNA (GenePharma, Shanghai, China) at a final concentration of 100 nmol/l. 48 h post transfection, the efficiencies of overexpression or knockdown were detected by Western blot. The sequences of KRT19 specific siRNAs were as follows: KRT19 siRNA1: Sense: 5′-CCAGCCGGACUGAAGAAUUTT-3′; Antisense: 5′-AAUUCUUCAGUCCGGCUGGTT-3′; KRT19 siRNA2: Sense: 5′-GGAAGACACACUGGCAGAATT-3′; Antisense: 5′-UUCUGCCAGUGUGUCUUCCTT-3′; Negative control siRNA: Sense: 5′-UUCUCCGAACGUGUCACGUTT-3′; Antisense: 5′-ACGUGACACGUUCGGAGAATT-3′.

Western blot

Total proteins were extracted from cultured cells and lysed by RIPA buffer (Solarbio, Beijing, China) containing 1% protease inhibitors (Roche, Mannheim, Germany) for 20 min on ice. BCA assay (Beyotime, shanghai, China) determined the protein concentrations. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Merck, Darmstadt, Germany) with wet transfer. The membranes were blocked with 5% skimmed milk powder in Tris Buffered saline Tween (TBST) for 1 h at room temperature and incubated with a diluted solution of primary antibodies at 4°C overnight. The next day, membranes were incubated with secondary antibodies (1:5000, Zsbio, China) for 50 min at room temperature. After washing with TBST for three times, the blots were visualized by ECL reagent.

The primary antibodies and dilutions were described: antibodies for KRT19, SIRT4, Flag, HA were purchased from Proteintech Group (Wuhan, China), for p-S6K, p-AKT, S6K and AKT were obtained from Cell Signaling Technology (CST, USA) and the dilutions were 1:1000. GAPDH (1:5000, Zsbio, China) was utilized as an endogenous control. The band intensities of target proteins were quantified using ImageJ software and normalized to GAPDH. All results were presented as relative expression levels based on three independent replicates.

qRT-PCR

To synthesize cDNA, the total RNA from cells was extracted by Trizol reagent (Thermo Fisher Scientific, USA), and reverse transcribed with Rever Tra Ace qPCR RT Master (Code No. FSQ-201, TOYOBO, Japan). Then the obtained cDNAs were amplified with a SYBR Green Master mix (Roche, Mannheim, Germany) using the ABI 7500 Fast Real-time PCR Detection System (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were 95°C for 15 s, followed by 30 s at 60°C and 30 s at 72°C for 40 cycles. GAPDH was used as the internal control for normalization. The relative mRNA expression was calculated by $2^{-\Delta\Delta Ct}$ method. All procedures were carried in triplicate. The specific primer sequences were listed as follows: KRT19-F: 5′-TCGAGCATGAGGTATCCAGT-3′; KRT19-R: 5′-GTAGCGGTTCTTCGTGTCTT-3′; GAPDH-F: 5′-CATGTTCGTCATGGGTGTGAA-3′; GAPDH-R: 5′-GGCATGGACTGTGGTCATGAG-3′.

Colony formation assay

Cells were planted onto six-well culture plates in triplicate and incubated in a 10% FBS-containing medium for about 10 days. The colonies were fixed in methanol for 10 min and stained by crystal violet for 20 min, then observed under a microscope and photographed. For quantification, colonies containing more than 50 cells were counted manually in three independent experiments, and the average colony number was plotted using GraphPad Prism 7.0.

Transwell migration and invasion assays

5×10^5 CRC cells *per* well were seeded in the upper transwell chambers (Corning, USA) containing 200 μl pure medium. The Matrigel (1:8, BD Biocoat, San Jose, CA, USA) was pre-coated on the top chambers to take the invasion assay and the migration assay was performed without Matrigel. The lower chambers were replenished with RPMI-1640 medium with 20% FBS 600 μl. After incubation at a constant temperature of 37°C for 48 h, the cells that invaded to the lower chambers through the filter pores were washed with PBS, fixed by 4% methanol and then stained with 1% crystal violet. The cotton swabs were used to gently remove the non-migratory or non-invasive cells on the top chambers. Then, cells from randomly selected fields were counted and photographed with a microscope at least in three fields. Three independent experiments were performed. The number of migrated or invaded cells in five randomly selected fields *per* chamber (under 200×magnification) was counted using ImageJ software, and results were presented as the mean ± SD from three independent experiments.

For the migration assay, CRC cells were seeded in the upper chambers of Transwell inserts without Matrigel coating, allowing cells to migrate through the pores. For the invasion assay, the upper chambers were pre-coated with diluted Matrigel (1:8, BD Biocoat, San Jose, CA, USA), simulating the extracellular matrix barrier. In both assays, 5×10^5 cells were seeded in serum-free medium in the upper chamber, while the lower chamber contained 600 μl medium supplemented with 20% FBS as a chemoattractant. After 48 h incubation at 37°C, the migrated or invaded cells were fixed, stained with 1% crystal violet, and counted under a microscope.

Co-immunoprecipitation (co-IP)

After 48 h transfection, cells were washed three times with PBS and lysed with 500 μl RIPA buffer supplemented with 1% protease inhibitors. The supernatants obtained from lysates by centrifugation (12000×*g* 10 min, 4°C) and 50 μl of the supernatants were collected as whole cell lysate for Western blot analysis. Then, the rest of supernatants were incubated with 1 μg of antibodies (Flag, HA or Ig-G) overnight at 4°C on a rocking platform. The next day, appropriate amounts of Protein G Sepharose beads (GE Healthcare, Uppsala, Sweden) were applied to the samples followed by incubation for 4 h 4°C on a shaker. The immunocomplexes

were then centrifuged (2000×*g*, 4°C) for 2 min and washed three times with 200 µl of lysis buffer. After the addition of 6×SDS loading buffer, sepharose beads containing immunocomplexes were boiled for 8 min and subjected to Western blot.

Statistical analysis

All data were analyzed by GraphPad Prism 7.0 statistical software (GraphPad, San Diego, CA, USA). Two tailed Student's *t*-tests were performed to examine the statistical relevance between groups. For the association between KRT19 expression and clinical pathologic characteristics, Pearson chi-square test (χ^2) statistic was utilized to analyze categorical variables across groups. Kaplan-Meier method was used to plot survival curves which were statistically analyzed by the log-rank test. Univariate survival analyze was evaluated using a Cox proportional hazards regression model. Results obtained from at least three separate experiments are presented as the mean ± SD. *p*-values < 0.05 were considered statistically significant (*), $p < 0.01$ was considered more statistically significant (**), and $p < 0.001$ was considered the most statistically significant (***).

Results

KRT19 is expressed at high levels in CRC

To confirm the efficiency of KRT19 overexpression and knockdown in CRC cell lines, Western blot analysis was performed. In HT29 cells transfected with KRT19-Flag, the protein level of KRT19 was increased approximately 3.5-fold compared with endogenous levels in wild-type HT29 and HCT116 cells, as detected by an anti-KRT19 antibody. Additionally, anti-FLAG antibody detected a protein band corresponding to KRT19-Flag, co-migrating with the endogenous KRT19 band, confirming the specificity of the anti-KRT19 antibody. For silencing experiments, both siRNA-1 and siRNA-2 effectively reduced KRT19 protein levels in HCT116 cells compared with scrambled siRNA controls, with siRNA-1 showing slightly higher knockdown efficiency. The residual KRT19 protein level in siKRT19 HCT116 cells was markedly lower than in wild-type HT29 cells, further validating the knockdown effect and antibody specificity. To investigate the KRT19 expression status in CRC, we first performed Western blot and qRT-PCR to compare the endogenous KRT19 expression patterns in

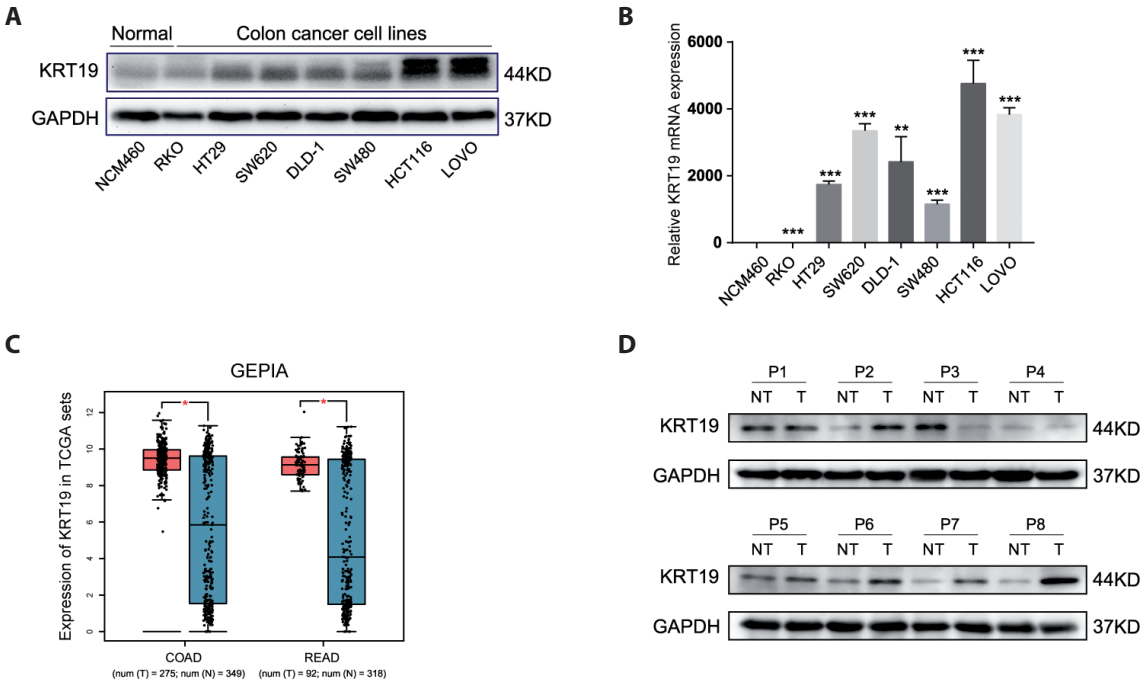

Figure 1. KRT19 is highly expressed in colorectal cancer (CRC). **A.** Western blot analysis of KRT19 protein expression levels in the normal colorectal cell line NCM460 and various CRC cell lines (RKO, HT29, SW620, DLD-1, SW480, HCT116, and LOVO). GAPDH was used as an internal control. ** $p < 0.01$ and *** $p < 0.001$ *vs.* NCM460 cells. **B.** Relative mRNA expression levels of KRT19 in NCM460 and CRC cell lines determined by qRT-PCR. Data are presented as mean ± SD from three independent experiments. ** $p < 0.01$ and *** $p < 0.001$ *vs.* NCM460 cells. **C.** Comparison of KRT19 expression between CRC tissues ($n = 367$) and normal colon tissues ($n = 667$) using the GEPIA database. **D.** Western blot analysis of KRT19 expression in 8 pairs of CRC clinical specimens (T) and matched 543 adjacent normal tissues (NT). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* NCM460 544 cells.

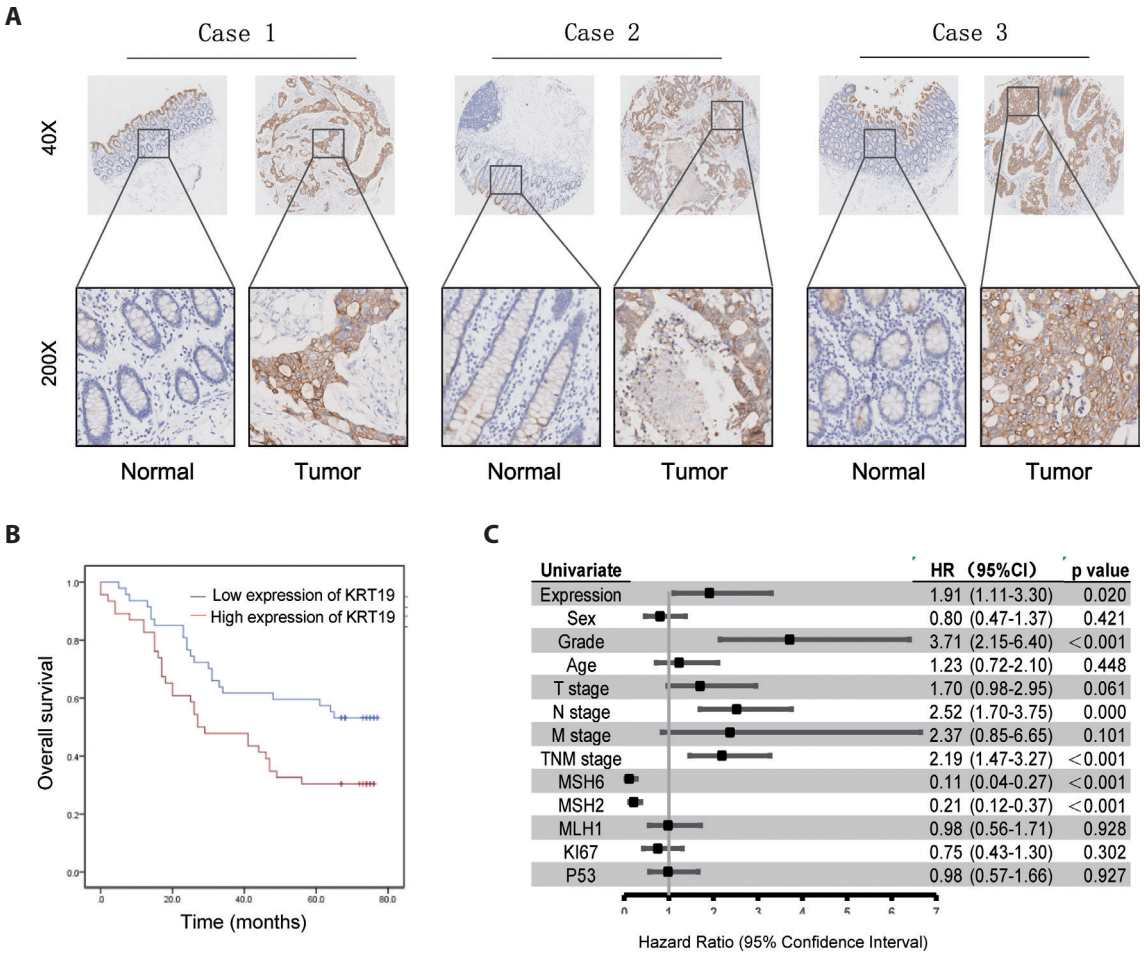

Figure 2. High KRT19 expression correlates with unfavorable clinicopathologic features and poor prognosis in CRC. **A.** Representative immunohistochemistry images of KRT19 staining in CRC patient tissue microarrays. Images show representative staining in normal and tumor tissues at 40× and 200× magnifications. **B.** Kaplan-Meier survival curves showing overall survival of CRC patients with high versus low KRT19 expression levels. Statistical significance was determined by the log-rank test. **C.** Forest plot of univariate Cox regression analysis evaluating the association between KRT19 expression, other clinical features (age, sex, grade, stage), and overall survival. Results are presented as Hazard Ratio (HR) with 95% Confidence Interval (CI).

normal colorectal cell line NCM460 and various CRC cell lines. As Figure 1A showed, in comparison to NCM460, KRT19 expression was moderately upregulated in several CRC cell lines; however, RKO cells showed relatively lower protein expression compared to others. Meanwhile, the CRC cell lines also had upregulated mRNA expression (Fig. 1B). Following which, KRT19 expression status in CRC was explored through online database GEPIA (https://gepia.cancer-pku.cn/index.html) and clinical samples from our cohort. Data from GEPIA confirmed the enhanced KRT19 expression in 367 CRC tissues in comparison to 667 normal colon tissues (Fig. 1C). As a further validation, we utilized Western blot to determine KRT19 expression from 8 paired CRC samples and matched peritumoral tissues. As indicated in Figure 1D, Of the 8 pairs of CRC specimens, KRT19 expression was found upregulated in most CRC samples. Our data above implied that KRT19 is expressed at high levels in CRC.

High KRT19 expression in CRC correlates with unfavorable clinicopathologic features and poor prognosis

KRT19 has been reported as a diagnostic marker in several cancers, such as lung, breast and pancreatic cancer (Yao et al. 2016; Saha et al. 2017; Saha et al. 2019; Yuan et al. 2021). However, the diagnostic value of KRT19 in colorectal cancer (CRC) remains unclear. With this aim, we detected CRC patients from TMAs by IHC. Three representative KRT19 images of immunostaining from CRC patients were displayed in Figure 2A. IHC analysis showed that the positive

expression of KRT19 protein was mainly localized at the cell membrane and cytoplasm, mainly in the cell membrane. The immunoreactivity of KRT19 was higher in tumors than in matched adjacent normal tissues. Based on staining score, the correlation analysis using χ^2 between KRT19 expression and clinicopathological characteristics in CRC revealed that higher KRT19 expression was significantly correlated with N stage (p = 0.039), tumor-node-metastasis (TNM) stage (p = 0.052), MSH6 (p = 0.035) and MSH2 (p = 0.028), but not with age, sex, grade, T stage, M stage, P53, MLH1 and Ki67 (Table 1). Furthermore, survival curves of CRC patients were plotted using Kaplan-Meier method and determined by log-rank statistical analysis. As indicated in Figure 2B, the overall survival analysis described that the patients with higher KRT19 expression status correlated with poorer clinical outcome in CRC. Moreover, univariate Cox regression model was built to assess the role of KRT19 expression on overall survival (OS) along with other clinical features (age, sex, grade, T stage, N stage, M stage, TNM stage, MSH6 and MSH2, P53, MLH1 and Ki67), which were selected based on their established roles in CRC progression (Brenner et al. 2007b). Results of Cox regression model were displayed as the HR with 95% CI. Our univariate analysis demonstrated that high level of KRT19, Grade, N stage, TNM stage, MSH6 and MSH2 were significantly associated with worse survival of CRC patients (Fig. 2C). Consequently, we can conclude that the increased level of KRT19 in CRC was closely associated with unfavorable clinicopathologic features and may serve as a dominating prognostic indicator for CRC patients.

KRT19 facilitates CRC cells proliferation, migration, and invasion

To illustrate the potential biological functions of KRT19 in CRC, we carried out various the tumorigenic phenotyping tests such as colony formation and Transwell assays to screen whether it affects cancer cell progression. Based on the KRT19 expression levels in CRC cells, HT29 was selected for gain-of-function analyses, while HCT116 was chosen for loss-of-function assays. Those siRNAs, KRT19-HA plasmids and matched controls were carried out to down or up regulate KRT19. Initially, we proceed to verify CRC cells proliferation ability by colony formation assay and we found that overexpressed KRT19 enhanced cell proliferation in HT29 while silenced KRT19 attenuated colony formation ability in HCT116 (Fig. 3A). In addition, we conducted Transwell analyses to evaluate the exact roles of KRT19 on CRC cells migratory and invasive properties. As showed in

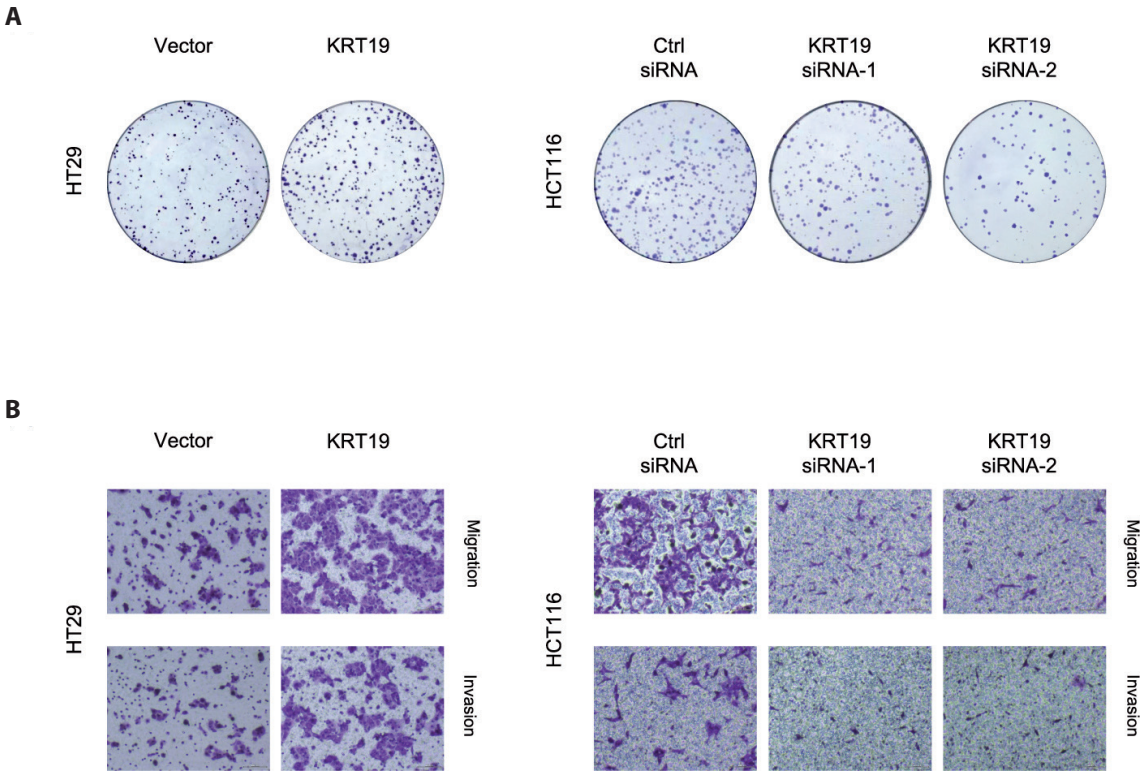

Figure 3. KRT19 facilitates CRC cell proliferation, migration, and invasion. **A.** Representative images of the colony formation assay in HT29 cells (overexpressing KRT19) and HCT116 cells (KRT19 knockdown *via* siRNA). **B.** Representative images of Transwell migration and invasion assays in HT29 and HCT116 cells with corresponding treatments.

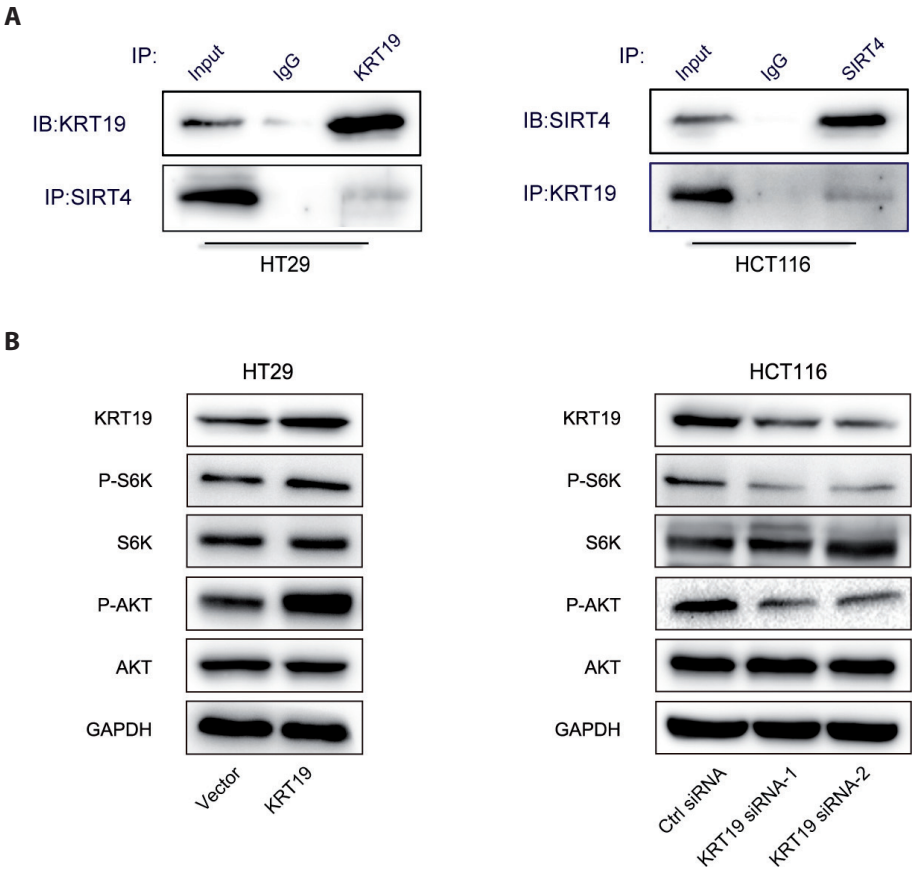

Figure 4. KRT19 activates the mTOR signaling pathway *via* interaction with SIRT4 IB, immunoblotting. **A.** Co-immunoprecipitation assay demonstrating the endogenous binding between KRT19 and SIRT4 protein in HT29 and HCT116 cells. **B.** Western blot analysis of mTOR signaling-related proteins, including phosphorylated S6K (p-S6K), phosphorylated AKT (p-AKT), total S6K, and total AKT, in CRC cells following KRT19 overexpression or knockdown. GAPDH was used as a loading control. All Western blot results are representative of three independent experiments.

Figure 3B, KRT19 overexpression led to enhanced migratory and invasive behaviors in HT29 cells, while KRT19 knockdown suppressed these capabilities in HCT116. Migration ability was evaluated by assessing cell movement through the membrane, while invasion capability was tested through Matrigel-coated membranes mimicking the extracellular matrix barrier. Quantitative results showed significant differences ($p < 0.01$) between control and experimental groups (Fig. 3D), confirming that KRT19 promotes both migration and invasion of CRC cells. Quantitative analyses of colony numbers and migrated/invaded cell counts were also performed. As shown in Figure 3C and D, overexpression of KRT19 significantly increased colony numbers and migration/invasion abilities, while KRT19 knockdown led to a significant reduction ($p < 0.01$). These results were consistent across three biological replicates.

KRT19 activates mTOR signaling in CRC via SIRT4

From our previous study has been published, TMT proteomics had been taken to gain insight into the underlying protein functions of SIRT4 in CRC (Cui et al. 2021). We carried out LC-MS/MS based identification of SIRT4 related DEPs, and KRT19 was just one of these 612 DEPs. However, fewer studies have explored the association between KRT19 and SIRT4. So we performed co-IP assay to clarify the endogenous binding of KRT19 and SIRT4 protein in HT29/HCT116. Lysates of CRC cells were immunoprecipitated with anti-HA antibody, and SIRT4 protein was tested with Western blot. SIRT4 was detected in the immunoprecipitated KRT19 complex, but not in the IgG control sample. Then lysates of CRC cells were immunoprecipitated with anti-Flag antibody followed by Western blot to examine KRT19. KRT19 was detected in the immunoprecipitated SIRT4 complex, but not in the IgG control sample (Fig. 4A). It has been widely recognized mTOR plays a pivotal role in the regulation of many cellular processes of cancers, including cell proliferation and metabolism (Csibi et al. 2021). As reported in Wang et al. (2019) SIRT4 defection was related to activation of mTOR signal transduction. So, we speculate that KRT19 may activate mTOR signaling in CRC *via* SIRT4. For further confirm our hypothesis, we detected the expression of mTOR signaling related proteins (p-S6K, p-AKT, S6K and AKT) in CRC cells by Western blot. As exhibited in Figure 4B, overexpression of KRT19 in HT29 cells led to elevated phosphorylation levels of AKT and S6K, indicating enhanced mTOR signal-

ing activity. In contrast, silencing KRT19 in HCT116 cells markedly reduced mTOR pathway activation, as evidenced by decreased levels of phosphorylated AKT and S6K, with total protein levels remaining stable (Fig. 4B). However, no considerable changes were observed in total S6K and AKT protein levels. Collectively, we have implicated that KRT19 could regulate mTOR pathway *via* SIRT4, but the underlying molecular mechanism remains to be deep explored.

To validate the interaction between KRT19 and SIRT4, we performed co-IP assays in CRC cells. Specifically, HT29 cells were transfected with KRT19-HA plasmid, and HCT116 cells were transfected with SIRT4-Flag plasmid. For HA-based IP, the lysates of KRT19-HA-overexpressing HT29 cells were immunoprecipitated using anti-HA antibody, followed by immunoblotting for SIRT4. Conversely, lysates of SIRT4-Flag-overexpressing HCT116 cells were subjected to anti-Flag IP, and KRT19 was detected by immunoblotting. In both cases, approximately 5% of total lysates (Input) were loaded alongside IP lanes to assess the efficiency and specificity of the pulldown and interaction.

Although our current findings indicate that KRT19 can promote mTOR signaling activation and CRC progression, the precise role of SIRT4 as an intermediary remains to be elucidated. Given our previous findings that SIRT4 knockdown activates the AKT/GSK3β/CyclinD1 axis, we hypothesize that KRT19 may modulate mTOR activity by interfering with SIRT4's tumor-suppressive functions. However, whether KRT19 exerts its effects on mTOR independently or dependently of SIRT4 requires further experimental clarification. In particular, rescue experiments using SIRT4 knockdown followed by KRT19 overexpression – or *vice versa* – would be essential to determine whether KRT19 still activates mTOR signaling in the absence of SIRT4. These studies are currently underway in our laboratory and will help clarify the KRT19/SIRT4/mTOR regulatory axis in CRC.

Discussion

Latest GLOBOCAN data shows that there are 1.8 million new cases and 881,000 CRC deaths in 2018, causing a heavy economic and national burden worldwide (Bray et al. 2018). As is well-known, CRC is arising through three major pathways, including adenoma-carcinoma sequence, serrated pathway, and inflammatory pathway. Progression from precancerous lesions to cancer takes 5–10 years, providing an important time window for intervention (Brenner et al. 2007a). In addition, TNM stage of CRC is closely related to prognosis. Hence, CRC screening and early detection have gained increasing attention as important means to reduce the morbidity and mortality of CRC (Pan et al. 2021). Traditional CRC screening methods often include fecal immunochemical test (FIT), CT colonography (CTC) and so on (Pickhardt et al. 2021). However, these screening methods inevitably have some limitations. Therefore, the application of sensitive and highly specific markers for CRC screening is a clinical issue that needs to be solved urgently.

KRT19, known as a cytoplasmic intermediate filament protein, has been reported as a widely detectable tumor maker in various cancers and associated with a poor prognosis, including lung, breast, cervical and pancreatic cancer, et al (Mehrpouya et al. 2019; Wang et al. 2020). For the past few years, KRT19 is known to have a stemness-related and epithelial mesenchymal transition (EMT) features in HCC (Govaere et al. 2014; Yokomichi et al. 2019). Upregulated expression of KRT19 has been diffusely recognized as an essential influencing factor for enhancing the cancer phenotypes in a variety of human cancers. Hyungjin Rhee et al. discovered that KRT19 expression was upregulated in HCC cells derived by hepatocyte growth factor (HGF) from HSCs leading to the c-MET and the MEK-ERK1/2 pathway activation (Rhee et al. 2018). However, plenty of studies considered KRT19 displays contrasting effects on cell proliferation, invasion, migration, and apoptosis, depending on the cancer cell type (Ju et al. 2013; Saha et al. 2017; Alsharif et al. 2021). As reported by et al. (2019) KRT19 knockdown led to an increased cancer properties because of attenuated Wnt and enhanced Notch signaling in breast cancer, but in the colon cancer showed the opposite trend. The current study first explores the gene expression status and clinical prognostic value of KRT19. What is more, KRT19 exhibits aggressive role in CRC carcinogenesis and the underlying molecular basis that may contribute to the crosslinking of KRT19 in CRC has been illuminated.

Multiple studies have shown that tumor cells undergo metabolic reprogramming during the tumor carcinogenesis and progression to provide energy and nutrients, maintain cellular redox homeostasis, and regulate intracellular signal transduction for rapid proliferation and growth in the tumor microenvironment characterized by hypoxia, poor blood vessels and nutrient deficiency (Agnihotri and Zadeh 2016; Medina 2020). Therefore, in-depth exploration of the metabolic abnormalities in CRC cells can reveal a new intersection between metabolism and disease evolution and provide important theoretical basis for tumor intervention and new opportunities for clinical transformation. In recent years, mitochondrial NAD+ dependent ADP-ribose transferase SIRT4 has attracted more attention for its role in regulating tumor energy metabolism, such as glutamine metabolism by inhibiting glutamate dehydrogenase (GDH) (Haigis et al. 2006), glucose metabolism by repressing pyruvate dehydrogenase complex (PDH) (Jeong et al. 2014) and lipid metabolism by targeting malonyl-CoA decarboxylase (MCD) (Jeong et al. 2013). Our previous research has clarified the suppressive role of SIRT4 in CRC; we further identified the DEPs in the

SIRT4/Control comparison group through TMT quantitative proteomics analysis. The 612 DEPs from LC-MS/MS showed that KRT19 expression was significantly reduced in SIRT4 overexpression group. So, we ask for help with co-IP experiment, and ascertained there is an endogenous binding between KRT19 and SIRT4.

The mTOR pathway is one of the vital pathways known to regulate major cellular functions including proliferation in human cancers (Xu 2014). Recent data also indicates that mTOR could coordinate eukaryotic cell growth and metabolism with environmental inputs, including nutrients and growth factors (Saxton and Sabatini 2017). Alfred Csibi et al. (2013, 2021) demonstrated that the mTORC1 pathway in the stimulation of glutamine anaplerosis by promoting the activity of GDH which can be repressed by the transcription of SIRT4. We thus, proposed the scientific hypothesis that KRT19 interacts with SIRT4 to form a binary complex, which activates mTOR signaling axis by regulating tumor energy metabolism, thus promoting the malignant progression of CRC. Given the observed inverse relationship between SIRT4 and KRT19 expression and their respective effects on CRC cell proliferation and migration, we speculate that KRT19 may promote tumor progression partly by antagonizing the tumor-suppressive functions of SIRT4. However, the direct contribution of the KRT19-SIRT4 interaction to these phenotypic changes remains to be further investigated. Additionally, we also found activation of mTOR pathway through either silencing or overexpression of KRT19. We speculated that there may be a close association between KRT19 and metabolic reprogramming of CRC suggesting a developed direction for future work.

Several biomarkers have been identified and used clinically or experimentally in CRC, including carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and more recently, molecular markers such as KRAS, BRAF mutations, microsatellite instability (MSI), and CDX2. While these markers provide valuable prognostic or predictive information, their sensitivity and specificity, particularly in early-stage CRC or metastatic prediction, remain suboptimal. Compared with these conventional biomarkers, KRT19 appears to be significantly upregulated in CRC tissues and cell lines, as demonstrated in this study. Moreover, unlike mutational markers that require DNA sequencing, KRT19 expression can potentially be assessed *via* immunohistochemistry or RNA-based assays, enabling simpler clinical implementation. Importantly, the KRT19–SIRT4–mTOR axis may offer a novel mechanistic pathway not covered by existing markers, suggesting its potential use not only in diagnosis but also as a therapeutic target. Nevertheless, further validation in clinical specimens and larger patient cohorts is needed to confirm its robustness and clinical utility.

In summary, the present study provides novel visuals for the CRC screening. Our study is the first report indicating KRT19 is a potential prognostic marker for CRC screening, and the interaction between KRT19 and SIRT4 may be a breakthrough for future research. Additionally, our findings suggest that KRT19 could facilitate CRC cells proliferation, invasion and migration and induce mTOR signaling, but the underlying mechanisms about how KRT19 regulates tumor energy metabolism remains unclear. Nevertheless, our study was carried out only based on *in vitro* experiments, lacking further evidence *in vivo*. In the future, it still requires scientific molecular biology experiments and randomized clinical trials to prove the efficacy of KRT19 as a diagnostic marker for CRC patients. While our study demonstrates that KRT19 promotes CRC progression *via* activation of the mTOR signaling pathway, direct functional validation using mTOR inhibitors has not yet been performed. Given the established role of mTOR in tumor growth and metabolism, it would be of great interest to investigate whether pharmacologic inhibition of mTOR – using agents such as rapamycin or everolimus – can reverse the oncogenic effects of KRT19 overexpression in CRC models. Such experiments would provide direct evidence linking KRT19-driven mTOR activation to CRC progression and further support the therapeutic relevance of this axis.

Given the emerging demand for sensitive biomarkers in colorectal cancer screening, KRT19 holds potential as a candidate for point-of-care testing (POCT). However, in our current study, the exploration was limited to tissue-level expression and *in vitro* validation. Future studies should be designed to assess the feasibility of using KRT19 detection in minimally invasive samples such as blood, stool, or rectal swabs. Additionally, integration with rapid diagnostic platforms and validation in patient-derived samples are necessary to substantiate its clinical translational value. Until such data is available, the application of KRT19 in POCT should be considered preliminary and exploratory.

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