ORIGINAL RESEARCH

# TSC22D2 Regulates ACOT8 to Delay the Malignant Progression of Colorectal Cancer

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**Purpose:** Colorectal cancer (CRC) is one of the cancers with high incidence and mortality rates worldwide. In China, there are approximately 400,000 new CRC cases each year, seriously endangering people's life and health. Transforming growth factor  $\beta$ -stimulated clone 22 domain family, member 2 (TSC22D2) is widely expression in cancers, but the role of TSC22D2 in CRC are still unknown.

**Methods:** Real-time quantitative PCR (qRT-PCR) and Western blot were applied to determine the TSC22D2 levels. CCK-8, colony formation and transwell assays were used to determine the proliferation and metastasis abilities of CRC cells in vitro. In vivo metastatic potential was assessed using a subcutaneously injected mouse model and. Western-blot and immunoprecipitation experiments were used to study the mechanism of TSC22D2-mediated metastasis.

**Results:** We found TSC22D2 was deregulated in CRC tissues and cells and implied poor prognosis. Overexpression TSC22D2 significantly promoted CRC cells proliferation and tumorigenicity both in vitro and vivo, whereas knockdown TSC22D2 resulted in the opposite effects. Importantly using a co-immunoprecipitation (co-IP) assay combined with mass spectrometry analysis to identify TSC22D2-interacting acyl-coenzyme A thioesterases 8 (ACOT8), TSC22D2 maintained stability of ACOT8. Overexpression of TCC22D2 in CRC cells can promote the expression of ACOT8 and inhibit the proliferation and metastasis of CRC cells through EMT mechanism, highlighting the possibility of TSC22D2 as a potential target in CRC development.

**Conclusion:** In summary, the present study revealed the inhibitory effect of TSC22D2 on the proliferation of colorectal cancer cells, suggesting that TSC22D2 may be an important tumor suppressor and a potential therapeutic target during colorectal carcinogenesis. **Keywords:** colorectal cancer, TSC22D2, ACOT8, EMT

#### Introduction

Colorectal cancer is a human disease with high incidence and mortality rates, and is one of the most common malignant tumors worldwide.<sup>1</sup> With the improvement of people's living standards and changes in diet structure, the incidence rate of colorectal cancer is rapidly increasing.<sup>2</sup> At present, the treatment options for colorectal cancer mainly include surgical resection, radiotherapy, and palliative care. High nutrition and low fiber diet, genetic factors, and certain chronic intestinal diseases accompanied by intestinal mucosal hyperplasia are closely related to the onset of CRC.<sup>3</sup> According to the 2020 Chinese Society of Clinical Oncology (CSCO) guidelines for the diagnosis and treatment of colorectal cancer, molecular diagnosis of colorectal cancer mainly relies on the content of MMR protein,<sup>4</sup> mutation frequency of RAS, BARF genes, and gene fusion level of NTRK and Her2.<sup>5,6</sup> Although these measures have improved the accuracy of CRC diagnosis to some extent, the objective remission rate of CRC is still unsatisfactory. Therefore, better understanding

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of the molecular mechanisms of CRC will be beneficial for tumor typing and personalized treatment, and will improve the overall survival rate of CRC patients.

There is increasing evidence that many TSC22D2 family proteins interact with other proteins to form macromolecular complexes and participate in a wide range of biological processes. For example, TSC-22 acts as a tumor suppressor by safeguarding p53 from poly-ubiquitination mediated-degradation.<sup>7</sup> TSC22D3 inhibits NF-κB nuclear translocation and DNA binding.<sup>8</sup> Sequencing analysis of a family's genes revealed that TSC22D2 is associated with various cancers.<sup>9</sup> Lower levels of TSC22D2 indicate a poor prognosis.<sup>10</sup> TSC22D2 is in the cytoplasm of eukaryotic cells and belongs to the leucine zipper transcription factor TSC22 domain family. Previous studies have found that in colorectal cancer, TSC22D2 repressed the expression of cyclin D1 at both mRNA and protein levels. TSC22D2 physically binds to pyruvate kinase isoform M2 (PKM2), a glycolytic enzyme that has been reported to be associated with the growth and survival of multiple cancer cell types, overexpression of TSC22D2 decreased the level of nuclear PKM2.<sup>11,12</sup> TSC22D2 is found to be lowly expressed in CRC, and is negatively regulated by miR-543-3P, which enhances the malignant progression ability of CRC.

Tumor formation requires a lot of energy, and fatty acid is one of the main energy providers of cell metabolism. Studies have shown that fatty acid synthase (FASN) is involved in tumor development.<sup>13</sup> Acyl-CoA thioesterase 8 (ACOT8) is located on chromosome 20 (20q13.12), a type II thioesterase containing six exons and a peroxisome proliferator response element of about 438bp.<sup>14</sup> ACOT8 has a catalytic role in fat hydrolysis, and it participates in the occurrence and development of various tumors, ACOT8 promotes the growth of HCC cell lines to meet energy requirements by releasing free fatty acids.<sup>15</sup> ACOT8 may through the positive adjustment OXPHOS electron transport chain in ccRCC to influence the development and progress of ccRCC.<sup>16</sup> It has also been shown that ACOT8 synthesizes cholic acid via the classical pathway and is associated with dietary fat emulsification. Fatty acid binding protein (FABP)-1, -4, and -6, PPAR $\gamma$  are increased with the deposition of high unsaturated fatty acids (HUFA), which are involved in the emulsification and transport of fatty acids.<sup>17</sup> However, whether TSC22D2 can promote the malignant progression of CRC by regulating the stability of ACOT8 remains unclear.

Our experiment verified the expression level of TSC22D2 in CRC cells and further analyzed the biological significance of TSC22D2 in CRC. The results of in-vivo and in-vitro experiments showed that overexpression of TSC22D2 inhibited the malignant progression of CRC. Mechanistically, we confirmed that ACOT8 is a downstream target of TSC22D2, and TSC22D2 positively regulates the expression of ACOT8. Meanwhile, the TSC22D2-ACOT8 axis also enhanced CRC cell proliferation via EMT. Thus, the TSC22D2-ACOT8 axis is potentially a prognostic biomarker and a therapeutic target for treating CRC patients.

#### **Materials and Methods**

#### Cell Culture

Human colorectal cancer cell lines (HCT116, Caco2, colo205, SW620) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco), penicillin (100ug/mL), streptomycin (100u/mL) (Sigma, St-Louis, MO, USA), and maintained at 37°C in 5% CO<sub>2</sub> environment.

#### Infection and Transfection

TSC22D2 overexpression plasmid (TSC22D2), knockdown plasmid (si-RNA), empty vector plasmid (Vector), ACOT8 overexpression (ACOT8) and knockdown plasmid empty vector plasmid (si-NC) along with TSC22D2 knockdown lentivirus (sh-TSC22D2) and inserted nonsense sequence (sh-NC) were all purchased from Shandong Weizhen Biological Co., Ltd. Cell transfection experiments were performed using Lipofectamine2000 according to the manufacturer's instructions. For lentiviral infection,  $1 \times 10^5$  SW620 cells were seeded in a 6-well plate 12 hours in advance, infected according to the cell infection procedure provided by the biological company, and infected cells were selected with puromycin for two weeks.

# Quantitative Real-Time PCR (qRT-PCR)

Total RNA in CRC cells was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), RNA concentration was measured using a UV spectrophotometer, reverse transcription was performed using cDNA Synthesis SuperMix, and then real-time fluorescence quantitative PCR was performed using SYBR Green PCR Mix to amplify the mRNA levels. The primer sequences were shown in Table 1

#### Western Blot Assay

CRC cells were collected and total proteins were extracted from CRC cells using RIPA lysis buffer. The total protein concentration was determined using the BCA method (P0011, Beyotime, Shanghai, China) reagent kit. The proteins were loaded onto a 10% SDS-PAGE gel for electrophoretic separation and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk and incubated with primary antibodies TSC22D2 (25418-1-AP, Protechin, Wuhan, China), ACOT8 (CSBPA000805, CUSAB, Wuhan, China), E-cadherin (#3195, Cell Signaling Technology, Beverly, MA, United States), N-Cadherin (#13116, Cell Signaling Technology), Vimentin (#5741, Cell Signaling Technology),  $\beta$ -tubulin (ER60054, HUABIO, Hangzhou, China) overnight at 4°C on a shaker. The membrane was then washed three times with TBST and incubated with secondary antibody (#7074 Cell Signaling Technology) at room temperature for 1 hour, followed by three washes with TBST, and visualized with ECL reagent (Millipore). Blots were imaged using the Amersham<sup>TM</sup> Imager 680 from GE Healthcare Life Sciences.

# Cell Proliferation Assay

The proliferation of CRC cells was assessed using the Cell Counting Kit 8 (CCK-8) (Yeasen Biotechnology, Shanghai, China). We seeded  $5 \times 10^3$  SW620 cells and HCT116 cells evenly in a 96-well plate. At 0h, 24h, 48h, and 72h, we added 10ul CCK-8 reagent to each well, and incubated the plate at 37°C in a 5% CO2 incubator for 1.5 hours. The absorbance at 450nm was measured using a microplate reader. For the colony formation assay,  $3 \times 10^3$  CRC cells were spread in a 6-well plate, the culture medium was gently mixed and changed every three days. After 10 days in the cell culture incubator, the culture medium was discarded, and the cells were fixed with 4% paraformaldehyde for 20 minutes, followed by crystal violet staining. The colonies were then washed, dried, photographed, and counted.

#### Transwell Migration and Invasion Assays

Transwell chambers (8  $\mu$ m, Corning, NY, United States) were placed in 24-well plates, with or without Matrigel (#4234, Corning). We added 150ul of 1×10<sup>5</sup> CRC cell suspension without FBS to the upper chamber of each chamber, and added 750ul of DMEM culture medium with 10% FBS to the lower chamber. After incubating the cells in the cell incubator for 48 hours, the chambers were removed, washed with PBS, fixed with 4% paraformaldehyde, stained with crystal violet, and photographed under an inverted microscope.

#### Immunofluorescent Staining

For immunofluorescence staining experiments, SW620 and HCT116 cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% BSA, and then the cells were incubated with the TSC22D2 antibody at 4°C overnight. Afterward, the cells were incubated with FITC-labeled secondary antibody at room

	Forward Primer	Reverse Primer
TSC22D2	ATGAAGACAGTGCATCTGGGG	TACCAGGATTGGCCTGTTGG
ACOT8	GAGGCTGTTTGGTGGTCAGA	CACGTGGACGTCTTCACTCA
GAPDH	GGCTGAGAACGGGAAGCTTGTCAT	CAGCCTTCTCCATGGTGGTGAAGA

#### Table I Primer

temperature for 1 hour, washed three times with PBS, stained with DAPI, and finally photographed with a fluorescence inverted microscope.

# Tumor Formation Experiments in Mice

Male nude mice were obtained from the Zhejiang Province Experimental Animal Center, and the review and approval of the Institutional Animal Care and Use Committee (IACUC) of ZJCLA were obtained. Nude mice of similar physiological status were divided into two groups for in vivo tumor growth size measurement.  $1 \times 10^6$  HCT116 cells with knockdown or control group TSC22D2 were inoculated in the nude mice. The tumor tissue in the nude mice was removed and analyzed three weeks after inoculation.

# Statistical Analysis

All the above experiments were repeated three times. Data were presented as the mean  $\pm$  SD of which the statistical analyses were performed using GraphPad Prism (GraphPad Software 8.0; San Diego, CA, USA). Unpaired, two-tailed Student's *t*-tests was used to analyze the significance between two groups, whereas one-way ANOVA analysis was used to compare the differences among multiple groups. Differences were defined as statistically significant if P<0.05.

# Results

# The Expression of TSC22D2 in CRC Cell Lines

Using the TCGA GEPIA database found that the expression of TSC22D2 in colorectal cancer tissue is significantly lower than that in normal tissue (Figure 1A). To verify the expression of TSC22D2 in CRC, qRT-PCR and Western blot were used to detect the mRNA and protein expression levels of TSC22D2 in CRC cells, respectively. We found that TSC22D2 was differentially low expressed in four types of CRC cells (Figure 1B and C). To better understand the expression location of TSC22D2 in CRC cells, we confirmed through immunofluorescence that TSC22D2 is expressed in the cytoplasm of cells (Figure 1D). Kaplan-Meier survival analysis showed that the lower expression of TSC22D2 indicates



Figure I TSC22D2 expression in CRC cell lines predicted poor survival. (A) Expression of TSC22D2 in tumor and non-tumor tissues in TCGA database. (B and C) mRNA and protein expression of TSC22D2 in CRC cells. (D) Immunofluorescent images show the TSC22D2 protein primarily localized to the cell cytoplasm. (E and F) TSC22D2 expression and CRC patients' clinical prognosis.

a poor prognosis for CRC patients (Figure 1E and F). Altogether, these results strengthened possible of TSC22D2 be a promising prognostic biomarker for CRC.

#### Inhibition of TSC22D2 Enhances CRC Cell Proliferation and Invasion in vitro

To achieve specific knockdown of TSC22D2, HCT116 cells with higher expression of TSC22D2 were transfected (Figure 2A). CCK-8 results showed that knocking down TSC22D2 enhanced the proliferation ability of CRC cells (Figure 2B). Colony formation experiments showed that knocking down TSC22D2 enhanced the ability of CRC cells to form clones (Figure 2C). Furthermore, through transwell experiments, we found that knocking down TSC22D2 significantly promoted the migration and invasion ability of CRC cells (Figure 2D). To further study the effect of TSC22D2 on the biological behavior of CRC cells, we chose SW620 cells with low expression of TSC22D2 for stable overexpression. Western blot experiments showed that it was significantly overexpressed in SW620 cells (Figure 2E). Then, using CCK-8 to detect the proliferation ability of CRC cells after overexpression of TSC22D2, it significantly decreased after overexpression of TSC22D2 (Figure 2G). In addition, through transwell experiments, we found that the migration ability of SW620 cells stably overexpressing TSC22D2 were significantly weakened (Figure 2H). These research results are sufficient to prove that TSC22D2 inhibits the growth of CRC cells.



Figure 2 TSC22D2 inhibits the proliferation, migration and invasion of CRC cells. (A) HCT116 cells were transfected with control si-RNA (si-NC) or TSC22D2 si-RNA and subjected to Western blot for TSC22D2expression. (B and C). CCK8 assay and Colony assay verified that TSC22D2 knockdown enhanced the capacity of proliferative and colony formation of HCT116 cells. (D). The number of migrating and invading HCT116 cells were increased by TSC22D2 know down. (E–H). SW620 cells transfected with pcDNA/TSC22D2 or empty vector and measured by Western blot for TSC22D2 expression. Proliferation and migration capability of cells were also assessed. \*P < 0.05.

# TSC22D2 Induces EMT to Regulate the Progression of CRC Cells

Epithelial-mesenchymal transition (EMT) is an important marker of the invasion and metastasis process of tumor cells.<sup>18</sup> Next, we investigated the effect of TSC22D2 on EMT. We used Western blot to verify the changes in typical EMT markers in SW620 and HCT116 cells. The results showed that in HCT116 cells with TSC22D2 knocked down, the protein expression level of E-cadherin was significantly downregulated, while the levels of N-cadherin and vimentin increased (Figure 3A). In contrast, in SW620 cells with stable overexpression of TSC22D2, the protein expression level of E-cadherin and vimentin decreased (Figure 3B). These results imply that TSC22D2 may promote the migration and invasion ability of CRC cells by enhancing the EMT process.

#### TSC22D2 Suppresses Tumor Growth in vivo

To study the role of TSC22D2 in tumor development, we constructed a HCT116 cell line with TSC22D2 knocked down and injected it into Male nude mice. The health status of the mice and the growth of tumors in the mice were monitored every three days. Four weeks later, the mice were euthanized, and the tumor tissues were removed and weighed.

As shown in (Figure 4A), compared with control group, HCT116 cells stable knockdown TSC22D2 effectively increased CRC tumorigenesis. The growth curves of subcutaneous tumors formed by HCT116 cells indicated knockdown TSC22D2 markedly promoted tumor growth in vivo, and TSC22D2 knockdown increase the weight of subcutaneous tumors formed by HCT116 cells (Figure 4B and C). The above dates confirm the function of TSC22D2 suppressing CRC occurrence, TSC22D2 knockdown promotes CRC cells growth in vivo.

#### TSC22D2 Triggers the Malignant Progression of CRC Cells via Regulating ACOT8

To better study the specific mechanism by which TSC22D2 regulates the progression of CRC, we found through the GEPIA database that TSC22D2 and ACOT8 are positively correlated. This was also confirmed by immunoprecipitation-tandem mass spectrometry (IP-MS) (Figure 5A and B). The role of ACOT8 in cancer has been previously reported.<sup>19,20</sup>



Figure 3 TSC22D2 altered the expression of EMT-related proteins. ( $\mathbf{A}$  and  $\mathbf{B}$ ) E-cadherin, Vimentin, N-cadherin were detected by Western blot in HCT116 cells and SW620 cells transfected with si-RNA or TSC22D2 plasmid. \*P < 0.05.



Figure 4 TSC22D2 inhibits CRC growth in mice. (A). Subcutaneous tumorigenesis in mice was evaluated after PPP1R14B knockdown. (B and C) Both tumor volume and tumor weight in TSC22D2 knockdown group (n=5) were obviously higher than those in control group (n=6). \*P < 0.05.



Figure 5 Identification of TSC22D2-interacting proteins using co-IP/MS. (A). There were 8 overlapping TSC22D2 binding proteins identified. (B). Correlation of TSC22D2 with ACOT8 in the GEPIA database. (C). ACOT8 was immunoprecipitated by TSC22D2 in HCT116 cells. (D). Expression of ACOT8 mRNA in tumor and non-tumor tissues in TCGA database.

There is growing evidence that ACOT8 can serve as a novel cancer marker.<sup>16,21</sup> The interaction between ACOT8 and TSC22D2 was further confirmed by immunoprecipitation (IP) analysis (Figure 5C). To investigate the role in CRC, we use TCGA database to evaluate ACOT8 mRNA expression in CRC (Figure 5D). ACOT8 is expressed to varying degrees in four different CRC cell lines as we showed before (Figure 1C). When we knocked down TSC22D2 as described above, we found that the protein expression level of ACOT8 also showed a downward trend. Conversely, overexpression of TSC22D2 increased the protein level of ACOT8 (Figure 2A and E). To explore the function of ACOT8 in CRC, we knocked down or overexpressed ACOT8 in corresponding CRC cells and used Western blot to detect the knockdown or overexpression effects. The experimental results showed that knocking down ACOT8 promoted the proliferation ability of CRC cells (Figure 6A–D). Conversely, overexpression of ACOT8 inhibited the proliferation, migration, invasion, and clone formation of CRC cells (Figure 6E–H). Altogether, these dates support the notion that TSC22D2 suppresses the CRC cells growth by regulates ACOT8.

#### Dsicussion

Colorectal cancer is one of the common malignant tumors of the digestive tract.<sup>22</sup> CRC research has attracted much attention. Though numerous therapeutic strategies have developed to improve the treatment outcomes of CRC, it is remaining a devastating disease with complex and poorly elucidated physiopathology.<sup>23,24</sup> It is urgent to explore the potential therapeutic target for CRC. In the study, we demonstrated TSC22D2 lower expression in CRC tissues than normal liver tissues. Mechanistically, we found TSC22D2 promoted CRC progression via regulating ACOT8 stability.

The TSC22 family has been widely verified to have this function, and members of the TSC-22 family can bind with other proteins to form large biomolecules and function.<sup>25</sup> For example, TSC22D1, as a transcription factor, forms a complex with FGFR2 to inhibit the occurrence and development of CRC.<sup>26</sup> TSC22D1 can induce cell senescence and thus play an anti-tumor role, and TSC22D1 has also been found to promote cell apoptosis in cervical cancer.<sup>27</sup> TSC22D3



Figure 6 ACOT8 inhibits the proliferation, migration and invasion of CRC cells. (A). HCT116 cells were transfected with control si-RNA (si-NC) or ACOT8 si-RNA and subjected to Western blot for TSC22D2 expression. (B and C). CCK8 assay and Colony assay verified that ACOT8 knockdown enhanced the capacity of proliferative and colony formation of HCT116 cells. (D). The number of migrating and invading HCT116 cells were increased by ACOT8 know down. (E–H). SW620 cells transfected with pcDNA/ACOT8 or empty vector and measured by Western blot for ACOT8 expression. Proliferation and migration capability of cells were also assessed. \*P < 0.05.

has also been frequently confirmed to be beneficial for the treatment of diabetic retinopathy after activation by glucocorticoids.<sup>28,29</sup>

However, there is very little research on TSC22D2 in tumors. In this study, we demonstrated that TSC22D2 was associated with CRC patients' poor prognosis. We investigated the expression and biological functions of TSC22D2 in CRC by analyzing the data from the publicly available databases. We verified the mRNA and protein expression levels of TSC22D2 in CRC by qRT-PCR and Western blot. Knockdown of TSC22D2 enhanced the proliferation, migration, invasion and EMT of CRC cells in vitro and significantly promoted the growth of CRC tumors in vivo. While TSC22D2 overexpression decreased the proliferation of CRC cells, indicating TSC22D2 could function as a tumor suppressor in CRC. Moreover, we used tandem liquid chromatography to identify proteins that interact with TSC22D2 and confirmed that TSC22D2 inhibits the growth of CRC cells by regulating ACOT8. ACOT8 is involved in the proliferation, survival, and metabolic reprogramming of tumor cells.<sup>19</sup> In the process of cell apoptosis, ACOT8 inhibits tumor cell apoptosis.<sup>30</sup>

In this study, we found that the stability of ACOT8 protein was subjected to TSC22D2. In addition, using the GEPIA database, we found that TSC22D2 is positively correlated with ACOT8. Western blotting revealed that the level of ACOT8 protein was affected upon TSC22D2 expression. We further demonstrate that TSC22D2 regulation of ACOT8 retarders CRC progression mediated by EMT. This suggests that TSC22D2 by influencing the ACOT8 state to promote the progress of CRC.

However, our work also had some limitations, and raised questions that would be solved by further experiments. First, we performed an IP assay to identify the interacting partner of TSC22D2. Nevertheless, it could not be concluded that the

other protein candidates were not able to bind with TSC22D2. Second, we determined the downstream destination of TSC22D2, but we did not determine the upstream translation mechanism of TSC22D2, mining the specific proteins and mechanisms that drive the translation of TSC22D2 will be part of our future studies.

# Conclusion

In summary, our research confirmed the expression of TSC22D2 in CRC and its ability to predict poor clinical prognosis. Furthermore, ACOT8 is recognized as a novel target of TSC22D2 and participated in TSC22D2-induced tumor progression of CRC. Those findings suggest that TSC22D2 may represent a novel biomarker for CRC and serve as a potential therapeutic target for CRC treatment.

# **Institutional Board Statement**

The animal study protocol followed the Guide for the Care and Use of Laboratory Animals and was approved by the Zhejiang Provincial Animal Care and Use Committee. (Ethics Code: ZJCLA-IACUC-20040166).

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# Disclosure

The authors report no competing interests in this work.

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