

Research on the Mechanism of CHDH in the Metastasis of Colorectal Cancer

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Abstract Colorectal cancer (CRC) is currently one of the most serious types of cancer in the world, with one of the highest morbidity and mortality rates of all cancers. Such a high fatality rate makes CRC a research hotspot in recent years. Cancer metastasis is one of the main reasons leading to the high mortality rate of cancer, CRC is no exception, CRC is very easy to develop liver metastasis, nearly half of the patients will suffer from liver metastasis. Therefore, the transfer of CRC was studied in this study. Choline dehydrogenase is a vital enzyme in the human body, and its metabolites can act as carbon units in carbon metabolism. Previous research has shown that CHDH is a breast cancer marker. As a result, the involvement of the Choline Dehydrogenase (CHDH) gene in CRC metastasis was investigated in this work. The findings indicated that CHDH might influence colorectal cancer invasion and migration by influencing the Epithelial-mesenchymal Transition (EMT) process of CRC. **Abbreviations:** CRC = Colorectal Cancer, CHDH = Choline Dehydrogenase, EMT = Epithelial-mesenchymal Transition, FDA = Food and Drug Administration, SEPT9 = Receiver Operating Characteristic, NDRG4 = NDRG4 (Human) Recombinant Protein, BMP3 = Bone Plasticin 3, DMEM = dulbecco's modified eagle medium, FBS = Fetal Bovine Serum

Keywords: choline dehydrogenase, colorectal cancer, Epithelial-Mesenchymal transition

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1. Introduction

Colorectal cancer (CRC) is now one of the most serious cancers. According to the statistics of the 2020 study, the incidence and mortality of colorectal cancer were 10% and 9.4%, respectively, ranking among the top three cancers [1]. And, when statistics from 2015 to 2020 were compared, it was shown that the incidence and death of colorectal cancer rose the greatest [2,3]. The high morbidity and mortality of colorectal cancer has much to do with its special development process. There are no obvious symptoms of colorectal cancer in the early stage, and because hospitals' primary detection method is colonoscopy, this invasive detection method causes many potential patients to disregard the routine examination, resulting in the vast majority of colorectal cancer patients developing to stage III or stage IV once diagnosed, or having metastasized to other tissues, such as the liver [4-9]. Because of the current severity of colorectal cancer, study into its pathophysiology is becoming increasingly crucial. A greater understanding of colorectal cancer pathophysiology will make it easier to find treatment targets in the future.

Studies on the mechanism of colorectal cancer are being conducted increasingly frequently as the disease's

severity increases. It is exceedingly challenging to make an early diagnosis of CRC since there are no clear symptoms in the early stages. As a result, several research are seeking for early CRC diagnostic indicators. For example, SEPT9 and the combination of NDRG4 and BMP3 have been approved by the FDA for the diagnosis of colorectal cancer based on DNA methylation [10]. And a novel cell-free DNA (cfDNA) for early diagnosis of CRC. And a novel cell-free DNA (cfDNA) based methylation could help in the early diagnosis of CRC [11]. At the same time, various non-coding RNAs, including as miR-21, miR-31, miR-92a, lncRNA NEAT1, and others, can be used as markers for the early diagnosis of CRC to improve specificity [12-15]. Some biomarkers are more likely to be associated with the early diagnosis of CRC, but since CRC is also a cancer that spreads easily, our study wanted to explore the mechanism of CRC spread.

Choline is critical for the maintenance of life activities and is required for the maintenance of cellular structure and function. Choline is another key component in carbon metabolism. Choline dehydrogenase (CHDH) is an enzyme that catalyzes the dehydrogenation of choline to betaine aldehyde, which then acts as a methyl donor in carbon metabolism [16]. Aberrant carbon metabolism will result in aberrant DNA methylation, increasing the progression of cancer. As an essential enzyme in carbon metabolism, the content of CHDH may thus have a role in

the onset and progression of cancer [17]. However, research into the mechanism of CHDH in cancer is still in its early stages. According to several research, CHDH can be utilized as a prognostic biomarker for breast cancer [18]. Furthermore, choline metabolism has been revealed to have a significant role in the etiology and progression of breast cancer [19]. There is currently no report on the study of CHDH in colorectal cancer. This article focuses on the role of CHDH in the occurrence and development of colorectal cancer.

2. Methods

2.1. Cell Lines and Cell Culture

Four colorectal cancer cell lines (HT29, LOVO, SW480 and HCT116) and normal colon epithelial cells (NCM460) were purchased from the cell bank of the Shanghai Institute of Biological Sciences (Shanghai, China). SW480 and HCT116 were grown in DMEM medium (MA0212, Meilunbio, China) with 10% FBS (PWL001, Meilunbio, China) and 1% penicillin/streptomycin (MA0110, Meilun, China), whereas HT29, LOVO, and NCM460 were cultured in RPMI-1640 medium (MA0215, Meilunbio, China) with 10% FBS and 1% penicillin/streptomycin. All cells were grown at 37°C in a humidified atmosphere with 5% CO₂.

2.2. Bioinformatics Analysis

To forecast the potential role of CHDH in colorectal cancer, we first utilized the public databases GEPIA2 (<http://gepia2.cancer-pku.cn/>) and Kmplot (<http://www.kmplot.com/>) to assess the expression level, survival, and staging of the CHDH gene in colorectal cancer.

2.3. RNA Extraction and Reverse Into cDNA

Trizol (BS258A, Biosharp, China) was used to extract RNA from normal and colorectal cancer cells according to the instructions provided by the reagent manufacturer. The quality of RNAs was measured by NanoDro 2000 c (Thermo Scientific, USA). Then, 2 µg RNA was used to reverse the extracted RNA into cDNA using MonScrip™ RTIII Super Mix with dsDNase (Two-Step) (MR05201, Monad, China). The cDNA was stored at -20°C for subsequent experiments.

2.4. Agarose Gel Electrophoresis Assay

cDNA was amplified by PCR using MonAmp™ 2x Taq Mix Pro(+Dye) (MP05401, Monad, China). PCR amplification reaction procedure was 94°C for 5min, followed by 32 cycles of 94°C for 15 s, 65°C for 15 s, 72°C for 10s, last, 72°C for 5min. According to the size of the target gene fragment, we selected 1.5% agarose gel for electrophoresis to detect gene expression. The electrophoresis was stopped when the bromophenol blue indicator moved to the middle of the agarose gel and was developed and photographed by the developer.

2.5. Quantitative Real-Time PCR (qRT-PCR) Assay

cDNA was mixed with primers and MonAmp™ ChemoHS qPCR Mix (MQ00401, Monad, China) for qPCR, three repeat holes were set, and the experiment was repeated three times. The reaction procedure of qPCR was 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s. The melting curve uses the instrument's default acquisition procedure. Primer sequences for PCR and qPCR are listed in the table below.

Table 1. The sequences of primers and siRNA for PCR and qPCR

Gene	Primers sequences	
	Forward (5'-3')	Reverse (5'-3')
GAPDH	gtcagtgggtggacctgacct	tgctgtagccaaattcgttg
CHDH	agggtccatcaactctccac	gcatacctgctgaatgtaga
E-cad	ttgagtgtctgcacagagg	gaggagctgagtgaacctg
N-cad	cagagagtcgccaatgtca	ttcacaagtctcgccctctt
VIM	gagaactttgccgttgaagc	tccagcagcttctgtaggt
siCHDH	ggacaugaccauccauggaatt	uucauggauggucaugucctt

2.6. Western Bolt Assay

RIPA reagent (MA0151, Meilun, China) was used to extract proteins from cells, followed by BCA reagent to determine protein concentration, and 10% SDS-PAGE was chosen based on the intended protein band size. The protein was then transferred to a PVDF membrane and blocked for one hour and thirty minutes at room temperature with 5% milk. GAPDH (10494-1-AP, PTG, 1:20000), CHDH (17356-1-AP, PTG, 1:5000), E-cad (ab231303, Abcam, 1:10000), N-cad (ab76011, Abcam, 1:1000), and VIM (ab92547, Abcam, 1:5000) primary antibodies were incubated with the protein overnight at 4°C. The membrane was then treated for 1 hour at room temperature with anti-mouse secondary antibody and anti-rabbit secondary antibody, with the results being analyzed with an ECL kit and ImageJ software. All Western Blot results were determined by repeating the experiment three times to obtain the same death result.

2.7. Silencing of CHDH

Knockdown experiments on the CHDH gene were performed using short RNAs (siRNA) and transfection using Lipofectamine 2000 (Invitrogen, USA) reagents. The medium was changed to complete medium 6h after transfection. After 48 hours of siRNA transfection, cells can be resetting or used to extract RNA/ protein extracted for further experiments. Following an effective determination of the interference impact of siRNA on the CHDH gene by three replicated studies, siRNA was employed in additional tests.

2.8. Wound Healing Test

The transfected cells were re-suspended and counted, and re-inoculated into 6-well plates with 1×10⁶ cells per well. After 24 hours, the line was crossed and photographed. After that, cell migration was observed and recorded under the microscope every 24 hours.

2.9. Transwell Test

Matrix glue (BD Biosciences, MA) was added to the upper chamber of the invasion group. After 30 minutes in the incubator, the excess matrix glue solution was sucked out. The cells after transfection were resuspended using serum-free medium, and the number of cells was adjusted to 1×10^6 cells/ml. 200 μ l was added to the upper chamber of the chamber, and 600 μ l complete medium was added to the lower chamber of the chamber. The chamber was removed 24 hours later, the excess cells and matrix glue in the upper chamber were wiped off, cleaned twice with PBS, and then fixed with paraformaldehyde for 10 minutes, stained with 0.1% crystal violet (C8470, Solarbio, China) for 30 minutes, and finally observed and photographed under microscope.

2.10. Statistical Analysis

The results are expressed as the mean $\pm$ SEM of at least three independent experiments unless stated otherwise. Paired data were evaluated by a Student's *t* test. A one-way ANOVA was used for multiple comparisons. Statistical analyses were

performed with GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). A *p* value of <0.05 was considered significant.

3. Results

3.1. Bioinformatics Results Showed that CHDH Was Highly Expressed in Cancer Cells

We searched some basic data of CHDH gene in colorectal cancer through public database. Firstly, pan-cancer analysis found that the expression level of CHDH gene was up-regulated in five types of cancer, and the differences in colorectal cancer were relatively significant (Figure 1 A). Then, we searched the expression level, survival and cancer stage of CHDH gene in colorectal cancer in GEPIA2, and found that CHDH gene was highly expressed in colorectal cancer (Figure 1 B, $*P < 0.05$). At the same time, it was found that the CHDH gene is significant in the survival and cancer cycle analysis of colorectal cancer, so the research of CHDH gene in colorectal cancer was of great significance (Figure 1 C, D).

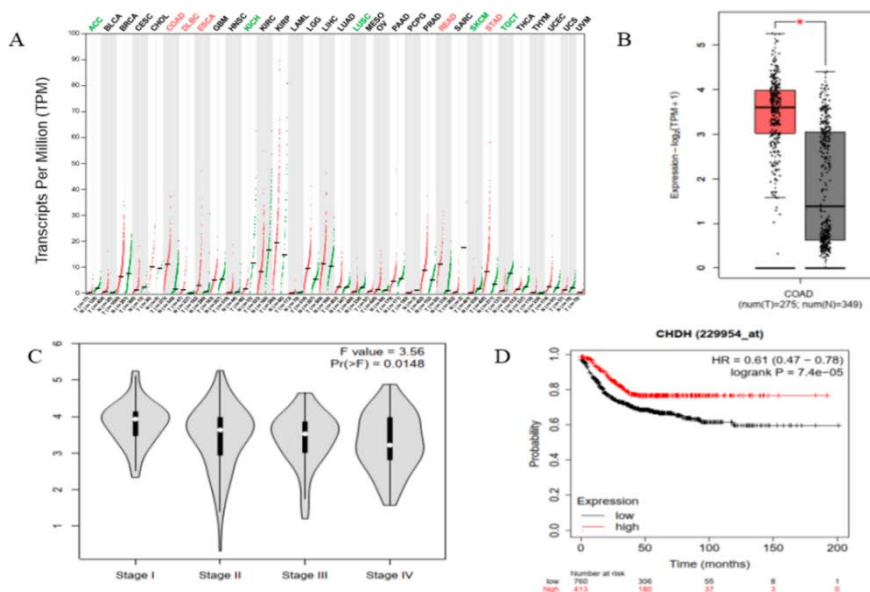


Figure 1. Bioinformatics analysis of CHDH gene. (A) The level of CHDH gene expression in all cancers was determined by pan-cancer assays. (B) Expression of CHDH gene in normal and CRC tissues. (C) The effect of CHDH gene on the clinical staging of CRC. (D) Effect of CHDH gene on survival of CRC patients. $*P < 0.05$.

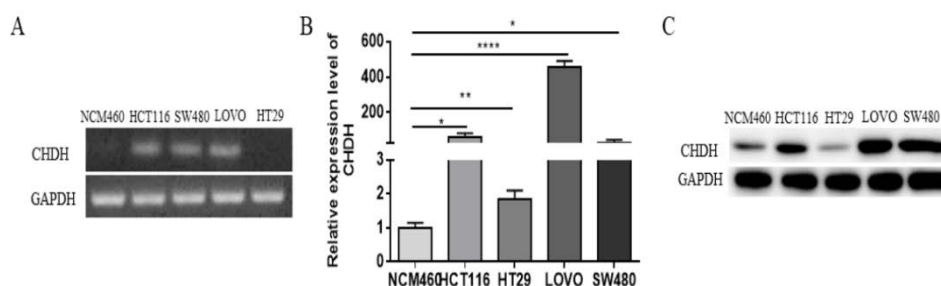


Figure 2. Expression of CHDH gene in colon epithelial cells and colorectal cancer cells. (A) Agarose gel electrophoresis of CHDH expression in normal colon epithelial cells (NCM460) and four CRC cells (HCT116, SW480, HT29, LOVO). (B) qPCR results of CHDH expression in normal and CRC cells, $*P < 0.05$ versus NCM660 and HCT116, $**P < 0.01$ versus NCM460 and HT29, $***P < 0.001$ versus NCM460 and LOVO, $*P < 0.05$ versus NCM660 and SW480. (C) WB results of CHDH expression in normal and CRC cells.

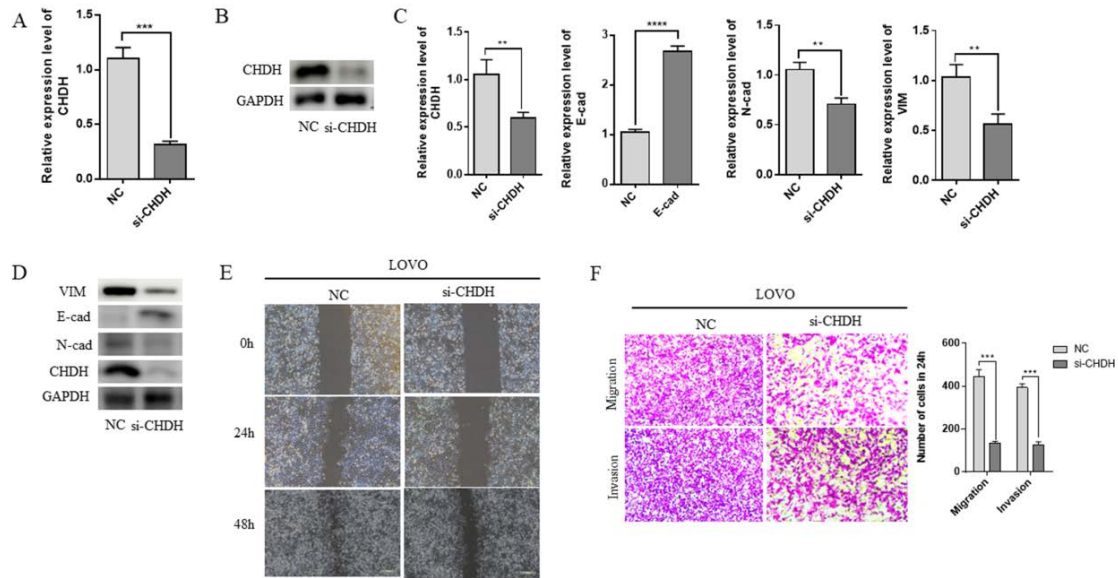


Figure 3. Effect of CHDH gene on tumor EMT process. (A, B) Effect of siRNA knockdown on CHDH gene, *** $P < 0.001$. (C, D) Changes in RNA and protein expression levels of several classic EMT genes after CHDH gene knockdown. ** $P < 0.01$, *** $P < 0.001$. (E) Wound healing test results after knocking down CHDH. (F) Transwell results were obtained after CHDH was knocked down.

3.2. CHDH Gene Is Highly Expressed in Colorectal Cancer

The level of CHDH expression in NCM460 and four types of colorectal cancer cells was checked using agarose gel electrophoresis, qPCR, and WB. The results of agarose electrophoresis and qPCR all demonstrated that CHDH expression was up at the RNA level. There was no significant change in the expression level of HT29 cells in WB results, but the expression level of the other three CRC cells was dramatically raised, which was compatible with the bioinformatics analysis results (Figure 2 A, B, C). As a result, we confirmed the high level of CHDH expression in CRC and postulated that CHDH was a carcinogenic component.

3.3. CHDH Gene Affects Tumor Progression by Influencing EMT Process

We chose LOVO cells for the upcoming experiment because earlier research revealed that LOVO cells had a high and largely steady level of CHDH gene expression. To examine the impact of CHDH gene knockdown on the development of colorectal cancer, we employed short interfering RNA to silence the CHDH gene. The wound healing test revealed that following CHDH gene suppression, LOVO migration slowed considerably. Then we detected the expression levels of E-cad, N-cad and VIM, and the results showed that after the silencing of CHDH gene, the expression levels of RNA and protein of E-cad were increased, while the expression levels of RNA and protein of N-cad and VIM were decreased. We then performed the transwell experiment and wound healing test using LOVO cells. We examined the cell migration at 0h, 24h and 48h under the microscope in Wound healing test. The results showed that the migration ability of LOVO cells was weakened when CHDH gene was

knocked down, indicating that high expression of CHDH gene was more conducive to the migration of cancer cells (Figure 3 E). Transwell results showed that the number of LOVO cells invaded or migrated decreased after CHDH was knocked down in both migration and invasion experiments, which also proved that CHDH gene can promote the migration of cancer cells (Figure 3 F).

These findings demonstrated that CHDH gene knockdown suppressed tumor EMT. Therefore, the tumor migration was inhibited after CHDH gene knockdown, thus inhibiting the tumor process. Therefore, in CRC, CHDH can promote EMT process, thus promoting cancer migration and invasion.

4. Discussion

Human choline dehydrogenase (CHDH), a key enzyme in choline metabolism, has been linked to the etiology of several illnesses, including cancer and metabolic disorders. After mitochondrial damage, CHDH can take part in mitochondrial autophagy [20,21]. According to studies, single nucleotide polymorphisms (SNPS) of CHDH are significant contributors to the development of cancer. SNPS can influence choline metabolism, which in turn influences cancer. Low expression of CHDH has been linked to a high risk of recurrence after taking moxifen monotherapy, according to research on breast cancer, which also indicated that low expression of CHDH is more favorable to the advancement of breast cancer [22,23]. In addition, CHDH inhibits the occurrence of pancreatic cancer, but promotes the progression of gastric cancer, hepatocellular carcinoma, head and neck squamous cell carcinoma, and clear cell renal cell carcinoma [24]. Current studies have found that the expression of CHDH in different tumors is different, but these studies have not involved colorectal cancer, as the incidence and mortality of cancer in recent years, second

only to breast cancer, the severity of colorectal cancer is self-evident, so our study has also taken colorectal cancer as the object to explore the important role of CHDH gene in the occurrence and development of colorectal cancer.

In this study, colorectal cancer was selected as the research object, and the role of CHDH gene in colorectal cancer metastasis was revealed, and it was found that CHDH could promote colorectal cancer migration by promoting tumor EMT process. CHDH is an important enzyme in the body, choline under the action of CHDH can be converted into betaine, betaine contains three methyl groups, can use one methyl group to produce methionine, and then S-adenosine methionine (SAM), the other two methyl groups can participate in folate mediated carbon metabolism of a carbon unit [25]. Carbon metabolism plays an important role in the energy cycle and other processes in the body, and can participate in the methylation process of DNA, RNA and protein. If this process is faulty, it is easy to lead to cancer. Therefore, this is also one of the reasons why CHDH plays a role in tumors. Choline is also an important nutrient for human metabolism, which plays a crucial role in the synthesis of cell membranes, and abnormal choline metabolism can also lead to changes in cell homeostasis. The role of the CHDH gene in various cancers has been reported in recent years, but the role in CRC has not been reported. Because colorectal cancer is prone to liver or lung metastasis, our study focused on whether CHDH has an effect on CRC metastasis. Our study found that CHDH is highly expressed in colorectal cancer. Therefore, we first knocked down CHDH in CRC cells, and then detected changes in some EMT-related markers and regulators at the RNA level and protein level. Our results suggest that the CHDH gene may contribute to tumor progression by promoting EMT progression in colorectal cancer.

The present study demonstrated that CHDH gene knockdown by siRNA can be used to influence tumor migration by affecting EMT processes. However, the role of CHDH gene in CRC migration and invasion still needs to be further studied. The next step is to further explore the specific mechanism by which CHDH affects EMT process. In the next step, RNA sequencing was conducted on cells with CHDH gene knockdown. Through sequencing analysis, enrichment of differential genes was screened and related signaling pathways or transcription factors were selected. The expression of these differential genes was detected by qPCR and WB, and appropriate genes were selected for further knockdown or overexpression verification, so as to refine the specific mechanism of CHDH affecting the EMT process of colorectal cancer.

Although the mechanism of action of CHDH gene in tumor has been reported, and it has been found to be closely related to tumor proliferation and prognosis, there is no research on CHDH as a serum marker for the diagnosis of cancer. As an important enzyme in the body, CHDH also plays an important role in the process of cancer. Therefore, the future research on CHDH is still very meaningful, and the research on CHDH gene is still promising and significant. Despite the fact that there have been few research on the physiological and biochemical properties of CHDH, it is clear from the ones that have been done so far that it has the potential to be used as a

biomarker for malignancies. The structure of CHDH and the design and manufacturing of particular small molecule inhibitors or CHDH activators can be the focus of future research, resulting in new targets and treatment approaches for metabolic diseases and malignancies. On the other hand, molecular diagnostics that targets the SNPS of the CHDH gene can also offer a fresh approach of detecting cancers or other metabolic illnesses, as well as their prognosis.

5. Conclusion

In summary, we detected the expression levels of several EMT-related genes by knocking down CHDH gene, and verified the results of cell migration and invasion by scratch assay and Transwell assay. Finally, we concluded that CHDH gene can promote tumor metastasis by promoting the EMT process of CRC.

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Availability of Data and Material

The data supporting the findings of this work are available from the corresponding author upon reasonable request.

Conflicts of Interest

There is no conflict of interest between authors.

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