Original Article
CCND1 as an unfavorable factor mediated miR-340 suppression of chemotherapy resistance for 5-Fu in colon adenocarcinoma

Jing Wen Jiang¹,²*, Yi Yu Chen¹,³*, Xue Wu Chen², Wei Yi Fang¹, Rong Cheng Luo¹

¹Cancer Center, TCM-Integrated Hospital, Southern Medical University, Guangzhou 510310, PR China; ²Hainan Province Hospital of Traditional Chinese Medicine, Hainan 570203, PR China; ³Cancer Research Institute, Southern Medical University, Guangzhou 510515, PR China. *Equal contributors.

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Abstract: Aims: In this investigation, we explored the expression of CCND1 and the correlation between nuclear expression of CCND1 and clinicopathologic features including survival prognosis in colon adenocarcinoma (CRC). Further, we investigated CCND1 as a target of miR-340 participating in miR-340-induced inhibition of chemotherapy resistance for 5-Fu in colon adenocarcinoma. Methods: The mRNA and protein expression of CCND1 in CRC tissues and colon tissues was examined by real-time PCR, western blot, and immunohistochemistry respectively. The relationship between the nuclear expression levels of CCND1 and clinical features including survival prognosis was analyzed. Further, siCCND1 or miR-340-induced chemotherapy sensitivity to CRC was investigated. Finally, CCND1 as a direct target of miR-340 in CRC was examined. Results: The mRNA levels of CCND1 was markedly higher in CRC tissues than their pair colon tissues (P=0.0017). Further, elevated CCND1 protein expression was indicated in cell nucleus of CRC tissues compared to colon tissues. High levels of CCND1 nuclear protein were positively correlated with T stage (P<0.001) of CRC patients. Patients with higher CCND1 nuclear expression had a significantly shorter overall survival time than patients with low CCND1 nuclear expression. Multivariate analysis indicated CCND1 nuclear expression is an independent prognostic indicator (P=0.004) for patients with CRC. Furthermore, overexpression of CCND1 or suppression of miR-340 significantly decreased chemotherapy sensitivity of 5-Fu to CRC cells. Finally, CCND1 was validated as a direct target of miR-340 in CRC. Conclusion: CCND1 as an unfavorable factor mediated miR-340 suppression of chemotherapy resistance for 5-Fu in colon adenocarcinoma.

Keywords: CCND1, CRC, nuclear expression, 5-Fu, miR-340

Introduction
Cyclin D1 (CCND1) belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. In the previous studies, increased CCND1 expression has been frequently found in a variety of tumors including epithelial liver cancer, ovarian cancer, nasopharyngeal carcinoma, gastric cancer, and lung cancer and may contribute to tumorigenesis [1-6].

Colorectal cancer (CRC) (also known as colon cancer, rectal cancer or bowel cancer) is the development of cancer in the colon or rectum (parts of the large intestine). It has become one of the most common malignant gastrointestinal carcinomas with a rising morbidity and mortality currently. Over one million cases was diagnosed and 0.6 million people died (8.1% of all cancer deaths) in 2008 [7], and approximately 3~4% of all CRC [8], and 5-15% of CRC diagnosed before age 50 years [9, 10]. Colon adenocarcinoma as a predominantly histologic subgroup in colorectal cancer is one of the leading causes of cancer death in developed countries. In previous study, CCND1 was observed to be co-existed with VEGF indicating poorer disease-free survival rates and overall survival rates in colorectal cancer [11]. Furthermore, it was also found to be positive correlation with P-Stat5 expression, which was associated with shorter survival in patients with...
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CCND1 is a target of miR-340 in CRC [12]. However, the expression pattern of CCND1 and its correlation with clinical features including prognosis were seldom reported in colon carcinoma alone.

miR-340 is a tumor-inhibitory miRNAs that has been documents in some tumors. Furthermore, it can reverse cisplatin resistance of hepatocellular carcinoma cell lines by targeting Nrf2-dependent antioxidant pathway [13]. However, whether miR-340 induced chemotherapy sensitivity of 5-Fu to tumor cells was never been reported [14, 15].

In this study, we investigated the expression pattern of CCND1 in colon cancer tissues at mRNA and protein levels and evaluated the correlation of its nuclear expression with clinicopathologic features and patient survival. Our results hint CCND1 as an unfavorable prognostic factor promoting the pathogenesis of colon cancer patients.

Materials and methods

Sample collection

20 fresh paired colon adenocarcinoma and colon tissues were collected from the People’s Hospital of Zhongshan City, China, at the time of diagnosis before therapy. All fresh samples were preserved immediately in liquid nitrogen. A tissue array including 90 paired paraffin-embedded colon cancer samples and colon samples was were purchased from the National Engineering Center for BioChips in Shanghai, China. Furthermore, we also collected 41 paraffin-embedded colon adenocarcinoma samples from the People’s Hospital of Zhongshan City.

For the use of these clinical materials for research purposes, prior consent from the patients and approval from the Ethics Committees of this hospital was obtained. All specimens had confirmed pathological diagnosis and were staged according to the 2010 colorectal cancer staging system of the AJCC/UICC.

Real-time polymerase chain reaction (PCR)

Differential mRNA expression of CCND1 was measured using Real-time PCR in 20 paired colon cancer tissues and colon tissues using a Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) and SYBR Premix Ex Taq (Takara, Shiga, Japan), as described previously [16]. The sense primer was 5’-GCAGCAGAAA-GCGAGAC-3’, and the anti-sense primer was 5’-ACTCTGTTCCTGCGAC-3’. The ACTB gene was amplified as an internal control using the sense primer 5’-TAAGGAGAACGTGCTAGC-3’ and anti-sense primer 5’-GACTGCATCTCCT-GCTT-3’.

Protein extraction

For four paired colon adenocarcinoma samples and colon samples were frozen using liquid nitrogen and then respectively ground by hand to a fine powder with mortar and pestle. Subsequently, nuclear protein was extracted from these tissue powders according to the instruction of nuclear protein extraction kit (Gaiji Inc, Nanjing, China). Cell protein extraction had been described in our previous report [5].

Western blot

Protein lysates were resolved on 10% SDS polyacrylamide gel, electro transferred to polyvinylidene fluoride membranes (Invitrogen, Inc. Carlsbad, CA, USA), and blocked in 5% non-fat dry milk in Tris-buffered saline, pH 7.5 (100 mM NaCl, 50 mM Tris and 0.1% Tween-20). Membranes were immunoblotted overnight at 4°C with an CCND1 antibody at a dilution of 1:400 (Abcam, USA) and a histone3 antibody or GAPDH at a dilution of 1:1000 (Cell Signaling Technology Inc, USA), followed by their respective horseradish peroxidase (HRP)-conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Immunohistochemistry

Immumohistochemistry was done as described [17] previously with a rabbit anti-human CCND1.
**Figure 2.** Elevated nuclear expression of CCND1 promoted the progression and poor prognosis in CRC. A. Increased CCND1 protein was predominantly expressed in CRC tissues compared to colon tissues by western blot assays. B. CCND1 protein was examined between CRC and colon tissues by immunohistochemistry assay. Case 1: Negative of nuclear CCND1 protein in CRC and colon tissues; Case 2 and 3: Positive expression of nuclear CCND1 protein in CRC, but negative expression in colon tissues. C. Positive nuclear expression of CCND1 protein was negatively correlated with the overall survival time for CRC patients. D. Nuclear CCND1 expression in clinical stage III+IV, but not clinical stage I+II, was shown to promote the poor prognosis and lead to the shorter overall survival time for CRC patients by strata analysis.

**Table 1.** The expression of CCND1 in colon cancer (CRC) and colon tissue

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<th>N</th>
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<th>P value</th>
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<tr>
<td>Colon adenocarcinoma</td>
<td>131</td>
<td>High 71 Low 60</td>
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</tr>
<tr>
<td>Colon tissue</td>
<td>90</td>
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polyclonal antibody at concentration of 1:100 (Santa Cruz Biotechnology, USA) (Santa Cruz Biotechnology, USA). Sections were visualized with DAB and counterstained with hematoxylin, mounted in neutral gum, and analyzed using a bright field microscope. The stained tissue sections were reviewed separately by two pathologists blinded to the clinical parameters and evaluated for the presence of nuclear staining. Tumor cells with nuclear staining more than or equal to 10% were considered as positive nuclear expression. Less than 10% staining was regarded as negative nuclear expression.

**MTT cytotoxicity assay**

Colon cells (HCT116 and SW-480) were respectively seeded in 96-well plates in 100 μl 1640 medium supplemented with 10% FBS at 5×10³ cells/well. Once the cells attached, they were respectively treated by siCCND1 or miR-340 with 5-Fu in 5, 10, 20, 40, 60 or 80 μM (250 mg/ml) and incubated at 37°C in 5% CO₂ for 48 h. Subsequently, 10 μl of MTT (5 mg/ml) (Sigma, StLouis, MO, USA) was added to each well, and the plates were incubated at 37°C for 4 h. Further, the supernatants were removed and 100 μl of DMSO (Sigma) was added to each well. The absorbance value (OD) of each well was measured at 490 nm and half maximal inhibitory concentration (IC50) was calculated. Experiments were performed three times.

**Transient transfection with miR-340 mimics and its inhibitor**

miR-340 mimic and its inhibitor were designed and synthesized by Guangzhou RiboBio (RiboBio Inc, China). Twenty-four hours prior to transfection, colon cancer cells were plated onto a 6-well plate or a 96-well plate (Nest, Biotech, China) at 30-50% confluence. They were then transfected into cells using Turbo-
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FectTM siRNA Transfection Reagent (Fermentas, Vilnius, Lithuania) according to the manufacturer’s protocol. Cells were collected after 48 hr for further experiments.

miRNA target validation

CCND1 were predicted to be directly regulated targets of miR-340 miRwalk softwares (University of Heidelberg, Mannheim, Germany). A 312 fragment of CCND1 3’UTR amplified by PCR primers was cloned into psiCHECK-2 vectors (named wt). Site-directed mutagenesis of the miR-340 binding site in CCND1 3’UTR was performed using GeneTailor Site-Directed Mutagenesis System (Invitrogen; named mt). For reporter assays, wt or mt vector and the control vector psiCHECK-2 vector were cotransfected into SW480 cells with miR-340 mimics or inhibitor. Luciferase activity was measured at 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

Statistical analyses

All statistical analyses were carried out using the spss software program (version 13.5; SPSS, Inc., Chicago, IL, USA). The test was used to analyze the differential expression of CCND1 mRNA between tumor tissues and normal tissues. The χ² test was utilized to analyze the relationship between CCND1 nuclear expression and clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. The significances of various variables in survival were analyzed using multivariate Cox proportional hazards model. A P value of less than 0.05 was considered statistically significant.

Results

CCND1 mRNA was elevated in colon cancer

In order to understand the role of CCND1 in colon cancer, real-time PCR was used to measure the expression of CCND1 mRNA transcripts in 20 freshly collected paired colon cancer and colon tissues. Compared with their control normal tissues, CCND1 mRNA level was significantly elevated in CARC tissues (P=0.0017) (Figure 1).

Nuclear protein expression of CCND1 promoted clinical progression and poor prognosis in colon cancer

In order to further evaluate the action of CCND1, we examined the differential nuclear expression of CCND1 protein in 4 paired CARC and colon tissues by western blot analysis. The result indicated that CCND1 expression was significantly increased in cell nucleus of CARC tissues compared to those colon tissues (Figure 2A). Further, we examined the expression levels CCND1 protein in 131 CARC and 90 colon tissues using immunohistochemical staining (Figure 2B). We found that specific CCND1 protein was predominantly stained in the nuclei of tumor cells. Furthermore, we observed that 54.2% (71/131) (Table 1) cases showed positive nuclear expression of CCND1. However, in colon tissues, only 8.89% (8/90) cases indicated positive nuclear CCND1 staining (P<0.001). Furthermore, the correlation between CCND1 nuclear expression and clinical characteristics was also analyzed. As shown in Table 2, a significant correlation between CCND1 nuclear expression with patient’s age, gender, N classification, distant metastasis (M classification), and clinical stage (I-II vs. III-IV) in 131 colon cancer cases was not observed, but the nuclear expression of CCND1 was significantly positively correlated with T classification (T₁-T₂ vs. T₃-T₄) (P<0.001) in CARC patients. Finally, we assessed the po-

<table>
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CCND1 is a target of miR-340 in CRC

Table 3. Summary of univariate and multivariate Cox regression analysis of overall survival duration

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<tr>
<td>CCND1 expression</td>
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<td>0.484</td>
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Figure 3. Knocking down CCND1 suppresses chemotherapy resistance of 5-Fu in CRC. A. Real-time PCR examined the interfering efficiency of siCCND1s in CRC. B. Western blot confirmed the interfering efficiency of siCCND1-2 by western blot assay in colon cancer. C and D. Knocking down CCND1 suppresses chemotherapy resistance of 5-Fu in CRC HCT116 and SW480 cells.

The possible prognostic value of CCND1 expression for CARC patients. Kaplan-Meier analysis with the log-rank test was used to analyze the association between the levels of CCND1 expression and patient survival. We observed that the level of CCND1 nuclear protein expression was negatively correlated with the overall survival time of CARC patients. Patients with nuclear expression had worse prognosis than those with negative nuclear expression of CCND1 (Figure 2C) (P=0.019). Interestingly, we further observed that nuclear CCND1 expression in clinical stage III+IV, but not clinical stage I+II, was shown to be worse prognosis and lead to the shorter overall survival time for CARC patients (Figure 2D) (P=0.001) by strata analysis.

Nuclear expression of CCND1 acted as an independent prognosis factor for CARC patients

To investigate the potential of CCND1 nuclear expression as independent prognosis marker, we used multivariate Cox proportional hazards model to analyze the significances of various variables in survival. Univariate analyses indicated that age, N classification, and CCND1 nuclear expression level were significantly associated with patient survival (P=0.002, P=0.002, and P=0.002 respectively). Further, multivariate analysis of CCND1 nuclear protein expression levels adjusting for age and N classification of CARC patients showed CCND1 nuclear expression as an independent prognostic marker for CARC patients (P=0.004) (Table 3).

Knocking down CCND1 decreased the chemotherapy resistance of 5-Fu to CARC

To the effect of CCND1 on 5-Fu chemotherapy resistance to CARC, we firstly constructed siCCND1 colon cell model. The results indicated that siCCND1-2 showed the best interfering efficiency among 3 siCCND1s based on real-time PCR (Figure 3A) and western blot (Figure...
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Figure 4. miR-340 mimics induced the chemotherapy sensitivity of 5-Fu in CRC. A and B, miR-340 induced the chemotherapy sensitivity of 5-Fu in CRC HCT116 and SW480 cells.

Figure 5. CCND1 was a direct target of miR-340 in CRC. A, miR-340 mimics suppressed the CCND1 expression in CRC cells. B, miR-340 inhibitor induced the CCND1 expression in CRC cells. C, Luciferase activity indicated that miR-340 suppressed 3’UTR of CCND1 in CRC.

3B) examinations. Further, knocking down CCND1 significantly reduced IC50 value and increased the sensitivity of 5-Fu to CARC HCT116 and SW480 cells (Figure 3C, 3D).

miR-340 increased the chemotherapy sensitivity of 5-Fu to CARC

In previous documents, miR-340-induced chemotherapy sensitivity of 5-Fu to CARC had never been reported. In this study, we observed that miR-340 mimic could markedly reduce IC50 value and increased the sensitivity of 5-Fu to CARC cells HCT116 and SW480 (Figure 4A, 4B).

miR-340 directly targeted CCND1 in CARC

To further study the mechanism of miR-340 in inducing chemotherapy sensitivity of 5-Fu, we used bioinformatics to predict the targeted genes of miR-340. Interestingly, CCND1 was predicted as a directly target gene. We observed that miR-340 mimics or inhibitor could respectively inhibit or increase the expression of miR-340 colon cancer cells (Figure 5A, 5B). Further, Wild-type (wt) or mutant (mt) 3’UTR vector of CCND1 and miR-340 mimics or inhibitor colon cells were co-transfected with colon SW480 cells. The results showed a significant decrease of luciferase activity in wt vector transfected with miR-340a mimics (Figure 5C, lanes 1; P<0.001) or an obvious increase of luciferase activity by using miR-340 inhibitor (Figure 5C, lanes 3; P=0.008) when compared with miR control, whereas the activity of mt vector was unaffected (Figure 5C, lanes 5 and 6) by using miR-340 mimics or inhibitor. Taken together, these results strongly supported that CCND1 was the direct target of miR-340 in CRC cells.

Discussion

CCND1 as a key oncogene has been reported to be cooperated with CDK4 and CDK6 driving cell cycle progression from G1 to S phase through the phosphorylation and subsequent inactivation of Rb protein and its abnormal expression has been indicated in tumors and promotes the disease progression and poor prognosis in these patients. Nevertheless, the expression pattern of CCND1 and its nuclear expression correlation with clinical features and the prognosis value were rarely reported in CRC.

In this study, we firstly found that CCND1 mRNA expression was upregulated in CRC tissues compared to colon tissues. This finding was similar to Jares and Molenaar et al’s documents respectively from mantle cell lymphomas and neuroblastoma [18, 19], but was inversed with
Hu's investigation in human hepatocellular carcinoma [20]. The discrepancy between our data and Hu et al's data would be most likely due to the different tumor samples. Our study preliminarily suggested the involvement of CCND1 in the pathogenesis of CRC.

Further, we observed that CCND1 was a coexpressed factor of nuclear and cytoplasm in CRC and colon tissues by immunohistochemistry staining assay. Interestingly, we observed that CCND1 expression was predominantly increased in cell nucleus of CRC, but not cytoplasm, compared to colon tissues. Our data was similar to Seiler, Huang, and Dekanić et al's investigations respectively in bladder cancer, oral cavity squamous cell carcinoma, and colorectal carcinomas [21-23], which suggested that elevated nuclear CCND1 expression might significantly stimulate the pathogenesis of CRC. Finally, the high nuclear expression of CCND1 in CRC tissues compared to their respectively adjacent colon tissues was confirmed by western blot. Further, our data presented the strong evidence that although nuclear expression of CCND1 was not correlated with age, gender, N classification, M classification, and clinical stage, it was significantly positively related to T classification. This result was similar with CCND1 reports from prostate cancer [24], papillary carcinoma in thyroid [25], colorectal cancer [26] et al, which indicated the key of nuclear CCND1 protein inducing cell growth in the pathogenesis of colon cancer.

Increased nuclear expression of CCND1 had been documented as an unfavorable factors in these tumors including pancreatic cancer [27], gastric adenocarcinoma [28], colorectal cancer [29] et al. Similar to these reports, we observed that nuclear expression of CCND1 was markedly negatively correlated with the survival time of CARC patients. Patients with nuclear CCND1 expression had an overall shorter survival time than those of patients with negative nuclear CCND1 expression. Our data demonstrated the significance of nuclear CCND1 expression and further supported CCND1 as a key oncogene in colon cancer. Interestingly, Mylona found that high nuclear expression of CCND1 was found as an favorable factor for prolonging survival time in breast cancer patient subgroups with aggressive phenotypes [30]. This data was inconsistent with our report from CRC, which may attribute to the suppressive role of CCND1 in tumor metastasis [31]. However, the effect of CCND1 on suppressing breast metastasis was still to be determined. In further strata analysis against clinical stage of CRC, we interestingly observed that nuclear CCND1 expression nuclear CCND1 expression in clinical stage III+IV, but not clinical stage I+II for colon cancer patients, was indicated to promote the worse prognosis and lead to the shorter overall survival time. This data suggested CCND1 as a preferable biomarker for evaluating the prognosis of advanced CRC patients. Finally, we observed that nuclear expression of CCND1 protein represented an independent prognostic marker for overall survival of CRC patients according to multivariate analysis. This result demonstrated the significance of CCND1 in predicting the prognosis of colon cancer patients.

5-Fu is a key chemotherapy drug that affects pyrimidine synthesis by inhibiting thymidylate synthetase thus depleting intracellular dTTP pools for colon cancer patients. Further, we observed that knocking down CCND1 significantly reduced chemotherapy resistance of 5-Fu to colon cancer cells. This result suggested the importance of CCND1 in chemotherapy resistance in colon cancer.

miR-340 had been demonstrated as a tumor suppressor in tumors [29, 30]. However, its role in regulating chemotherapy resistance of 5-Fu was still undetermined in colon cancer. In this study, miR-340 was observed to induce 5-Fu chemotherapy sensitivity in colon cancer. Yet, its detailed mechanism in modulating chemotherapy sensitivity had been documented in colon. Interestingly, we observed that CCND1 was predicted as a direct target of miR-340 by bioinformatics analysis. Subsequently, we confirmed the prediction by western blot and luciferase activity assay.

Taken together, our study demonstrated that the mRNA and protein level of CCND1 was markedly increased in CRC tissues compared to colon tissues. Furthermore, our data suggested that nuclear expression of CCND1 was positively correlated with the clinical progression and acted as a poor prognosis factor for CRC patients. Finally, CCND1 was validated as a direct target of miR-340 participating in miR-340-mediated suppression of chemotherapy resistance for 5-Fu in colon cancer.
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Disclosure of conflict of interest

None.

Authors’ contribution

JWJ and YYC performed the studies as well as assisted in the editing of manuscript. YWF and RCL designed this study and wrote this paper.

Address correspondence to: Wei Yi Fang and Rong Cheng Luo, Cancer Center, TCM-Integrated Hospital, Southern Medical University, Guangzhou 510310, Guangdong, PR China. E-mail: fangweiyi1975@163.com (WYF); luorc01@163.com (RCL)

References

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