Role of C9orf140 in the promotion of colorectal cancer progression and mechanisms of its upregulation via activation of STAT5, β-catenin and EZH2

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C9orf140 is a newly identified and characterized gene which is associated with cell proliferation and tumorigenicity. Expression of C9orf140 is upregulated in human gastric cancer and colorectal cancer (CRC); however, little is known about its role in CRC progression. We have investigated the clinical significance, biological effects and mechanisms of C9orf140 signaling. We found that the expression of C9orf140 is dramatically increased in a subset of CRC and correlates significantly with vascular invasion and lymph node metastasis. Our finding showed that knockdown of C9orf140 significantly reduced cell proliferation and invasion in vitro and dramatically increased overall survival and decreased lung metastasis in vivo. Conversely, overexpression of C9orf140 significantly increased lung metastasis and shortened overall survival when compared with control tumors. C9orf140-induced CRC cell invasion may depend on promoting the epithelial–mesenchymal transition progression. STAT5 may directly interact with the enhancer of zeste homolog 2 (EZH2) and β-catenin to enhance C9orf140 gene transactivation. Furthermore, C9orf140 may participate in cell invasion which is induced by STAT5, EZH2 or β-catenin activation. We describe the role of C9orf140 in CRC progression and find that C9orf140 overexpression may be regulated by STAT5, EZH2 and β-catenin interaction.

Introduction

Although colorectal cancer (CRC) mortality has decreased in the past decade, it remains one of the leading causes of morbidity and mortality in the world (1,2). Despite advances in early diagnosis and therapeutic treatments for CRC in recent years, overall patient survival has not significantly improved. The development of effective prevention and treatment strategies is limited by an incomplete understanding of the critical signaling pathways involved in CRC pathogenesis.

C9orf140, also known as p42.3, is a novel gene that has been recently isolated and identified by the mRNA differential display (mRNAADD) technique (3). The full-length cDNA of C9orf140 is ~4.0 kb, with an estimated molecular mass of 42.3 kDa and encodes a protein containing 389 amino acids. Further research has revealed that C9orf140 expression is cell cycle-dependent in gastric cancer cell lines. In addition, the same group reported that C9orf140 may also play important roles in malignant transformation (4). In our previous study, we found that mir-29a may downregulate the expression of C9orf140 via direct targeting the 3′-UTR region of C9orf140 gene, and the expression of C9orf140 was inversely correlated with mir-29a expression in human gastric cancer tissues (5). Furthermore, another group has illustrated that the expression of C9orf140 is elevated in CRC (6).

Recent studies have established a model of the protein structure spatial conformation of its domain and functional information and analyzed the regulatory function and mechanism of C9orf140 protein in malignant cell proliferation and tumor generation (7). Studies have also predicted that Ras protein, Raf-1 protein and MAPK may be involved in the regulation of C9orf140 expression and its functions. Although overexpression of C9orf140 has been detected by immunohistochemical staining and real-time PCR in various clinical tissue samples associated with tumor development (3,8), the role of C9orf140 in CRC progression and the mechanisms of C9orf140 overexpression remain unclear.

In our study, we show how C9orf140 mediates the progression of CRC both in vitro and in vivo. MAPK, JAK/STAT and the Wnt pathway may be responsible for C9orf140 overexpression in CRC cell. Moreover, C9orf140 overexpression can be induced by activated STAT5, enhancer of zeste homolog 2 (EZH2) and β-catenin. STAT5 may directly bind to the C9orf140 promoter and recruited EZH2 and β-catenin to the promoter region to activate the transcription of C9orf140. Knockdown of C9orf140 significantly decreased STAT5, EZH2 or β-catenin-induced cell invasion. To the best of our knowledge, this is the first report demonstrating that STAT5, EZH2 and β-catenin may be responsible for C9orf140 overexpression in CRC.

Materials and methods

Cell culture and treatment and quantitative real-time PCR

The details were described in Supplementary materials and methods, available at Carcinogenesis Online.

Cell proliferation

HCT116 cells were seeded into 96-well plates (2000 cells per well), cultured for 24 h, and then transfected with C9orf140/control siRNA. Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, at each time point, the cells were incubated with 10 µl of CCK-8 in 100 µl medium per well for 1 h at 37°C, and then absorbance was measured at 450 nm. The percentage of viable cells was calculated as: relative viability (%) = [A450 (treated) − A450 (control)]/A450 (control) − A450 (blank)] × 100%. All experiments were performed in triplicate.

Western blot and antibodies

The details were described in Supplementary materials and methods, available at Carcinogenesis Online.

Tissue microarray and immunohistochemical staining

Tumor specimens were obtained from 165 patients with primary CRC who underwent surgery at Shanghai Renji Hospital from 2006 to 2008. Meanwhile, 165 specimens of normal colonic epithelium taken from the same patients were also used. The study protocol was approved by the ethics committee of Shanghai Jiao-Tong University School of Medicine, Renji Hospital, and was in accordance with the provisions of the Helsinki Declaration of 1975. None of the patients had received preoperative treatments such as radiotherapy or
chemothrapy. Tissue microarray analysis was performed by Outdo Biotech (Shanghai, China) using standard techniques (9). The expression of C9orf140 was determined using primary antibodies (3) with LSAB kit (DakoCytomation, Copenhagen, Denmark), according to the manufacturer’s instructions. For the expression of EZH2, β-catenin and pSTAT5, we used the specific antibodies from Cell Signaling Technology (Beverly, Massachusetts).

The prepared tissue slides were examined independently by two investigators blinded to the clinical and the pathologic data. Protein expression was quantified using a visual grading system based on the extent of staining (percentage of positive tumor cells; graded on a scale of 0–4 where 0: none; 1: 1–25%; 2: 26–50%; 3: 51–75%; 4: >75%) and the intensity of staining (graded on a scale of 0–3 where 0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining). For further analysis, the product of the extent and intensity grades was used to define the cut-off value for elevated protein expression; protein expression was thus classified into two categories: high level (grades 4–12) and low level (grades 0–3).

Plasmids construction, transfections and lentiviral transduction

The shRNA against C9orf140 (GenBank accession number: NM_178448, STAT5 (L41142), β-catenin (NM_001098209) and the control shRNA/siRNA were purchased from Dharmacon RNA Technology (Lafayette, CO). The recombinant plasmids were constructed based on pGL-3 basic or pCDNA3.1-Flag plasmids, and an empty vector was used as control. Transfection of shRNA/siRNAs was carried out according to the manufacturer’s instructions. For lentiviruses transfections, CRC cells were transfected with 2 µg of lentivirus C9orf140-virus or lentivirus-control. Methods used for lentivirus production and infection were performed as described previously (10).

Transwell assay

We used chambers with 8 µm pore polycarbonate membranes, coated with Matrigel on the upper side (BD Biosciences). CRC cells with different treatment were then harvested, and 1 x 10^5 cells were seeded in serum-free medium into the upper chamber, whereas medium supplemented with 20% fetal bovine serum was applied to the lower chamber as a chemoattractant to induce invasion. After incubation for 48 h, migrated cells on the bottom surface of the filter were fixed, stained and counted.

Luciferase assay

The wild-type DNA fragment containing part of the promoter region (−1000 to +120) from transcription start site of the C9orf140 gene (GenBank® accession number NM_178448.3) was amplified from human genomic DNA with the following primers respectively: C9orf140 P-E 5′-GOGGTCACCT CAGG CCTGAG-3′ and C9orf140 P-R 5′-GAAGAT GTGGGC GCCCCGGGCT-3′, which introduced the cloning sites Kpn I and Bgl II (underlined), respectively. The DNA fragment obtained above was directly cloned into pGL3-basic (Promega, Madison, WI) between the Kpn I and Bgl II sites to obtain pGL3-basic-C9orf140P WT. The mutant DNA sequences of the C9orf140 promoter region encompassing all the four putative binding sites of STAT3 (−1000 to +120 from transcription start site) were synthesized and inserted into pGL3-basic vector. The mutant type constructs were designated as pGL3-basic-C9orf140P MT. T was replaced into A in each STAT3-binding site of pGL3-basic-C9orf140P WT and other mutant constructs with shRNA/siRNAs or plasmids at 1.0 µg and 100 ng of phRL. (Renilla luciferase) TK plasmid (Promega) for monitoring transfection efficiency were transiently transfected CRC cells in triplicate with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions. Luciferase activity was measured by using a BD Monolight 3010 luminometer (BD Biosciences). Promoter activity is reported as the mean ± SD.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit (Upstate, Charlottesville, VA) following the manufacturer’s protocol. Antibodies against C9orf140, EZH2, β-catenin, STAT5, pSTAT5 (Cell Signal Technology) and normal rabbit/mouse IgG (Upstate) were used.

Real-time PCR was performed in triplicate. Each PCR assay was carried out in a 20 µl reaction volume by using 6 µl of the eluted immunoprecipitated DNA. The amount of genomic DNA co-precipitated with the specific antibody was calculated as follows: CBRp − CBRq (genomic input) − CBrs (specific antibody), where CBRp (genomic input) and CBRq (specific antibody) are the mean threshold cycles of PCR performed in triplicate on DNA samples from the genomic input samples and the specific antibody samples, respectively.

Immunofluorescence assay

CRC cells were plated in four-well chamber slides (Nunc, Denmark) 24 h before transfection and then transiently transfected with C9orf140 overexpression/control plasmids for HT29 cells, and C9orf140/control siRNA for HCT116 cells. The cells were probed with corresponding antibodies for 1 h at room temperature and incubated with Alexa 546-conjugated donkey anti-goat IgG, followed by Alexa 488-conjugated donkey anti-mouse IgG (Invitrogen). Afterwards, the slides were mounted in 4',6-diamidino-2-phenylindole Fluoromount-G (SouthernBiotech, Birmingham) and images were captured using a laser-scanning confocal microscope (LSM-710; Zeiss, Germany).

Statistical analysis

Statistical analysis was performed with SPSS 17.0 software (SPSS, Chicago, IL). Data are expressed as means ± SE. Statistical differences between two groups were determined by Student’s t-test. For clinicopathological analysis, the chi-square test or Fisher’s exact test (two sided) was performed, and Spearman correlation was used to evaluate the correlation between the expressions of specific proteins. The Kaplan–Meier method was used to estimate the overall survival, and the log-rank test was used to evaluate the differences between survival curves. Two-sided P values <0.05 were considered statistically significant.

Results

Expression of C9orf140 in CRC tissues and cells

To evaluate the expression of C9orf140, real-time PCR and western blot analysis were performed. As shown in Figure 1A and 1B, C9orf140 was not detectable in normal human colon epithelial cells CRL-1790, but its expression was significantly increased in all CRC cell lines, especially in those highly invasive CRC cells lines. Immunofluorescent analysis showed that C9orf140 was mainly present in the cytoplasm of CRC HCT116 and HT29 cells (Figure 1C). Immunohistochemical staining was also performed in cancerous and normal tissues from the 165 CRC patients. As shown in Figure 1D, expression of C9orf140 was significantly increased in CRC tissues when compared with normal colon tissues. Moreover, the staining of C9orf140 is gradually increased from well-differentiated tissue, moderate tissue to poor differentiated tissue. The clinicopathological data of our study are shown in Table 1. High C9orf140 expression was significantly associated with CRC in female patients (73.8%, P = 0.006); patient over 60 years old (68.6%, P = 0.028); tumor proximal location (72.0%, P = 0.011); pathological classification (P = 0.001); degree of infiltration (P < 0.001) and lymph node metastasis (74.7%, P = 0.005).

C9orf140 plays an important role in progression of CRC cells

As described in the introduction, C9orf140 overexpression has been reported in various cancers (3,5) including CRC tissues (6). However, the role of C9orf140 in the progression of CRC remains
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Therefore, we firstly introduced lenti-C9orf140 shRNA-1/2 viruses and lenti-C9orf140 overexpression virus into CRC cells. Lenti-C9orf140 shRNA-1/2 viruses successfully decreased C9orf140 expression, respectively (Supplementary Figure 1A, available at Carcinogenesis Online). Introduction of lenti-C9orf140 overexpression virus remarkably increased C9orf140 expression (Supplementary Figure 1B, available at Carcinogenesis Online), whereas transduction of control shRNA or control virus had no significant effect on C9orf140 expression.

We then first analyzed whether C9orf140 participate in the cell proliferation of CRC cells by CCK-8 assays and BrDU proliferation assays. Knockdown of C9orf140 significantly decreased

![Graph showing expression of C9orf140 in CRC cells](image)

**Table I.** Clinicopathologic characteristics of C9orf140 expression in CRCs

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C9orf140 expression</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High (N=165)</td>
<td>Low (N=165)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>105 (63.6%)</td>
<td>60 (36.4%)</td>
<td></td>
</tr>
<tr>
<td>Cancer tissue</td>
<td>36 (21.8%)</td>
<td>129 (78.2%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>45 (52.9%)</td>
<td>40 (47.1%)</td>
<td>0.006*</td>
</tr>
<tr>
<td>Female</td>
<td>59 (73.8%)</td>
<td>21 (26.2%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>22 (50.0%)</td>
<td>22 (50.0%)</td>
<td>0.028*</td>
</tr>
<tr>
<td>≥60</td>
<td>83 (68.6%)</td>
<td>38 (31.4%)</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Proximal</td>
<td>67 (72.0%)</td>
<td>26 (28.0%)</td>
<td>0.011*</td>
</tr>
<tr>
<td>Distal</td>
<td>38 (52.8%)</td>
<td>34 (47.2%)</td>
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<tr>
<td>Pathological classification</td>
<td></td>
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<td></td>
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<tr>
<td>Phase I</td>
<td>7 (36.8%)</td>
<td>12 (63.2%)</td>
<td>0.001*</td>
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<tr>
<td>Phase II</td>
<td>59 (61.5%)</td>
<td>37 (38.5%)</td>
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</tr>
<tr>
<td>Phase III</td>
<td>39 (78.0%)</td>
<td>11 (22.0%)</td>
<td></td>
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<tr>
<td>Degree of infiltration</td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Muscularis mucosae</td>
<td>1 (16.7%)</td>
<td>5 (83.3%)</td>
<td></td>
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<tr>
<td>Muscular layer</td>
<td>4 (25.0%)</td>
<td>12 (75.0%)</td>
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<tr>
<td>Serosa</td>
<td>87 (71.9%)</td>
<td>34 (28.1%)</td>
<td></td>
</tr>
<tr>
<td>Extraserosa</td>
<td>13 (59.1%)</td>
<td>9 (40.9%)</td>
<td></td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>46 (53.5%)</td>
<td>40 (46.5%)</td>
<td>0.005*</td>
</tr>
<tr>
<td>Present</td>
<td>59 (74.7%)</td>
<td>20 (25.3%)</td>
<td></td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>97 (63.0%)</td>
<td>57 (37.0%)</td>
<td>0.562</td>
</tr>
<tr>
<td>Present</td>
<td>8 (72.7%)</td>
<td>3 (27.3%)</td>
<td></td>
</tr>
<tr>
<td>Prognosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival</td>
<td>37 (57.8%)</td>
<td>27 (42.2%)</td>
<td>0.137</td>
</tr>
<tr>
<td>Death</td>
<td>37 (71.2%)</td>
<td>15 (28.8%)</td>
<td></td>
</tr>
</tbody>
</table>

Data were showed as Number (%) or Number.

*Significant difference.
cell proliferation compared with negative control (Figure 2A and Supplementary Figure 2, available at Carcinogenesis Online), suggesting that C9orf140 may mediate CRC cell proliferation. As the mechanism of C9orf140-induced cell proliferation has been well explored before (3,5), here we mainly focus on the role of C9orf140 in CRC cell invasion. In transwell cell invasion assays, representative data showed that overexpression of C9orf140 dramatically increased the invasive ability of CRC cells (Figure 2B and Supplementary Figure 3A, available at Carcinogenesis Online). Conversely, knockdown of C9orf140 expression significantly reduced the invasive ability of CRC cells (Figure 2B and Supplementary Figure 3B, available at Carcinogenesis Online), indicating that C9orf140 may have a significant effect on cell migration and invasion in CRC cells. In wound healing assays, less wound closure was observed in CRC cells with knockdown of C9orf140 when compared with control (Figure 2C and Supplementary Figure 3C, available at Carcinogenesis Online). These data further suggest that C9orf140 plays an important role in cell invasion. Furthermore, knockdown of C9orf140 expression significantly decreased N-cadherin and increased E-cadherin expressions in HT29 and HCT116 cells by western blot analysis (Figure 2D) or immunofluorescence staining (Figure 2E), suggesting that C9orf140 may mediate epithelial–mesenchymal transition (EMT) initiation and CRC progression. In contrast, overexpression of C9orf140 dramatically reduced E-cadherin and enhanced N-cadherin expressions in

Fig. 2. The functional impact of C9orf140 overexpression and knockdown on CRC cell metastatic potential in vitro. (A) CCK-8 assay was performed in CRC cells after transfection of control/C9orf140 siRNA. (B) Transwell Matrigel invasion assays were performed in CRC cells transfected with control/C9orf140 shRNA and with control/C9orf140 overexpression viruses. Cells were observed under a light microscope and photographed (×200 magnification). Cells were counted from five random microscopic fields per insert in triplicate. The migrated cell numbers were normalized to that of the control group. Data are shown as mean ± SD from three separate experiments. (C) Wound healing assays were performed in CRC cells after transfection of control shRNA virus or C9orf140 shRNA virus. (D) Western blots analysis and (E) immunofluorescence data showing influences of C9orf140 on the expression of E-cadherin and N-cadherin. n = 3, analysis of variance, *P < 0.01.

n = 3, analysis of variance, *P < 0.01.
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CRC cells (Figure 2D and 2E). The data indicate that the C9orf140 may mediate EMT during CRC progression and promote CRC invasion.

The functional impact of C9orf140 overexpression and knockdown in CRC cell invasion in vivo

We then explored the functional impact of C9orf140 overexpression and knockdown in vivo using a nude mouse metastatic tumor model. Different stable cells were transplanted into BALB/c-nu/nu mice via tail vein injection. We found that more subcutaneous metastases were detected after injection of HT29-GFP-C9orf140 cells when compared with the injection of control cells (Figure 3A). More importantly, mice inoculated with HT29-GFP-C9orf140 cells had a shorter overall survival time (mean: 65.7 days) when compared with those injected with control cells (mean: 76.4 days; Figure 3C). Hematoxylin–eosin staining showed that fewer metastatic CRC cells were observed in the lungs of nude mice at 17 weeks after injection of HT29-GFP-C9orf140 and the HT29-GFP-control group. (F) Metastatic CRC cells were detected in the lungs of nude mice at 17 weeks of the HT29-GFP-C9orf140 shRNA and HCT116-GFP control shRNA group.

Fig. 3. The functional impact of C9orf140 overexpression/knockdown on CRC cell metastatic potential in vivo. (A) Assessment of subcutaneous metastatic capacity of 2.5 x 10^6 HT29-GFP-control (n = 15) or HT29-GFP-C9orf140 (n = 15) cells by inoculating nude mice via tail vein injection. Biofluorescence images and summarized data showed subcutaneous metastasis development at 40 days with a 1-s exposure and total photon flux for each treatment group (left-sided mice: PBS, middle mice: HT29-GFP-control stable cells, right-sided mice: HT29-GFP-C9orf140 stable cells). n = 15, analysis of variance (ANOVA), *P < 0.0001. (B) Left-sided mice: PBS, middle mice: HCT116-GFP-control shRNA stable cells, right-sided mice: HCT116-GFP-C9orf140 shRNA stable cells. n = 15, ANOVA *P < 0.0001. (C and D) Comparison of survival curves of mice. n = 15, ▲P < 0.05 (Student’s t-test), compared with HT29-GFP-control cells or HCT116-GFP-control shRNA cells. (E) Metastatic CRC cells were detected in the lungs of nude mice at 17 weeks of the HT29-GFP-C9orf140 and the HT29-GFP-control group. (F) Metastatic CRC cells were detected in the lungs of nude mice at 17 weeks of the HCT116-GFP-C9orf140 shRNA and HCT116-GFP control shRNA group.
subcutaneous metastases were detected after downregulation of C9orf140 (Figure 3B). Knockdown of C9orf140 remarkably increased the overall survival time and significantly decreased lung metastasis of tumor cells in vivo (Figure 3D and 3F; Supplementary Table 3, available at Carcinogenesis Online). These data suggest that C9orf140 expression plays a critical role in CRC cell motility and metastasis.

Effects of inhibition of MAPK, JAK/STAT and Wnt signaling pathway on C9orf140 overexpression in CRC cells

To explore the mechanism of C9orf140 overexpression in CRC cells, we introduced different inhibitors of signaling transduction pathways into CRC cells. Western blot analysis (Supplementary Figures 4A and 5A, available at Carcinogenesis Online) showed that C9orf140 expression was significantly decreased after treatment of AG490 (an inhibitor of JAK/STAT signaling pathway), U0126 (an inhibitor of MAPK signaling pathway) and PNU74654 (an inhibitor of Wnt signaling pathway) in HT29 and HCT116 cells. However, there is no significant change of C9orf140 expression after Rapamycin (an inhibitor of mTOR signaling pathway) treatment. The data suggest that the JAK/STAT, MAPK and Wnt signaling pathway may participate in the regulation of C9orf140 expression in CRC cells. Further analysis showed that knockdown of MEK1/2 significantly decreased the expression of p-MEK1/2, ERK1/2 and C9orf140, respectively (Supplementary Figures 4B and 5B, available at Carcinogenesis Online). The expression of C9orf140 was significantly decreased after ERK2 siRNA transfection in HT29 and HCT116 cells, respectively (Supplementary Figures 4C and 5C, available at Carcinogenesis Online). It has been reported that MAPK pathway regulates EZH2 overexpression in breast cancer (12). EZH2 may directly activate the transcription of oncogenes (13). Velichutina et al. have predicted that C9orf140 may be regulated by EZH2 (14). Therefore, we next detected whether EZH2 participates in the expression of C9orf140. Western blot analysis showed that knockdown of EZH2 expression significantly decreased the expression of C9orf140 in CRC cells (Supplementary Figures 4D and 5D, available at Carcinogenesis Online), indicating that EZH2 may play an important role in C9orf140 expression. In addition, knockdown of JAK-2 expression dramatically downregulated the expression of STAT5 and C9orf140 in CRC cells (Supplementary Figure 5E, available at Carcinogenesis Online) and knockdown of STAT5 significantly decreased C9orf140 expression in HT29 and HCT116 cells (Supplementary Figures 4E and 5F, available at Carcinogenesis Online). However, knockdown of STAT3 had no significant effect on the expression of C9orf140 (data not shown). These data indicate that STAT5, but not STAT3, may mediate the expression of C9orf140 in CRC cells. Moreover, we found that downregulation of the expression of β-catenin remarkably reduced the expression of C9orf140 in CRC cells (Supplementary Figures 4F and 5G, available at Carcinogenesis Online), suggesting that β-catenin may also play an important role in the expression of C9orf140 in CRC cells.

STAT5 enhanced the transactivation of C9orf140 by recruiting EZH2 and β-catenin

To investigate the relationship among STAT5, EZH2 and β-catenin activation in CRC cells, we analyzed the promoter region of C9orf140 (Figure 4A). The ~1000 to +120 long 5′ flanking region of C9orf140 was obtained by PCR amplification using human genomic DNA as a template (the transcription start site designated as +1). DNA sequence analysis of the C9orf140 promoter regions revealed four putative STAT5-binding sites. The DNA fragment was then subcloned into the promoter-less luciferase reporter vector pGL3-basic. Knockdown of STAT5 dramatically decreased the transcriptional activity of the C9orf140 promoter in HCT116 cells (Figure 4C and Supplementary Figure 6A, available at Carcinogenesis Online). Furthermore, the transcriptional activity was significantly decreased after mutation of STAT5-binding sites in the C9orf140 promoter (Figure 4B).

In addition, a dual-luciferase assay with different siRNAs in combination was used to measure the putative promoter activity. As shown in Figure 4C, knockdown of STAT5, EZH2 or β-catenin dramatically decreased the expression of the reporter genes driven by the C9orf140 promoter, respectively. We also found that cotransfection of STAT5 plus EZH2 siRNAs, STAT5 plus β-catenin siRNAs or EZH2 plus β-catenin siRNAs induced a much lower level of C9orf140 reporter activity in CRC cells than transfection of STAT5, EZH2 or β-catenin siRNA alone. Cotransfection of all three siRNAs STAT5, EZH2 and β-catenin caused the lowest increase of C9orf140 reporter activity in CRC cells, further supporting a possible connection between STAT5, EZH2 and β-catenin in gene activation.

We next investigated the molecular mechanism underlying the transcription potential of STAT5, EZH2 and β-catenin in respect to C9orf140 expression. To assess the ability of STAT5 to recruit EZH2 and β-catenin, a ChIP assay and western blot analysis were performed. Nuclear extracts were then subjected to ChIP with EZH2, STAT5 and pSTAT5 specific antibodies. Figure 4D shows that C9orf140 genomic DNA was detectable in the immunoprecipitate. ChIP real-time PCR analysis demonstrated that knockdown of EZH2 and STAT5 significantly decreased pSTAT5 recruitment to the C9orf140 promoter (Figure 4E). The data indicate that STAT5 may directly bind to the C9orf140 promoter and EZH2 may participate in the recruitment of pSTAT5 to the promoter. In addition, the same ChIP immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transfected to membranes and probed with pSTAT5, STAT5, EZH2 or β-catenin specific antibodies. Western blot analysis showed that both EZH2 and β-catenin were readily detectable in the immunoprecipitates with STAT5 or pSTAT5 antibodies, respectively, in HCT116 cells (Figure 4F). These data suggest that pSTAT5 may bind to genomic DNA of C9orf140 promoter and recruit EZH2 and β-catenin. We next further examined that STAT5, EZH2 and β-catenin interacted with each other at endogenous cellular levels in CRC cells via immunoprecipitation studies (Figure 5A), suggesting that the interaction between STAT5, EZH2 and β-catenin is necessary for pSTAT5-induced transactivation of the C9orf140 promoter. In addition, in protein binding assays in vitro, western blot showed that STAT5 and β-catenin were pulled down by the Flag-EZH2 fusion protein with Flag gel, respectively (Figure 5B and Supplementary Figure 6C, available at Carcinogenesis Online). The similar result was obtained from the protein binding assay in vitro by incubating Flag-β-catenin plus Flag gel with commercial purified EZH2, STAT5 proteins (Figure 5B and Supplementary Figure 6C, available at Carcinogenesis Online), indicating that STAT5 may directly interact with EZH2 and β-catenin.

Since STAT5, EZH2 and β-catenin may mediate CRC cell invasion, we next detected whether C9orf140 participates in these protein-induced cell invasion in CRC. Overexpression of STAT5, EZH2 or β-catenin significantly increased CRC cell invasion under basal conditions. Knockdown of C9orf140 dramatically blocked these protein-mediated CRC cell invasions, respectively (Figure 5C–E and Supplementary Figure 6B, available at Carcinogenesis Online), indicating that STAT5, EZH2 or β-catenin-induced CRC cell invasion may depend on upregulation of C9orf140 expression. Analysis of clinicopathology also showed significant correlation between the expression of C9orf140 and the expression of EZH2, β-catenin and pSTAT5 in CRC tissues (n = 75), respectively (all P < 0.001) (Figure 5F).

Discussion

The C9orf140 oncogene is highly conserved in mammals and has been reported to play an important role in the progressive transformation of normal gastric epithelium cells to cancer cells (3). It expresses differently during different stages of the cell cycle, indicating that C9orf140 may be involved in regulation of cell cycle. This notion is also supported by our previously results showing that C9orf140 may stimulate cellular proliferation. It has been reported
Fig. 4. STAT5 directly interacts with EZH2 and β-catenin to enhance C9orf140 gene transactivation. (A) Bioinformatic analysis of STAT5 transcriptional factor binding sites in part of C9orf140 gene promoter region. The numbers on the left side indicate the locations upstream of the first base of the initial translation site. STAT5-binding sites are highlighted, and the DNA sequence encompassed by two arrows was amplified in ChIP assay. (B) WT, wild-type; MT, mutant type. Mutation of STAT5-binding sites significantly decreased the transcriptional activity of C9orf140 promoter in the luciferase assay. \(n = 3\), ▲▲\(P < 0.01\) (Student’s t-test). (C) The C9orf140 promoter activity was measured by a dual-luciferase assay combined with different siRNAs. \(n = 3\), analysis of variance, *\(P < 0.05\), compared with control siRNA; **\(P < 0.05\), compared with EZH2/STAT5/β-catenin siRNA; ▲\(P < 0.05\), compared with β-catenin siRNA + EZH2 siRNA. (D) The C9orf140 DNA was detected in the chromatin sample immunoprecipitated from HCT116 cells using an antibody against EZH2, STAT5 and pSTAT5. (E) Real-time PCR of the ChIP samples shows that knockdown of EZH2 and STAT5 dramatically decreased the binding efficiency of STAT5 to the C9orf140 promoter in CRC cells, respectively. Another Real-time PCR of the ChIP assay was performed using GAPDH as negative control for STAT5 binding. \(n = 3\), ▲▲▲\(P < 0.01\) (Student’s t-test). (F) ChIP was performed from HCT116 cells using anti-pSTAT5, anti-STAT5 or control antibodies, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and membranes probed with anti-STAT5, anti-EZH2 or anti-β-catenin antibodies.
Fig. 5. Coimmunoprecipitation of EZH2 with pSTAT5/β-catenin or pSTAT5 with β-catenin in CRC cells and the role of C9orf140 in STAT5, EZH2 and β-catenin-induced cell invasion. (A) Immunoprecipitation of endogenous proteins from CRC cells were performed using anti-STAT5, anti-EZH2 and anti-β-catenin antibodies. (B) Protein binding assay in vitro by incubating Flag-EZH2 fusion plus flag gel / Flag-β-catenin fusion plus flag gel with commercial purified EZH2, STAT5 and β-catenin proteins. (C–E) Transwell Matrigel invasion assays were performed in HCT116 cells infected with different plasmids and shRNAs as indicated. Cells were observed under a light microscope and photographed. Cells were counted from five random microscopic fields (×200) per insert in triplicate. The migrated cell numbers were normalized to that of the control group. Data are shown as mean ± SD from three separate experiments. n = 3, analysis of variance *P < 0.05, compared with control plasmid + control shRNA, **P < 0.05, compared with EZH2 overexpression plasmid + control shRNA, ▲P < 0.05, compared with STAT5 overexpression plasmid + control shRNA, ▲▲P < 0.05, compared with β-catenin overexpression plasmid + control shRNA. (F) Immunohistochemical analysis of correlation between the expression of C9orf140 and the expression of EZH2, β-catenin and pSTAT5 in CRC tissues (×200 magnification). n = 75, Spearman correlation.
that C9orf140 silencing may alter the expression of two key proteins, CHK2 and cyclin B1, which are involved in cell cycle regulation (5). Furthermore, C9orf140 has been shown to be overexpressed in CRC tissues and associated with low total cholesterol levels (6). However, the molecular mechanisms underlying aberrant expression of C9orf140 gene in CRC are poorly understood. In the present study, we found that C9orf140 may play an important role in CRC cell motility and metastasis for the following reasons: (i) expression of C9orf140 was significantly increased in CRC cell lines and cancer tissues, (ii) overexpression of C9orf140 in CRC tissues was associated with increased aggressiveness of the tumor, (iii) down-regulation of C9orf140 significantly decreased the invasive ability in CRC cells and (iv) overexpression of C9orf140 markedly increased the metastatic ability of CRC cells and decreased overall survival in an in vivo model of CRC metastasis. Conversely, downregulation of C9orf140 significantly decreased the metastatic ability of CRC cells and increased overall survival in vivo.

It is well known that the depth of tumor invasion, distant metastasis and lymph node metastasis are the major prognostic factors in CRC (8,15,16). Recent reports have indicated that the expression of different oncogenes in various malignant tumors may act as biological markers of malignant potential (17–19). In our current study, we found that C9orf140 expression was significantly increased from early CRC to advanced CRC (Figure 1D and Table 1), with the highest expression of C9orf140 observed in cases with lymph node metastasis (74.7%; P = 0.005). These results suggest that the higher expression of C9orf140 may imply an aggressive CRC phenotyp.

Our study also showed that C9orf140 may participate in the invasiveness of CRC cells through promotion of EMT during CRC progression. As a calcium-dependent cell adhesion molecule, E-cadherin is predominantly expressed in epithelial tissues (20) and is an important tumor suppressor gene which may lose the expression and function in tumor progression and invasion (21–24). The loss of E-cadherin expression is a crucial step in the initiation of tumor metastasis and a fundamental event in EMT (25). In our present study, we found that C9orf140 may mediate EMT initiation and CRC progression since knockdown of C9orf140 significantly downregulated the expression of N-cadherin (the mesenchymal marker of EMT) and upregulated the expression of E-cadherin in CRC cells (Figure 2D); overexpression of C9orf140 dramatically increased the expression of N-cadherin and decreased the expression of E-cadherin (Figure 2D).

We have previously shown that the expression of C9orf140 may be regulated by mir-29a (5), which inhibits the cell proliferation and blocks the cell cycle via repression of C9orf140 expression. In the present study, we further explored the mechanism of C9orf140 overexpression in CRC. We found that C9orf140 expression was significantly reduced by inhibition of the JAK/STAT pathway with an inhibitor AG490, of the MAPK signaling pathway with an inhibitor U0126 and of the Wnt signaling pathway with an inhibitor (26). However, inhibition of the mTOR signaling pathway with Rapamycin had no significant effect on the expression of C9orf140 (Supplementary Figure 4, available at Carcinogenesis Online). The data indicate that the expression of C9orf140 may be regulated by a JAK/STAT pathway, a MAPK pathway and a Wnt signaling pathway. Furthermore, we found that STAT5, EZH2 and β-catenin may cooperate with each other to regulate the expression of C9orf140. This result is supported by our following observations: (i) knockdown of STAT5, EZH2 or β-catenin expression significantly decreased the expression of C9orf140, respectively; (ii) STAT5 may directly bind to the C9orf140 promoter region and activate its transcription; (iii) STAT5, EZH2 and β-catenin may have an additive effect in activation of the C9orf140 promoter; (iv) STAT5, EZH2 or β-catenin-induced CRC cell invasion may depend on upregulation of C9orf140 expression and (v) the expression of STAT5, EZH2 or β-catenin showed significant correlation with the expression of C9orf140 in CRC tissues.

In addition, EZH2 is a well-characterized histone methyltransferase that specifically targets histone H3-K27 for methylation, which is a hallmark for gene silencing. In this study, we found that STAT5, EZH2 and β-catenin may interact with each other to trans-activate the transcription of C9orf140 gene. Our data are consistent with recently report demonstrating that PAF recruits EZH2 to the β-catenin transcriptional complex and specifically enhances Wnt target gene transactivation in colon cancer cells (26). Furthermore, another study showed that EZH2 activates the transcription of c-Myc and cyclin D1 oncogenes by integrating estrogen and Wnt signaling pathway and promotes cell proliferation in breast cancer cells (13). These reports and our data indicate that EZH2 may not only act as a transcription repressor to inhibit tumor suppress gene transcription but also function as a transcriptional regulator to enhance gene transcription.

In conclusion, this study provides new evidence that C9orf140 may play an important role in tumor invasion in CRC via promoting EMT during tumor progression. Moreover, C9orf140 overexpression may be induced by activation of STAT5, EZH2 and β-catenin. STAT5 may directly bind to the C9orf140 promoter and recruit EZH2 and β-catenin to the promoter region to activate the transcription of C9orf140. Furthermore, C9orf140 may mediate STAT5, EZH2 and β-catenin-induced CRC cell invasion. These findings suggest that C9orf140 inhibition may be a novel potential target for CRC treatment. However, the specific function of C9orf140 still needs to be further explored in the future.

Supplementary material
Supplementary materials and methods, Tables 1–2 and Figures 1–6 and can be found at http://carcin.oxfordjournals.org/

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References

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