Original Article
Upregulation of C/EBPα contributes to colorectal cancer growth, metastasis and indicates poor survival outcome

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Abstract: The function and clinical implication of transcription factor CCAAT/enhancer-binding protein α (C/EBPα) in colorectal cancer (CRC) still remains undefined. In fact, C/EBPα has long been considered as a tumor suppressor in hematopoietic system and also found lowly expressed in numerous solid tumors. However, our results here for the first time showed that C/EBPα was unexpectedly upregulated and was an independent prognostic marker for patients with CRC. We therefore aimed to explore the detailed role and mechanisms of C/EBPα in CRC. Our investigation demonstrated that C/EBPα promoted tumor growth both in vitro and in vivo. In addition, suppression of C/EBPα inhibited cell proliferation by inducing G1 phase arrest and inducing apoptosis. Also, C/EBPα enhances CRC cells migration and invasion in vitro as well as metastasis in vivo through regulating EMT. Mechanistically, C/EBPα exerts its oncogenic role by targeting c-Myc/cyclin D1 mediated by β-catenin involved pathway and we provide evidence indicating that cytoplasmic exclusion of C/EBPα might contribute to its oncogenic function in tumor progression. In conclusion, C/EBPα acts as an oncogene in CRC and could be a potential biomarker of colon carcinogenesis.

Keywords: C/EBPα, colorectal cancer, proliferation, metastasis, EMT

Introduction

Transcription factor CCAAT/enhancer-binding proteins (C/EBPs) form a family of basic region leucine zipper (bZIP) transcription factors that includes six members (α, β, γ, δ, ε and ζ) with related sequences and functions [1-6]. They involved in the process of cell cycle, inflammation, metabolism, cellular proliferation and differentiation. C/EBPα is relatively high expressed in liver, adipose tissue, myeloid cells and skin, and its function has been best characterized in adipocytes and the hematopoietic system. C/EBPα has long been known as a major regulator of myeloid differentiation and functionally impaired in leukemia. Genetic alteration of CEBPA is a major etiology of human Acute myeloid leukemia (AML), with a mutation-rate frequency 10% among this disease [7]. Its role as a tumor suppressor in hematopoietic system has been well clarified, for it controls the expression of many myeloid genes, including genes encoding growth factor receptors. Comparable to involvement in myeloid development, C/EBPα is also a key transcription factor controlling adipogenesis, in the absence of C/EBPα, adipose tissue expansion and regeneration are impaired [8-10]. In recent years, the tumor suppressor role for C/EBPα in solid tumors has been reported by several studies. Downregulation of C/EBPα levels have been described in numerous solid tumors such as liver, breast, skin, lung, et al. [11-15], and shown to correlate with tumor size and progression as well as poor-prognosis outcome [14, 16, 17]. However, in distinct studies, increased expression of C/EBPα correlated with increased proliferation and disease progression [18-20].

Colorectal cancer (CRC) is one of the most common cancers and lead to the third cancer-related death across worldwide. Despite progress
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has been achieved in comprehensive treatments, late-stage CRC patients suffer from a poor 5-year survival [21]. Thus, understanding the mechanisms underlying the development and progression of CRC and identifying novel molecular target is of great significance. Till now, studies investigating the expression level and the functional significance of C/EBPα in CRC still remain absent. Therefore, we here for the first time report that C/EBPα was unexpectedly high expressed in tumor tissues and it was an independent prognostic marker for patients with CRC. In addition, we provide evidence indicating that cytoplasmic exclusion of C/EBPα might contribute to its oncogenic function in tumor progression. A series of assays were performed and our results revealed that C/EBPα promoted tumor growth and metastasis in CRC patients. It was also able to regulate cell cycle distribution and cell apoptosis. Mechanistically, C/EBPα can induce EMT and exerts its oncogenic role by targeting c-Myc/cyclin D1 mediated by β-catenin involved pathway.

Material and methods

Cell culture and reagents

Human CRC cell lines HT29, HCT116, SW480, SW620, SW1116 and RKO cells were purchased from the American Type Culture Collection (ATCC, VA, USA). All the cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C/5% CO₂ in a humidified incubator.

Patients and samples

All the 77 CRC specimens were collected from Ruijin Hospital, Shanghai Jiaotong University. Ethics approval for use of human specimen was obtained from the Biomedical Ethics Committee of Ruijin Hospital. All the tumor tissues and paired normal tissues were collected from patients undergoing operation from 2010 to 2011. Clinical and pathological data were collected. Pathological staging was performed in accordance to the TNM classification [22].

Immunohistochemistry (IHC)

Immunohistochemical staining was conducted according to the manufacturer’s instructions (Immunostain SP kit, DakoCytomation, USA). Slides were incubated with a 1:50 diluted anti-C/EBP antibody (Cell Signaling Technology, Danvers, MA) and a 1:200 diluted anti-Ki-67 antibody (Santa Cruz Biotechnology) overnight at 4°C, followed by a HRP secondary antibody for 30 minutes at room temperature. The results of immunostaining were determined by the number of positive cells (the percentage of cells stained: 0 = 0, 1 = 1-10%, 2 = 11-50%, 3 = 51-80%, 4 = 81-100%; and staining intensity: negative = 0, weak = 1, moderate = 2, strong = 3). The intensity of the immunostaining score and the percentage of immunoreactive cells score were multiplied to generate immunoreactive score (IRS) scores ranging from 0 to 12. Immunohistochemical scores were independently determined by two pathologists without access to patient characteristics.

Real-time quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from cell lines and tissues using Trizol (Invitrogen) according to the manufacturers’ instructions. cDNA was synthesized by using reverse transcription kit (Invitrogen, CA). Quantitative polymerase chain reaction (PCR) was performed by using SYBR Green PCR Master Mix (Applied Biosystems, UK). Primers for C/EBPα were as followed: forward 5’-TGTATACCCCTGGTGGGAGA-3’ and reverse 5’-TCATAACTCCGGTCCCTCTG-3’; GAPDH was used as the control: forward 5’-ATCTGTCCCCCTCTGCTGA-3’ and reverse 5’-GATGACCTTGCCCACAGCCT-3’.

Western blot

Western blot was performed as previously described [23]. The primary antibodies for C/EBPα (1:1000), E-cadherin (1:1000), N-cadherin (1:1000) and Vimentin (1:1000) were purchased from Cell Signaling Technology, Danvers, MA. The secondary antibodies such as horseradish peroxidase (HRP)-conjugated antirabbit (1:10000) antibodies were from Sigma-Aldrich.

Plasmid construction/transfection

The GFP-labeled C/EBPα-specific shRNA and C/EBPα overexpression plasmids were obtained from GeneChem Co. Ltd., Shanghai, China. Lentivirus transfection was performed as
Figure 1. Expression of C/EBPα in CRC cell lines and in CRC tissues. A. RT-PCR analysis showing C/EBPα expression in 15 paired CRC samples; B. C/EBPα expression in six CRC cell lines detected by western blot; C. Cell proliferation: proliferative abilities of six CRC cell lines were determined by CCK8 assays as described in the Materials and methods; D. Migration: migratory abilities of six CRC cell lines were determined by migration assays as described in the Materials and methods; E. C/EBPα expression level in tumor tissues and the paired normal tissues was evaluated by immunohistochemical staining with tissue microarray; F. Scores for NDRG1 expression are shown as box plots, with the horizontal lines representing the median; the bottom and top of the boxes representing the 25th and 75th percentiles, respectively; and the vertical bars representing the range of data. We compared CRC tissues with matched adjacent normal tissues; G. Representative images of C/EBPα expression in cytoplasm. Scale bars: 50 μm. Data represent the means ± SD from three independent experiments. ***P < 0.001.
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Table 1. Relationship between C/EBPα expression level and clinicopathologic variables in 77 CRC patients

<table>
<thead>
<tr>
<th>Clinicopathologic parameters</th>
<th>Case (n = 77)</th>
<th>C/EBPα expression</th>
<th>P value</th>
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<tbody>
<tr>
<td>Age</td>
<td></td>
<td>C/EBPα expression</td>
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<tr>
<td>≥ 65</td>
<td>32</td>
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<tr>
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<tr>
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<td>&lt; 5 ng/mL</td>
<td>33</td>
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***P < 0.001.

described previously [24]. The overexpression and interfering effect of these vectors/shRNAs were evaluated by immunoblot.

CCK-8 and colony formation assay

Cell proliferation was examined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). In short, cells (1.0 × 10⁴/well) were seeded in 96-well culture plates in triplicate and incubated for 4 days at 37°C/5% CO₂ in a humidified incubator. Viable cells were quantitated at each 24 h interval by measuring OD450 using microplate reader (Epoch; BioTek, Winooski, VT). For colony formation assay, 1000 tumor cells were seeded in a six-well plate and kept in an incubator until visible cloning appeared. Then the cloning was fixed with 4% formaldehyde for 30 min and later stained with crystal violet dye for 5 min.

Wound healing assay

Cells were cultured in serum-free medium for 24 h and wounded with pipette tips. Fresh medium was then replaced. 24 h later, the wound closing procedure was observed and photographed.

Apoptosis and cell cycle analysis

Cells (2 × 10⁶) were fixed with 75% ethanol at 4°C overnight and then washed with cold PBS and treated with RNase, followed by staining with propidium iodide for 30 min in dark. Cell cycle analysis was then performed by flow cytometry (FACSCalibur; Becton Dickinson, Sparks, MD). Apoptotic cells were stained using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) and analysed by flow cytometry.

In vivo tumour growth and metastasis assay

RKO/sh-C/EBPα and RKO/sh-NC cells (5 × 10⁶ in 100 ul of sterilized PBS buffer) were implanted subcutaneously into the left flank of 5-week-old male nude mice (6 mice per group). Tumour growth was determined by measuring the tumour volume (mm³, V = tumour length × tumour width²/2) every 2-3 days using calipers. Mice were euthanized after 6 weeks post-inoculation and the tumours were excised; The cells interpreted as the average number of cells ± standard deviation per field.
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(5 × 10⁶ in 300 ml PBS) were tail-vein injected into the nude mice and on the indicated day, the mice were killed and photographed. All the lung tissues were embedded in paraffin, followed by hematoxylin and eosin and IHC staining. Metastases were counted on five lobes of the lung. The animal studies were approved by the Institutional Animal Care and Use Committee of the Shanghai Jiao Tong University. All animal experiments were performed according to the guidelines on the care and use of animals for scientific use.

**Statistic analysis**

The data are expressed as mean ± s.d. of ≥3 independent experiments and were statistically analyzed using Student’s t-test. The results were considered significant when P < 0.05.

**Results**

*C/EBPα is expressed in CRC cell lines and is aberrantly up-regulated in CRC tissues*

To explore the potential roles of C/EBPα in CRC, its expression level was examined in CRC cell lines and in specimens from CRC patients. At first, we randomly selected 15 pairs of CRC specimens to evaluate the mRNA level of C/EBPα expression. Unexpectedly, C/EBPα transcript levels were significantly increased in tumor tissues compared to corresponding normal tissues (Figure 1A). Apparently, this result was adverse to the previous research conducted in other solid tumors [7]. Thus, we further analyzed a tissue microarray (TMA) containing 77 pairs of tumor and adjacent normal tissue by immunochemistry (Figure 1E). In these
cases, the significant higher expressions of C/EBPα were identified in cancer tissues compared to their adjacent normal tissues (Figure 1F). As C/EBPα is a transcription factor, its function relies on its nuclear localization. We observed that among the C/EBPα expressing tumor tissues, C/EBPα was expressed in the nuclear of almost all the cells, and C/EBPα-positive expression in the cytoplasm was evident in 17 (22%) cases. Interestingly, 15/17 (88%) patients with cytoplasm C/EBPα-high expression were at stage III and IV, compared to 21/60 (35%) patients with cytoplasm C/EBPα-low/negative expression (Figure 1G; Table 3).

Next, we evaluated the expression of C/EBPα in 6 colon cell lines and differential expression of C/EBPα was observed in a cell line dependent manner. It was shown that C/EBPα was highly expressed in HCT116, SW620 and RKO cells (Figure 1A), which show higher migratory capacity (Figure 1B); In contrast, C/EBPα showed a relatively lower expression in HT29 cells (Figure 1A) with low migratory activity (Figure 1B). Apparently, C/EBPα expression positively related to the cell migratory ability in these CRC cells (P < 0.001). In addition, C/EBPα expression was also consistently correlated to the cell proliferative ability (Figure 1C) in these examined CRC cell lines (P < 0.001).

Collectively, the results of C/EBPα expression level in CRC cell lines and CRC tissues seems not to support its tumor suppressor function as it used to be considered.

**Overexpression of C/EBPα independently predicts worse prognosis of CRC**

We next analyzed the associations between expression of C/EBPα and several clinical and pathologic factors (Table 1). While the expression of C/EBPα is not related to age (P = 0.196), gender (P = 0.832), tumor histology (P = 0.669) or tumor site (P = 0.769), the analysis reveals that the high expression of C/EBPα is significantly correlated with Dukes’ stage (P = 0.003), tumor invasion (P = 0.009), lymph node metastasis (P = 0.002), and liver metastasis (P = 0.022).

To clarify whether expression of C/EBPα is a prognostic factor of CRC, survival predicted cutoff optimization was applied for immunohistochemistry scores of C/EBPα (Figure 2).
Figure 3. C/EBPα contributes to increased tumor growth of CRC in vitro and in vivo. A. Cells transfected with sh-C/EBPα and C/EBPα, respectively, were detected using western blot; B. Representative images of colony formation assays for RKO, HCT116, and HT29 cells. Statistical results of Colony formation numbers are also shown; C. As assessed by CCK-8 assay, knockdown of C/EBPα inhibited CRC cells proliferation in vitro, whereas ectopic expression of C/EBPα accelerated the growth of CRC cells; D. Tumor volume measured per week in different groups, downregulation of C/EBPα in RKO cells inhibited tumor growth rate compared with the RKO/sh-NC control group; E. Images of xenografts in nude mice; F. Proliferation of tumor cells in vivo. The effect of C/EBPα on cell proliferation in tumors was assessed by examining the proliferative index of Ki-67. Immunohistochemistry staining showed that the Ki-67 staining was significantly decreased in the RKO/sh-C/EBPα tumors compared with the RKO/sh-NC tumors. Scale bars: 50 μm. **P < 0.01, ***P < 0.001.
overall and disease-free survival, a cutoff of C/EBPα score at 7 is identified with best sensitivity and specificity to separate cohort into C/EBPα-high (N = 28) and -low (N = 49) groups (Figure 2A, 2C). As we expected, with established cutoff, high expression of C/EBPα predicts clearly worse overall survival (Log-rank \( P = 0.0004 \)) and disease-free survival (Log-rank \( P < 0.0001 \)) (Figure 2B, 2D).

To further evaluate whether the expression of C/EBPα is an independent prognostic predictor, multivariate cox-regression survival analyses are further used with multiple clinical and pathologic parameters (Table 2). Strikingly, at the cutoff of 7, high C/EBPα immunohistochemistry scores are independently associated with worse OS (HR = 3.787, \( P = 0.027 \)) and DFS (HR = 4.600, \( P = 0.008 \)) of CRC.

Collectively, our results indicate that C/EBPα is potential to be an effective prognostic biomarker for CRC and might play an important role in the pathogenesis of CRC.

C/EBPα contributes to increased tumor growth of CRC in vitro and in vivo

In order to explore the function of C/EBPα in CRC cells, we employed lentivirus-mediated shRNA to knock down the expression of C/EBPα in HCT116 and RKO cells, and selected HT29 for exogenous C/EBPα overexpression. The effect of knockdown or overexpression was confirmed by western blot (Figure 3A). Given the apparent phenomenon that C/EBPα level positively relates to cell proliferative ability in the CRC cell lines (Figure 1C), the role of C/EBPα in growth was assessed. The results of CCK-8 assays shown in that HCT116/sh-C/EBPα cells remarkably inhibited growth at day 6 relative to the HCT116/sh-NC cells; RKO/sh-C/EBPα cells showed a remarkably inhibited growth at day 5 relative to the RKO/sh-NC cells. Conversely, expression of C/EBPα in the slow-growing HT29 cells remarkably enhanced their growth ability at day 5 over the HT29/vector cells (Figure 3C). On the other hand, the results of plate clonal assays also confirmed the effect of C/EBPα on proliferation of CRC cells (Figure 3B).

Based on the in vitro results described above, we examined the in vivo effect of C/EBPα by injecting RKO/sh-C/EBPα and RKO/sh-NC cells subcutaneously into flank of nude mice. As expected, downregulation of C/EBPα in RKO cells inhibited both tumor growth rate and tumor size (Figure 3D, 3E), compared with the RKO/sh-NC control group. To clarify whether the oncogenic role of C/EBPα was caused through regulating cell proliferation as observed in in vitro cell system (Figure 3B, 3C), tumor sections were stained with cell proliferation marker Ki-67. The proliferation index was calculated from five randomly selected microscopic fields in each tumor section. As shown in Figure 3F, the percentage of proliferative cells (Ki-67 positive) was significantly decreased in the tumors produced from RKO/sh-C/EBPα cells, compared to the tumors from RKO/sh-NC cells. Thus, a decreased proliferation rate by down regulation of C/EBPα is responsible for tumor suppression in vivo.

C/EBPα promotes cell proliferation by interfering cell cycle progression and regulating cell apoptosis

Next, to understand how C/EBPα affects proliferation, a flow cytometry assay was applied to examine the effects of C/EBPα on CRC cell-cycle progression and apoptosis. As shown in Figure 4A, the HT29-C/EBPα cells exhibited a significant decrease of cell populations at G0/G1 phase and a relative increase of cell populations at S phase, comparing to their respective negative control cells. In contrast, suppression of C/EBPα in RKO and HCT116 cells significantly blocked the cell cycle at the G1/S phase transition, concomitant with an increase in G1 phase. Due to the sub-G1 peak is indicative of apoptosis induction after prolonged mitosis [25, 26], we furtherly assessed apoptosis by flow cytometry. As shown in Figure 4B, suppression of C/EBPα drastically induced apoptosis as compared with that measured in non-silencing shRNA-transfected RKO and HCT116 cells. Furthermore, we observed that overexpression of C/EBPα significantly protected HT29 cells from apoptosis. After that, the activation of the downstream effectors, such as caspase-3 and PARP, was investigated. As shown in Figure 4C, elevated expression of total and/or cleaved caspase-3 and PARP were observed after C/EBPα down-regulation in RKO and HCT116 cells. Whereas, the expression level of caspase-3, c-caspase-3 and c-PARP were significantly lower in HT29/C/EBPα cells than in HT29/vector cells.
Figure 4. C/EBPα promotes colorectal cancer progression. A. Cell cycle was assessed by flow cytometry based on phycoerythrin-conjugated annexin V (PE-A). The HT29-C/EBPα cells exhibited a significant de-
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Taken together, these data indicated that C/EBPα promotes cell proliferation by interfering cell cycle progression and protecting cell from apoptosis.

C/EBPα enhances CRC cells migration and invasion in vitro as well as metastasis in vivo through regulating EMT

Transwell assays showed that migratory and invasive potential was dramatically impaired in RKO/sh-C/EBPα and HCT116/sh-C/EBPα cells compared to sh-NC cells (Figure 5B), while increased level of C/EBPα promoted migratory and invasive abilities of HT29 cells. Additionally, wound healing assays indicated that the distance between wound edges of RKO/sh-C/EBPα and HCT116/sh-C/EBPα cells was markedly longer than those of RKO/sh-NC and HCT116/sh-NC cells. A shorter distance was also observed in wound healing of HT29/C/EBPα cells compared with control group (Figure 5A). Thus, these results indicated that C/EBPα promotes migration and invasion of colon cancer cells.

We next evaluated the metastatic capacity of stable transfected RKO/sh-C/EBPα and RKO/sh-NC cells after intravenous injection. The weight of each group was monitored every week, and the weight loss was recorded, representing the metastatic tumor appearance (Figure 5C). After the sacrifice of mice, we assessed the number of metastatic lesions in H&E-stained sections of lungs. These results show that sh-C/EBPα tansfected cells metastasized to the lung less readily than did its corresponding control cells, for C/EBPα downregulation led to later metastases and evolved less and smaller nodules in the lung parenchyma (Figure 5D, 5E). Interestingly, the C/EBPα expression in the lung metastases of RKO/sh-NC group detected by immunohistochemistry (IHC) indicated that it was mainly expressed in the cytosol; Whereas, the tumor specimen from subcutaneously xenografts presented a nuclear and cytoplasm mixed expression of C/EBPα (Figure 5F), which suggested that C/EBPα protein experienced a relocalization process during distal metastasis. This phenomenon coincided with the fact that cytoplasm expression of C/EBPα appeared at a relative late stage of the disease as mentioned before (Figure 1G; Table 3).

After ectopically expressed C/EBPα in CRC cells, we observed morphological changes in these cells which is in agreement with epithelial–mesenchymal transition (EMT) program that is a crucial trait in cancer progression [27-29]. As illustrated in Figure 6A, HT29/C/EBPα acquired a mesenchymal, spindle-like morphology compared with the HT29/vector cells, which means it perhaps developed into a more aggressive phenotype. However, HCT116/sh-C/EBPα and RKO/sh-C/EBPα cells developed an epithelial cell like morphology and tended to accumulate in clusters (increase in cell-cell contacts). Therefore, we considered that C/EBPα promote CRC cell migration and invasion via inducing EMT.

C/EBPα exerts its oncogenic role by targeting c-myc/cyclin D1 mediated by β-catenin involved pathway

To elucidate how C/EBPα affects CRC tumorigenesis and metastasis, some key molecules related to the metastasis and cell cycle were examined. To confirm the hypothesis that C/EBPα induced EMT in CRC, we examined changes in EMT markers in our established cells models. The results demonstrated that the upregulation of C/EBPα in HT29 cells led to lower expression of the epithelial marker E-cadherin and higher expression of the mesenchymal markers N-cadherin and Vimentin. While elevated levels of E-cadherin as well as reduced expression of N-cadherin and Vimentin, were observed in the HCT116/sh-C/EBPα and RKO/sh-C/EBPα cells (Figure 6B). As we found that down-regulation of C/EBPα was able...
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A

RKO

Sh-NC

Sh-C/EBPα

0h 24h

HCT116

Sh-NC

Sh-C/EBPα

0h 24h

HT29

Sh-NC

Sh-C/EBPα

0h 24h

B

Migration Assay

RKO

HCT116

Vector C/EBPα

D

Sh-NC Sh-C/EBPα

HCT116

Vector C/EBPα

E

Invasion Assay

RKO

HCT116

Vector C/EBPα

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Figure 5. C/EBPα enhances CRC cells migration and invasion in vitro as well as metastasis in vivo. A. Representative images of wound-healing assays for RKO, HCT116, and HT29 cells. Statistical results of relative migrating distances are also shown; B. Transwell assays: cells were seeded in upper chamber of insert and the migrated/invaded cells were examined after 24~48 h. Data represent the means ± s.d. from three independent experiments. Representative photos of stained cells are shown; scale bar, 50 μm; C. The weight loss curve: the weight of each group was monitored and recorded every week; D. The representative images show macroscopic observations of metastases in lung at the time of killing; Metastases in hematoxylin and eosin (H&E)-stained lung sections; E. Metastases were counted on five lobes of the lung in all animal groups. The results are collected and analyzed from each group and the histograms represent mean ± s.d. number of metastatic nodules. ***P < .0001, relative to the respective control cells; F. The representative IHC staining images of C/EBPα in subcutaneous tumors and lung metastatic tissues; scale bar, 50 μm.
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to induce G1 phase arrest, cell cycle related proteins were then examined. Western blot analysis results showed the levels of cyclin D1, CDK4, and CDK6 decreased after knockdown C/EBPα in HCT16 and RKO cells (Figure 6B). In contrast, upregulated expression of C/EBPα in HT29 had the opposite effects. Since c-Myc has been shown to be a direct transcriptional target of C/EBPα [30] and elevated c-Myc levels can contribute to increased metastatic behavior through the upregulation of target genes involved in the induction of EMT, we next examined c-Myc expression in these cells. As shown in Figure 6B, C/EBPα positively regulates c-Myc expression in colon cells.

It was reported that c-Myc and cyclin D1 were both target genes of β-catenin, furtherly, we noticed that C/EBPα was able to upregulate β-catenin expression in colon cells. Thus we wondered whether β-catenin mediated the oncogenic role of C/EBPα in CRC. After down-regulating the expression of β-catenin in HT29/C/EBPα cells (Figure 6C), we found the inhibition of β-catenin eliminated the C/EBPα-enhanced proliferation (Figure 6D), migration and invasion (Figure 6E), eliminated the C/EBPα-increased c-Myc and cyclin D1 expression (Figure 6C).

Thus, all these data shown that C/EBPα may exert its oncogenic role by targeting c-Myc/cyclin D1 mediated by β-catenin involved pathway.

Discussion

The expression of C/EBPα in CRC has not been reported till now. In fact, C/EBPα has long been established as a tumor-suppressor gene in human acute myeloid leukemia [7]. In addition, diminished C/EBPα have been reported in numerous solid tumors such as liver, breast, skin, lung, prostate, pancreatic, gastric, bladder, head and neck, cervical, and endometrial [11-14], indicating that in non-hematopoietic tissues C/EBPα also function as a tumor suppressor. In our study, we showed that C/EBPα was unexpectedly overexpressed in CRC tissues compared with adjacent normal tissues. Furthermore, C/EBPα high expression is significantly correlated with a shorter 5-year overall survival and disease-free survival for CRC patients. Multivariate analysis showed that C/EBPα was an independent factor in predicting prognosis of patients with CRC. All these data suggest that C/EBPα seem to play a promoting role in colorectal tumorigenesis and progression. Subsequent in vitro and in vivo studies further confirmed oncogenic function of C/EBPα in CRC. In addition, to support our conclusion, we checked the specificity of the shRNA we used (Supplementary Figures 1 and 2), overexpression of C/EBPα-shRNA resistant-C/EBPα in RKO and HCT116 cells can rescue function altered by down regulating C/EBPα expression. In fact, C/EBPα in solid tumors has been shown to influence key processes related to tumor biology, including cell proliferation, apoptosis, migration and invasiveness [11-14]. Our explorations indicated that C/EBPα expression was inversely correlated to the cell proliferative and migratory ability in CRC cells. Furthermore, the results of plate cloning assays and CCK-8 assays revealed that C/EBPα contributed to proliferation abilities of tumor cell; wound healing and Transwell assays showed that C/EBPα promotes migration and invasion of colon cancer cells. We next confirmed that C/EBPα enhances CRC tumor growth and metastasis through xenografts and intravenous injection of established C/EBPα overexpression and downregulation colon cells into nude mice.

In agree with our results, some studies also report increased expression of C/EBPα correlated with increased proliferation and disease progression [14, 16, 17, 31, 32], which were contrary to the tradition view that C/EBPα has a tumor-suppressor role. It was reported that the introduction of C/EBPα in HCC cells, keratinocytes or lung carcinoma cells did not affect cell cycle or cell proliferation levels [31-33]. Differences in proliferation due to C/EBPα can be the result of several mechanisms. Zhang et al. show that C/EBPα expression is positively correlated with androgen receptor (AR) levels, in particular, in the more aggressive tumors. However, C/EBPα is localized in the cytoplasm of these tumor specimens [34]. Interestingly, in our study, we found that C/EBPα protein experienced a relocalization process during distal metastasis. We showed that the C/EBPα expression in the lung metastases was mainly expressed in the cytosol; Whereas, the tumor specimen from subcutaneously xenografts pre-
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sented a nuclear and cytoplasm mixed expression of C/EBPα. This phenomenon coincided with the fact that cytoplasm expression of C/EBPα appeared at a relative late stage of the CRC. On the other hand, post-translational modifications as well as differences of isoform ratio in certain tissues might also be related to the function transition of C/EBPα. In acute myeloid leukemia, genetic alterations of C/EBPα has been found to be responsible for the development of cancer, in contrast, no evidences of mutations that directly affect the function of C/EBPα in solid tumors has been demonstrated. Therefore, the detailed regulation of C/EBPα in CRC remains elusive and is of interest to investigate.

Figure 6. C/EBPα exerts its oncogenic role by targeting c-myc/cyclin D1 mediated by β-catenin involved pathway. A. Bright-field images were taken to show cell morphological changes after sh-C/EBPα or C/EBPα treatment; B. Some key molecules related to the metastasis and cell cycle were examined by Western blotting; C. The C/EBPα overexpression and relative control HT29 were incubated with or without β-catenin specific shRNA (SH-β-catenin); Whole-cell lysates were extracted and immunoblotting was performed to assess β-catenin, c-Myc and cyclin D1 expression in cells transfected with Vector/SH-Con, C/EBPα/SH-Con, Vector/SH-β-catenin and C/EBPα/SH-β-catenin, respectively; D. As assessed by CCK-8 assay, C/EBPα promote cancer cell proliferation through increasing β-catenin expression; E. C/EBPα enhance cancer cell migration and invasion through increasing β-catenin expression. Tranwell assays: SH-Con and SH-β-catenin results. Data represent the means ± s.d. from three independent experiments. Representative photos of stained cells are shown; scale bar, 50 μm. ***P < 0.001.
To understand the functional mechanism of C/EBPα, we performed several arrays and revealed that C/EBPα promotes cell proliferation by speeding G1/S phase transition and protecting cells from apoptosis in CRC. One mechanism by which C/EBPα may be transformed from a cell growth inhibitor to a growth promoter was that PI3K can activate PP2A which then dephosphorylates C/EBPα on ser193 in mice [35, 36]. This posttranslational modification abolishes the inhibitory interaction of C/EBPα with cell cycle proteins [35], and whether a similar mechanism exists in CRC remains to be furtherly investigated. Meanwhile, we found that C/EBPα has a potential regulatory role in EMT. Our studies showed that C/EBPα induced the expression of mesenchymal marker N-cadherin, vimentin and suppressed the expression of epithelial marker E-cadherin, and vice versa. Depletion of c-Myc has been shown to prevent migration of several metastatic cells, mostly by releasing the repression of genes involved in the inhibition of canonical Wnt signaling [37-39]. Elevated c-Myc levels can contribute to increased metastatic behavior through the upregulation of target genes involved in the induction of angiogenesis and EMT, including vascular-endothelial growth factor-A (VEGFA) [40], MiR9 [41] and BMI1 [42, 43]. Since we found that C/EBPα was able to upregulate β-catenin expression levels and consequent increase expression of β-catenin-induced target genes, including c-Myc and cyclin D1, also, we found the inhibition of β-catenin eliminated the C/EBPα-enhanced proliferation, migration and invasion these findings suggest an important role for C/EBPα in the regulation of cell migration, potentially exerting its effects through suppression of the pathway targeting c-myc/cyclin D1 mediated by β-catenin involved pathway.

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Disclosure of conflict of interest

None.

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Supplementary Figure 1. A. Cells transfected with si-NC, si-C/EBPα-1 and si-C/EBPα-2, respectively, were detected using western blot; B. As assessed by CCK-8 assay, knockdown of C/EBPα by si-RNA inhibited CRC cells proliferation in vitro; C. As assessed by transwell assay, knockdown of C/EBPα by si-RNA inhibited CRC cells migration in vitro.

Supplementary Figure 2. A. As assessed by CCK-8 assay, knockdown of C/EBPα by sh-RNA inhibited CRC cells proliferation in vitro; overexpression of C/EBPα-shRNA resistant-C/EBPα in RKO and HCT116 cells can restore inhibited proliferation caused by C/EBPα-shRNA. B. As assessed by transwell assay, knockdown of C/EBPα by sh-RNA inhibited CRC cells migration in vitro; overexpression of C/EBPα-shRNA resistant-C/EBPα in RKO and HCT116 cells can restore reduced migration caused by C/EBPα-shRNA. C. As assessed by western blot, overexpression of C/EBPα-shRNA resistant-C/EBPα in RKO and HCT116 cells can rescue the expression of some key molecules related to the metastasis and cell cycle altered by C/EBPα-shRNA.