

Association of the invasiveness of colon cancer with the expression of C/EBP α

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Abstract. The present study aimed to investigate the association of the invasiveness of colon cancer (CC) with the expression of CCAAT/enhancer binding protein α (C/EBP α). Immunohistochemistry was performed to determine the expression of C/EBP α in the cancer and adjacent tissue samples from 48 patients with CC. A pCDGFP-C/EBP α eukaryotic expression vector was constructed, and a wound-healing assay was performed to observe the effect of transfection on the migration of SW480 cells. In addition, the expression levels of tumor invasion-associated proteins, including Kruppel-like factor 5 (KLF5), matrix metalloproteinase (MMP)-2, MMP-9, and E-cadherin (ECD) were detected subsequent to transfection. Immunohistochemistry analysis demonstrated that the rate of low C/EBP α expression in normal tissue was 6.25%, whereas the rate in CC tissues was 68.75%; this difference was statistically significant ($P < 0.05$). The patients with lower C/EBP α expression exhibited statistically larger tumor diameters, more advanced tumor-node-metastasis (TMN) stages and a greater likelihood of lymph node metastasis. The overexpression of C/EBP α significantly reduced the mobility of SW480 cells, and the expression of KLF5, MMP-2 and MMP-9 was reduced, whereas the expression of ECD was increased. In conclusion, C/EBP α was downregulated in CC tissue samples, and associated with the TMN stage and metastasis of CC; in addition, the overexpression of C/EBP α significantly reduced the invasiveness of CC cells. This may be significant for the diagnosis and treatment of CC in the future.

Introduction

The morbidity and mortality rates for colon cancer (CC) rank third among all types of malignant tumor, worldwide (1). CC incidence is increasing annually, and occurring in increasingly younger populations (2,3). The early stages of CC are frequently asymptomatic, meaning that invasion and metastasis have often already occurred when CC is diagnosed, thus affecting how effectively the disease can be clinically treated. The identification of CC-specific molecular markers and strategies to inhibit tumor invasion has crucial importance towards its early diagnosis, and potentially in the development of gene-targeted therapy.

CCAAT/enhancer binding protein α (C/EBP α) is widely expressed in human tissue samples (4), particularly in the liver, lung, adipose tissue and placenta (5,6). C/EBP α is a transcription factor that has been demonstrated not only to serve a role in inhibiting cell proliferation and promoting cell differentiation (7,8), but also in inhibiting the invasion and metastasis of tumor cells (9). Therefore, it demonstrates the typical characteristics of a tumor suppressor gene.

The present study examined 48 CC tumor specimens and determined their C/EBP α expression level, as well as the association between its expression and clinicopathological parameters. Then the effect of the induced overexpression of C/EBP α on the invasiveness of the CC cell line, SW480, was investigated, with the aim of providing a theoretical basis for novel molecular markers and the gene-targeted therapy of CC.

Patients and methods

Clinical data. A total of 48 patients with CC admitted into the Affiliated Nanhua Hospital (Hengyang, China) between October 2013 and November 2014 were selected, including 30 males and 18 females aged 32 to 78 years, with a mean age of 54.2 years. A total of 22 cases exhibited a tumor diameter ≤ 5 cm, whereas the remaining 26 cases were > 5 cm; 22 cases were stage T1 or 2, and 26 cases were in stage T3 or 4, according to the National Comprehensive Cancer Network Guidelines for Colon Cancer Tumor-Node-Metastasis (TNM) staging system (10); 21 cases had no lymph node metastasis, whereas 27 cases were lymph node metastasis-positive. All patients were diagnosed as CC and had not received chemotherapy prior to surgery. Tumor and adjacent normal tissue samples

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(distance from the tumor margin, >5 cm) were extracted from all patients, fixed in formalin, and embedded in paraffin. The study was conducted in accordance with the declaration of Helsinki, and was approved by the Ethics Committee of the University of South China (Hengyang, China). Written informed consent was obtained from all participants.

Immunohistochemistry. Representative formalin fixed, paraffin-embedded tissue blocks were selected from each sample. Sections of 5- μ m thickness were cut, deparaffinized by xylene (twice) for 10 min and rehydrated through a graded ethanol series (95, 85, 75 and 50%, for 5 min each). Antigen retrieval was performed by heating the slides in citrate buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 98°C for 30 min in a water bath. Endogenous peroxidase activity was quenched for 10 min with a peroxidase blocking reagent (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). The sample was incubated with primary antibodies, including anti-C/EBP α (dilution, 1:200; cat. no. sc-61; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight, and then were incubated with secondary antibodies anti-mouse IgG (dilution, 1:200; cat. no. A21010; Amyjet Scientific, Wuhan, China) for 30 min at 37°C. Antibody staining was visualized using the ChemMate Envision detection system (Dako; Agilent Technologies, Inc.). Sections were counterstained with Meyer's hematoxylin solution at room temperature for 30–60 sec. Negative controls were run simultaneously using a pre-immune serum without any antibody diluted to 1:10 and 1:5 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The C/EBP α IHC signals were scored on the following scale, considering the proportion of cells stained as follows: Score 0, no cells stained; score 1, weak or absent nuclear staining in <5% of cells; score 2, nuclear staining in 5–25% of the cells; score 3, nuclear staining in 26–50% of the cells; score 4, nuclear staining in >50% of the cells. Two observers quantified the staining results independently.

Plasmid construction. A pCDGFP-C/EBP α eukaryotic expression plasmid was constructed. PrimeSTAR polymerase was used to amplify the C/EBP α coding sequence from a human C/EBP α -containing cDNA library using polymerase chain reaction (PCR) with the following primers: Forward, CGC GGATCCGCGAGCCACCATGGAGTCGGCCGACT and reverse, CCGGAATTCCGGCGCGCAGTTGCCCATG. Pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 sec, 62°C for 30 sec and extension at 72°C for 32 sec for 40 cycles. The PCR products were then purified by agarose gel electrophoresis followed by double-enzyme digestion (*Bam*HI and *Eco*RI); the pCDGFP vector was prepared with the same double-enzyme digestion. T4 ligase was used to connect the digested PCR products and the vector at 16°C for 12 h, followed by plasmid transformation into TG1 competent *E. coli* cells. The transformed cells were then incubated on inverted ampicillin agar plates at 37°C until clones were visible (~10 h). A sterilized toothpick was used to select several monoclonal colonies and seed them into LB medium with ampicillin for incubation with shaking (37°C, 250 RPM) until the broth appeared cloudy (~10 h), when the bacteria were collected. The alkaline lysis method was used to extract the plasmids. The extracted plasmids were digested with *Bam*HI and *Eco*RI and separated

with gel electrophoresis for verification; the plasmids were then DNA sequenced for further confirmation.

Cell culture. The human CC cell line SW480 was purchased from Biowit Technologies Ltd. (Shenzhen, China), and stored in liquid nitrogen until the experiments. The cells were then cultured in 5% FBS (cat. no. 10099158; Thermo Fisher Scientific, Inc.), 10% FBS, and mycillin-containing (50 U/ml) Dulbecco's modified Eagle's medium (DMEM; Merck KGaA, Darmstadt, Germany) at 37°C with 5% CO₂; the cells were observed and the culture medium replaced on a daily basis. When cell confluence was >90%, they were passaged and re-seeded to an appropriate density. Cells in the logarithmic phase were used for transfection and cryo-preserved.

Cell transfection. The liposomal transfection method was used. Opti-MEM™ (Thermo Fisher Scientific, Inc.) was used to dilute Lipofectamine 2000 (1:1; 15 μ l) and pCDGFP-C/EBP α plasmids (5 μ g; 500 ng/ μ l) with empty plasmid as the negative control; upon standing for 5 min, they were agitated gently, left to stand for a further 20 min, and added to SW480 cells in the logarithmic phase. After 4–6 h of incubation, the culture medium was replaced with fresh complete medium, followed by a further 24–72 h incubation; the medium was changed once every 24 h. Then the C/EBP α expression level was determined with reverse transcription-quantitative PCR (RT-qPCR).

Wound-healing assay. The pCDGFP-C/EBP α -transfected and non-transfected SW480 cells were used in a wound-healing assay. At 6 h after transfection, the medium was changed, a pipette tip was used to draw lines in the cell monolayer of culture dishes and PBS was used to rinse the surface 2–3 times to remove debris. The culture dishes were then incubated and the medium was replaced daily. The cell migration was observed at 4 timepoints (0, 24, 48 and 72 h); 10 observation points were selected at each timepoint, and the distance between one side of the wound to the farthest point was measured and recorded. The mean distance was then used, and the cell mobility was calculated using the following formula: Mobility=(initial scratch width-scratch width at each time point)/initial scratch width x100%.

RNA isolation and RT-qPCR. The mRNA expression levels for C/EBP α were examined by RT-qPCR using RNA isolated from pCDGFP-C/EBP α -transfected and non-transfected SW480 cells (TRIzol® kit; Thermo Fisher Scientific, Inc.). Taq polymerase (Qiagen GmbH, Hilden, Germany) was used to add bases to primers and a PCR kit (Thermo Fisher Scientific, Inc.) was used for amplification in qPCR, and the thermocycling protocol was as follows: Pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 sec and 62°C for 30 sec, and extension at 72°C for 32 sec for 40 cycles. Using 2 μ g RNA, cDNA was synthesized using a Superscript III first-strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using a StepOne™ Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). Reactions of 20 μ l were performed using 100 ng/ml cDNA; the amplification of the product was measured via SYBR-Green fluorescence

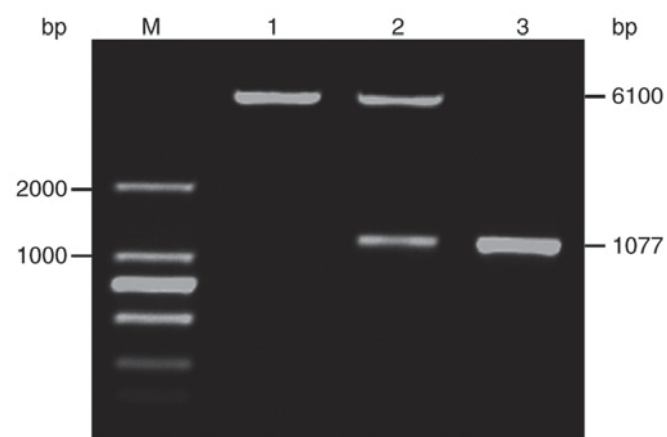


Figure 1. Double-enzyme digestion identification of recombinant plasmid pCDGFP-C/EBP α . M, DNA markers; 1, pCDGFP digested product; 2, pCDGFP-C/EBP α digested products; 3, C/EBP α polymerase chain reaction product. C/EBP α , CCAAT/enhancer binding protein α .

relative to endogenous cyclophilin, which was expressed in the comparative Cq format ($2^{-\Delta C_q}$) (11). The amplification thermocycling profile was 94°C for 0.5 min, 58°C for 1 min, and 72°C for 1 min, for 28 cycles. Primers for C/EBP α (forward, 5'-TCGCCATGCCGGGAGAACTCTAAC-3' and reverse, 5'-CTGGAGGTGGCTGCTCATCGGGG-3') were purchased from GenScript (Nanjing, China). β -actin was used as an internal control (forward, 5'-CCGACAGGATGCAGAAGGAG-3' and reverse, 5'-GGCACGAAGGCTCATCATTC-3').

Western blot analysis. Samples were centrifuged at 12,000 \times g for 5 min at 4°C (Centrifuge 5804/5804 R; Eppendorf, Hamburg, Germany) and the total protein content in the supernatant was determined using an Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China). A total of 30 μ g protein from the cell lysates was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was subsequently blocked with 5% skimmed milk at room temperature for 1 h, and incubated at 4°C overnight with KLF5 (cat. no. bs-2020R), MMP-2 (cat. no. bs-4599R), MMP-9 (cat. no. bs-20619R) or ECD rabbit polyclonal antibodies (dilution, 1:500; BIOS, Beijing, China), or a mouse anti-human anti- β -actin antibody (dilution, 1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequent to washing with 0.01 M Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated with horseradish peroxidase-labeled goat-anti-rabbit (cat. no. E030120-01) or anti-mouse (cat. no. E030110-01) IgG antibody (dilution, 1:1,000; EarthOx Life Sciences, Millbrae, CA, USA) at room temperature for 1 h. A standard enhanced chemiluminescence reaction (Sangon Biotech Co., Ltd., Shanghai, China) was performed, according to the manufacturer's protocol.

Statistical analysis. SPSS statistical software 10.0 (SPSS, Inc., Chicago, IL, USA) was used for all data analysis. Experimental data were expressed as the mean \pm standard deviation, and were analyzed with a paired t test. The association of clinicopathological parameters with expression status was

analyzed with the χ^2 test. $P < 0.05$ was considered to represent a statistically significant difference.

Results

Expression of C/EBP α in CC tumor and adjacent tissue samples. The CC tumor and adjacent tissue samples of from 48 patients with CC were analyzed with immunohistochemistry to assess the expression of C/EBP α . Among the 48 normal tissue samples, 3 cases (6.25%) exhibited the negative or low expression of C/EBP α , whereas the remaining 45 cases exhibited high C/EBP α expression; among the 48 CC tumor samples, 33 cases (68.75%) exhibited the negative or low expression of C/EBP α , whereas the remaining 15 cases exhibited high C/EBP α expression. Compared with the adjacent tissue samples, the expression of C/EBP α in CC tissue was significantly decreased ($P < 0.05$).

Associations between C/EBP α expression and CC clinicopathological parameters. Among the 48 cases, the C/EBP α expression was not associated with such clinicopathological parameters as the sex and age of the patients, whereas it was associated with the tumor size, the TNM stage and the lymph node metastasis status ($P < 0.05$; Table I).

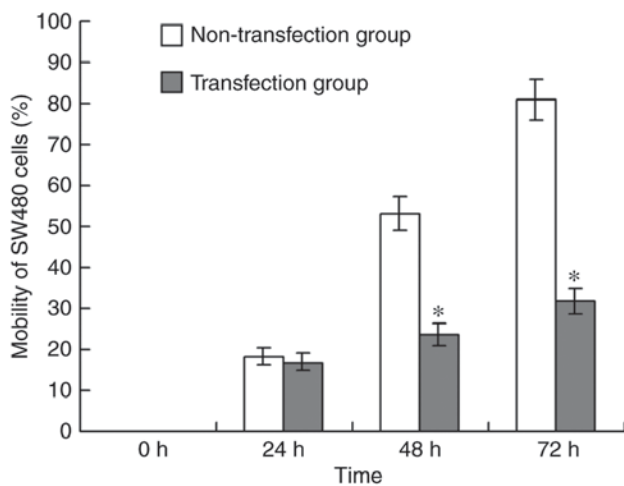
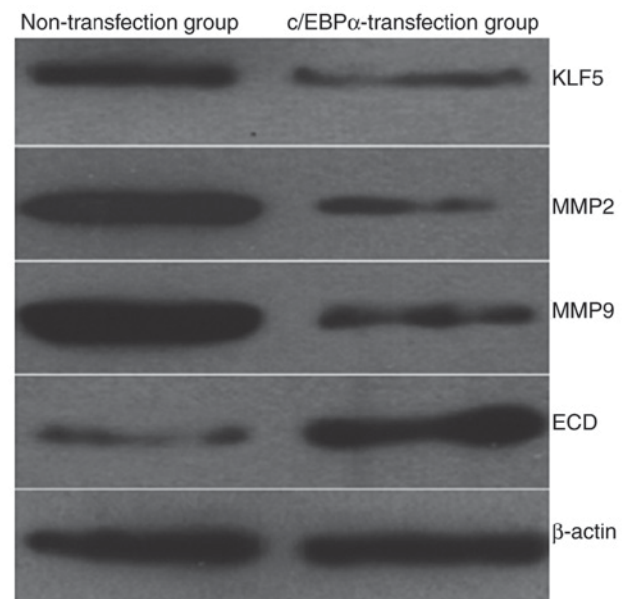
Identification of pCDGFP-C/EBP α plasmid. The recombinant plasmid pCDGFP-C/EBP α was digested using *Bam*HI and *Eco*RI to produce two bright bands. Comparison with the marker bands demonstrated that one band was the vector, pCDGFP (6,100 bp), and the other was the C/EBP α fragment (1,077 bp), indicating that the plasmid was successfully ligated. The subsequent sequencing results further verified the construct. The enzyme digestion results are displayed in Fig. 1. The expression level of C/EBP α in the transfected cells was detected using RT-qPCR. The relative expression level of C/EBP α was increased by >200-fold compared with the control group (238:1). This result indicated that C/EBP α was successfully expressed after transfection.

Impacts of overexpressed C/EBP α on the mobility of SW480 cells. At 24 h in the wound-healing assay, the cell mobility rate did not significantly differ between the non-transfection and transfection groups ($P > 0.05$), whereas at 48 and 72 h, the cell mobility rates were significantly different ($P < 0.05$), indicating the overexpression of C/EBP α significantly reduced the mobility of SW480 cells (Fig. 2).

Impacts of overexpressed C/EBP α on tumor invasion-associated proteins. At 48 h after plasmid transfection, western blotting analysis was applied to determine the effect of C/EBP α overexpression on the expression of tumor invasion-associated proteins, including KLF5, MMP-2, MMP-9, and ECD. The results revealed that, compared with the non-transfection group, the protein expression levels of KLF5, MMP-2 and MMP-9 in the C/EBP α -overexpression group were decreased, whereas the level of ECD was increased. The results demonstrated that the overexpression of C/EBP α reduced the expression of tumor invasion-promoting proteins, thus potentially inhibiting tumor invasion (Fig. 3).

Table I. Relationships between C/EBP α expression and the clinicopathological parameters of colon cancer.

Clinicopathological parameter	Total	Expression of C/EBP α , n (% of all patients)		χ^2	P-value
		Low	High		
Total	48	33	15		
Sex (%)				0.058	0.028
Male	30	21 (63.6)	9 (60.0)		
Female	18	12 (36.4)	6 (40.0)		
Age, years				0.076	0.074
≤ 50	27	19 (57.6)	8 (53.3)		
> 50	21	14 (42.4)	7 (46.7)		
Tumor diameter, cm				4.663	0.015
≤ 5	21	11 (33.3)	10 (66.7)		
> 5	27	22 (66.7)	5 (33.3)		
Tumor-node-metastasis stage				10.381	0.043
1/2	19	8 (24.2)	11 (73.3)		
3/4	29	25 (75.8)	4 (26.7)		
Lymph node metastasis				13.191	0.037
Yes	28	25 (75.8)	3 (20.0)		
No	20	8 (24.2)	12 (80.0)		

C/EBP α , CCAAT/enhancer binding protein α .Figure 2. Effect of CCAAT/enhancer binding protein α transfection on the mobility of SW480 cells, as determined with a wound-healing assay. *P<0.05 compared with non-transfection group.Figure 3. Effect of C/EBP α on the expression of tumor invasion-associated proteins. C/EBP α , CCAAT/enhancer binding protein α ; KLF5, Kruppel-like factor 5; MMP, metalloproteinase; ECD, E-cadherin.

Discussion

Tumor invasion and metastasis are processes in which cancer cells leave the primary lesion, invade surrounding tissue and continue to proliferate, ultimately resulting in the formation of new tumor lesions. This is the most critical biological property of malignant tumors. The C/EBPs are a family of transcription factors involved in the regulation of embryonic gut development in rodents that have also been detected in various types of malignancy; for example, Rask *et al* (12)

identified that the expression of C/EBP β was increased in all the assessed CC tumor samples compared with normal colon mucosa samples. Although the inter-patient variability was large, it was identified that liver-enriched inhibitory protein (LIP), the isoform of C/EBP β associated with the inhibition of transcription, was expressed at higher levels in Duke's stage B tumors compared with stage A, whereas stage C

tumors exhibited the lowest LIP expression (12). However, in the present study, it was identified that the expression of the C/EBP α subtype in CC tissues was significantly reduced relative to in the adjacent tissue. Thus, we hypothesize that C/EBP α may be a tumor suppressor in CC, whereas C/EBP β may increase CC invasiveness. This result is similar to other research in solid tumors; Yong *et al* (13) concluded that C/EBP α is a tumor suppressor in lung cancer, and that BMI1 is required for the oncogenic process downstream of C/EBP α loss.

The present study identified that C/EBP α was downregulated in CC, and was associated with tumor size, the TNM stage and the lymph node metastasis status, suggesting that the low expression of C/EBP α may affect the invasion and metastasis of CC. When observing the effect of C/EBP α expression on the invasiveness of SW480 CC cells, it was identified that the SW480 cells with C/EBP α overexpression exhibited significantly decreased invasiveness compared with untransfected cells. In addition, in SW480 cells with overexpressed C/EBP α , the expression of KLF5, MMP-2 and MMP-9 were reduced, whereas ECD was increased; this result was consistent with the patient data analysis. Previous studies have reported that the upregulation of KLF5, MMP-2 and MMP-9, and the low expression of ECD, promote the invasiveness of CC (14-18), which is consistent with the results of the present study.

C/EBP α has been demonstrated to inhibit cell growth via the direct repression of E2F-DP-mediated transcription (19). C/EBP α has also been observed to arrest cell proliferation through the direct inhibition of cyclin-dependent kinases 2 and 4 (20,21). However, the detailed mechanism for the C/EBP α -mediated inhibition of invasion-associated protein expression remains unclear, and should be considered in further research. In addition, the present study did not compare the pairs of CC tumor and adjacent tissues directly, so this is a limitation of the study that will be addressed in further research.

In summary, C/EBP α expression was associated with the occurrence and development of CC.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author, on reasonable request.

Authors' contributions

XJZ and LFL designed the experiment. XZL, LFL and SW reviewed the protocol. XJZ and LFL conducted the

experiment. WL and LJJ wrote the manuscript. WL and LJJ performed statistical analysis. XZL and SW reviewed the manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the declaration of Helsinki, and was approved by the Ethics Committee of the University of South China. Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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