CCAAT/enhancer-binding protein α decreases the viability of gastric cancer cells

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Received May 6, 2016; Accepted February 23, 2017

DOI: 10.3892/ol.2017.5987

Abstract. CCAAT/enhancer-binding protein (C/EBP) α, C/EBPβ and C/EBPδ are involved in inflammation and cell differentiation. In the present study, their roles in human gastric cancer cells were investigated. The human gastric cancer cell lines MKN45 and MKN74 were subjected to the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to analyze the expression levels of C/EBPα, C/EBPβ and C/EBPδ. The cells were transfected with expression plasmids for either C/EBPα or C/EBPδ, and subjected to a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS) assay and RT-qPCR for analysis of cyclin D1 expression. Expression levels of C/EBPα and C/EBPδ were decreased in MKN45 and MKN74 cells compared with in normal gastric tissue. Expression levels of C/EBPβ were decreased in MKN45 cells and increased in MKN74 cells. Viability of MKN45 cells was decreased by C/EBPα and C/EBPδ. Viability of MKN74 cells was decreased by C/EBPα, but increased by C/EBPδ. Expression levels of cyclin D1 were decreased in association with C/EBPα and C/EBPδ overexpression in MKN45 cells. Expression levels of cyclin D1 were decreased in association with C/EBPα overexpression, but increased in association with C/EBPδ overexpression, in MKN74 cells. The results of the present study indicate that C/EBPα is potentially useful for the treatment of gastric cancer.

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Key words: gastric cancer, CCAAT/enhancer-binding protein α, CCAAT/enhancer-binding protein β, CCAAT/enhancer-binding protein δ, reverse transcription-quantitative polymerase chain reaction, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt assay, episomal vector

Introduction

Gastric cancer (GC) is a leading cause of cancer-related mortality globally (1). Prognosis is poor in patients with advanced GC (2). Endoscopic surgical resection is currently used for the treatment of early-stage GC only (3). The lack of non-surgical alternatives in treatment approaches has resulted in a requirement to develop novel and targeted therapies to treat GC.

CCAAT/enhancer-binding proteins (C/EBPs) are transcription factors that belong to the family of basic leucine zipper proteins, and regulate gene expression in cell viability and inflammation (4,5). C/EBPα, C/EBPβ and C/EBPδ are involved in terminal differentiation of cells and inflammatory processes (6-8). Furthermore, the expression levels of C/EBPα and C/EBPβ are altered in cancerous tissue compared with in surrounding healthy tissues (9). Although it has been demonstrated previously that C/EBPδ is involved in GC (10), its specific role remains unknown. The specific roles of C/EBPα, C/EBPβ and C/EBPδ in GC remain incompletely understood.

Therefore, the expression levels of C/EBPα, C/EBPβ and C/EBPδ were investigated in GC cells as a potential treatment method to decrease the viability of GC cells. Experiments were conducted using the Epstein-Barr virus (EBV) episomal vector. EBV episomal vector containing the latent origin of replication, *oriP*, and nuclear antigen are able to efficiently transfect host cells, and stably express and amplify genes of interest in daughter cells following division (11).

Materials and methods

Cell culture. The GC cell lines MKN45 and MKN74 were purchased from RIKEN BioResource Center Cell Bank (Tsukuba, Japan). Cells were cultured in 10 cm dishes (Asahi Techno Glass Corporation, Funabashi, Japan) in RPMI-1640 medium (Sigma; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C, in a humidified chamber containing 5% CO_2.

Plasmid construction and transfection. A C/EBPα fragment, digested with *Nru*I-*Eco*RV from pG28B5.0 plasmid (kindly provided by Dr Kleanthis G. Xanthopoulos,

IRRAS AB, Stockholm, Sweden), was subcloned into *Eco*RV sites of the episomal vector pEBMulti-Neo (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to generate pEB/C/EBPα-Neo (12). A C/EBPδ fragment digested with *Eco*RI from IRCB003O22 (RIKEN BioResource Center DNA Bank, Tsukuba, Japan) was subcloned into pBluescript2SK(-) (Agilent Technologies, Inc., Santa Clara, CA, USA) to construct pBlue/C/EBPδ. An *Eco*RV-*Bam*HI-digested fragment of pBlue/C/EBPδ was subcloned into the *Eco*RV-*Bam*HI site of pEBMulti-Neo to generate pEB/C/EBPδ-Neo. Episomal plasmid vector constructs were transfected into GC cells and normal gastric mucosa cells. pEB/C/EBPα-Neo and pEB/C/EBPδ-Neo were transfected using Lipofectamine® LTX (Thermo Fisher Scientific, Inc.) into the cell cultures at concentrations of 10 or 100 ng/well in 96-well plates and at 2 μg/well in 6-well plates, according to the manufacturer's protocol. Mock transfection was carried out without nucleic acid material.

Cell viability analysis. Cells were trypsinized, harvested, centrifuged at 100 x *g* for 3 min at 4˚C and seeded in 96-well flat-bottom plates (Asahi Techno Glass Corporation) at a density of 1,000 cells/well, and incubated for 24 h in Dulbecco's modified Eagle's medium (Sigma; Merck KGaA) supplemented with 10% FBS. Following incubation for 24 h, the cells were transfected with pEB/C/EBPα-Neo or pEB/C/EBPδ-Neo. Following incubation for 72 h, cell viability was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS) assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. MTS is bioreduced by viable cells into a colored formazan product with an absorbance at 490 nm. Absorbance at 490 nm was analyzed using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Mock-transfected cells were used as a control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). GC and normal gastric mucosa cells were cultured in 6-well plates (Asahi Techno Glass Corporation). When the cells reached 70% confluence, they were transfected with 0, 0.25, 0.75 or 2.5 mg pEB/C/EBPα-Neo or pEB/C/EBPδ-Neo. Total RNA was isolated using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and 5 μg RNA was used for first-strand cDNA synthesis, using the SuperScript III First-Strand Synthesis system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was performed using Fast SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) for 40 cycles with 5 sec of denaturation at 95˚C and 5 sec of annealing/extension at 60˚C. PCR primers and amplicon lengths for qPCR are presented in Table I. Ribosomal protein L19 (RPL19), a constitutively expressed housekeeping gene (13), was used as an endogenous control to monitor the amount of mRNA. The gene expression levels were automatically analyzed using the MiniOpticon system (Bio-Rad Laboratories, Inc.), based on the ΔΔCq method (14). The relative expression levels were calculated as the expression level of a specific gene divided by that of RPL19. Human whole stomach RNA was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA) and used as a healthy control.

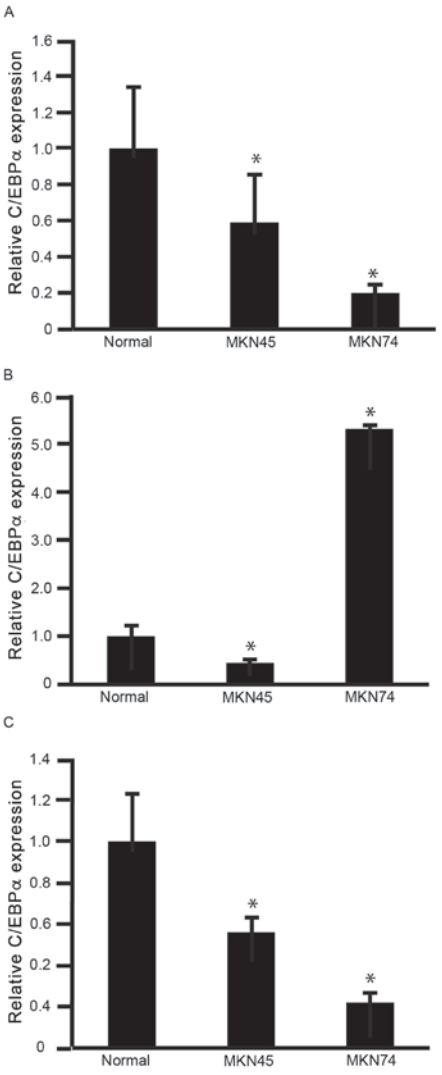

Figure 1. Expression levels of C/EBP mRNAs in gastric cancer cells. Total RNA was isolated from human gastric cancer MKN45 and MKN74 cells, and subjected to the reverse transcription-quantitative polymerase chain reaction to analyze the mRNA expression levels of (A) C/EBPα, (B) C/EBPβ and (C) C/EBPδ in gastric cancer cells compared with those of normal gastric mucosa (normal). *P<0.05 vs. normal cells (n=3). C/EBP, CCAAT/enhancer-binding protein.

Statistical analysis. One-way analysis of variance was calculated using JMP software (version 10.0.2; SAS Institute, Cary, NC, USA) to evaluate statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Results

To determine the expression levels of C/EBPα (Fig. 1A), C/EBPβ (Fig. 1B) and C/EBPδ (Fig. 1C) in GC cells, RNA was isolated from MKN45 and MKN74 cells for analysis using RT-qPCR. The expression levels of C/EBPα and C/EBPδ were significantly decreased in MKN45 and MKN74 cells, compared with normal stomach (gastric) mucosa (P<0.05; Fig. 1A and C). The expression levels of C/EBPβ were significantly decreased in MKN45 cells, but significantly increased in MKN74 cells, compared with normal stomach mucosa (P<0.05; Fig. 1B). These results indicated that C/EBPα and C/EBPδ warranted further investigation for their potential

Table I. Primer sequences and conditions for the quantitative polymerase chain reaction.

Primer name	Sequence	Description	Product size, kb	GenBank® accession no.
OMC321	5'-CGAATGCCAGAGAAGGTCAC-3'	RPL19, forward	157	BC095445
OMC322	5'-CCATGAGAATCCGCTTGTTT-3'	RPL19, reverse		
OMC351	5'-CGGACTTGGTGCGTCTAAGATG-3'	C/EBPα, forward	148	U34070
OMC352	5'-GCATTGGAGCGGTGAGTTTG-3'	C/EBPα, reverse		
OMC355	5'-AGAGGCGGAGGAGAACAAACAG-3'	Cyclin D1, forward	180	NM_053056
OMC356	5'-AGGCGGTAGTAGGACAGGAAGTTG-3'	Cyclin D1, reverse		
OMC569	5'-AAGCACAGCGACGAGTACAA-3'	C/EBPβ, forward	155	BC007538
OMC570	5'-AGCTGCTCCACCTTCTTCTG-3'	C/EBPβ, reverse		
OMC571	5'-AGAAGTTGGTGGAGCTGTCG-3'	C/EBPδ, forward	101	BC105109
OMC572	5'-CAGCTGCTTGAAGAACTGCC-3'	C/EBPδ, reverse		

RPL19, ribosomal protein L19, C/EBP; CCAAT/enhancer binding protein.

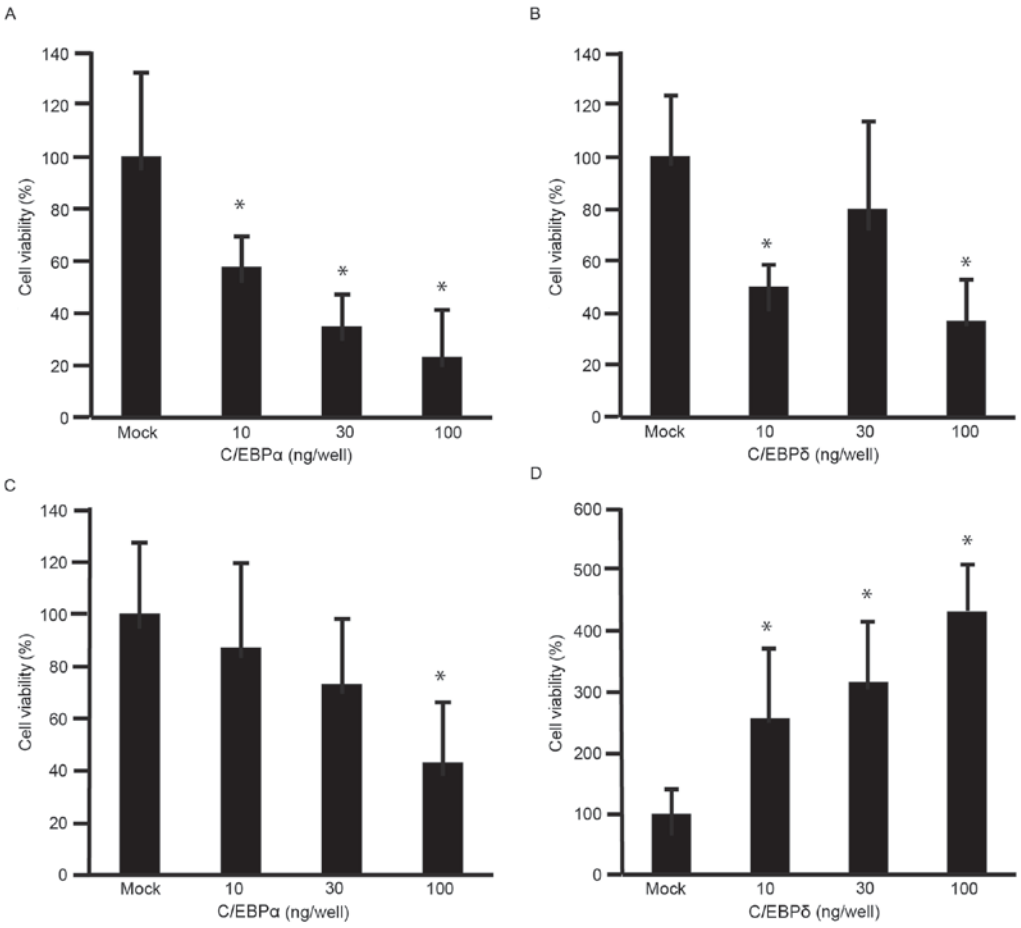

Figure 2. Cell viability assay. MKN45 cells were transfected with (A) C/EBPα or (B) C/EBPδ. MKN74 cells were transfected with (C) C/EBPα or (D) C/EBPδ. Transfection without nucleic acid material was used as a mock control. At 72 h after transfection, cells were subjected to a cell viability assay. Results are expressed relative to the mock control. *P<0.05 vs. mock-transfected cells (n=3). C/EBP, CCAAT/enhancer-binding protein.

involvement in decreasing GC cell viability. However, the biological significance of C/EBPβ in GC cells was not clear from these results.

The viability of MKN45 cells was decreased by C/EBPα (Fig. 2A) and C/EBPδ (Fig. 2B), compared with that of mock-transfected cells (P<0.05). Although the viability of MKN74 cells was significantly decreased by C/EBPα at 100 ng/well (P<0.05; Fig. 2C), it was significantly increased by C/EBPδ (P<0.05; Fig. 2D). These results indicated that C/EBPα decreased the viability of GC cells. To further

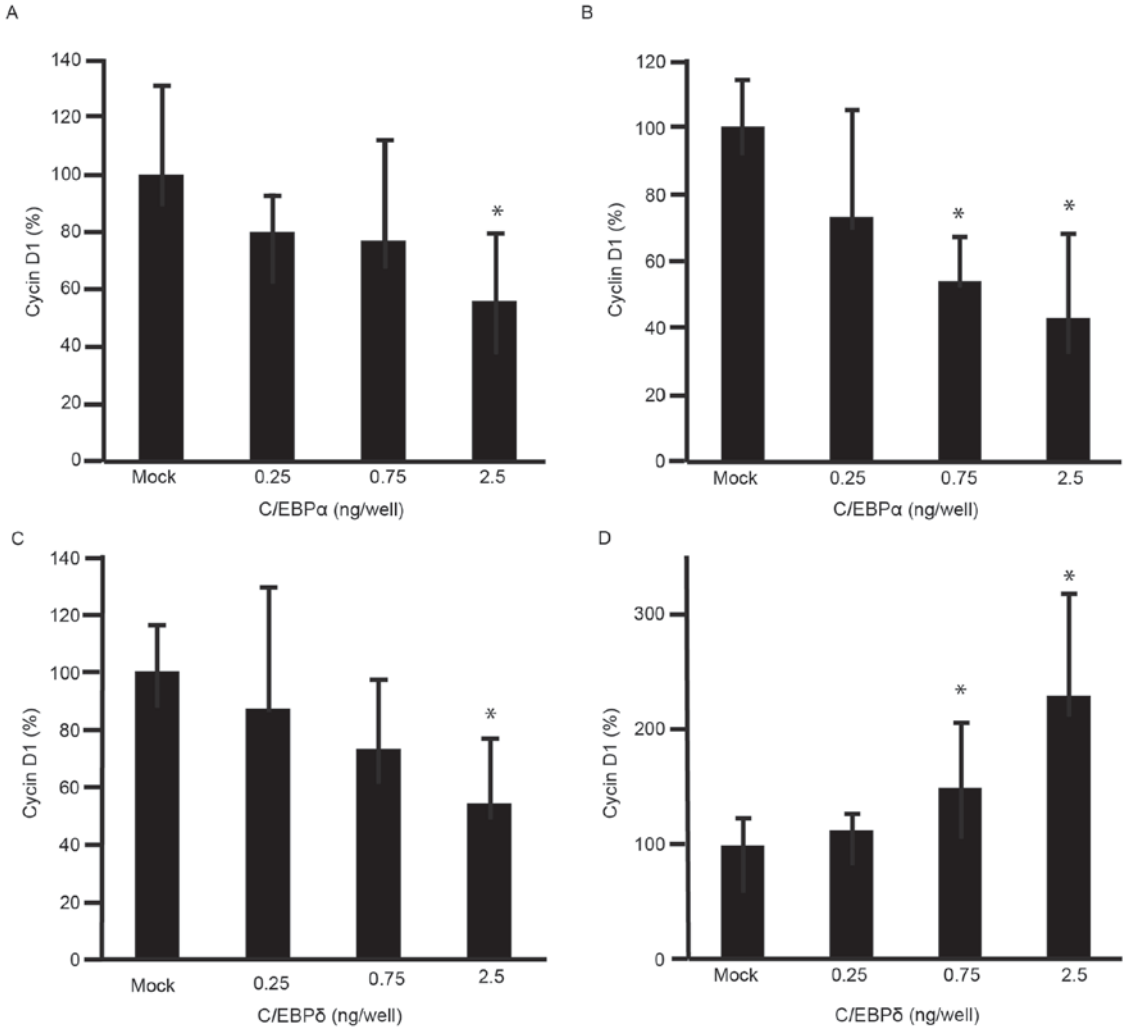

Figure 3. RT-qPCR analysis of cyclin D1 mRNA expression. (A) MKN45 cells or (B) MKN74 cells were transfected with C/EBPα, and subjected to RT-qPCR of cyclin D1. (C) MKN45 cells or (D) MKN74 cells (D) were transfected with C/EBPδ, and subjected to RT-qPCR of cyclin D1. Transfection without nucleic acid material was used as a mock control. Results are expressed relative to the mock control. *P<0.05 vs. mock-transfected cells (n=3). RT-qPCR, reverse transcription-quantitative polymerase chain reaction; C/EBP, CCAAT/enhancer-binding protein.

understand the effects of C/EBPα and C/EBPδ on cell viability, expression levels of cyclin D1 were analyzed using RT-qPCR. The expression levels of cyclin D1 were decreased in MKN45 and MKN74 cells transfected with C/EBPα (Fig. 3A and B). The expression levels of cyclin D1 were decreased in MKN45 cells transfected with C/EBPδ (Fig. 3C), but increased in MKN74 cells transfected with C/EBPδ (Fig. 3D).

Discussion

C/EBPα is expressed in normal gastric epithelium and downregulated in GC (15). Previous studies have also demonstrated that C/EBPα decreased the viability of hepatocellular carcinoma cells (16,17). In the present study, the expression levels of C/EBPα were identified to be decreased in GC cells. The results indicated that C/EBPα is able to facilitate tumor cell suppression in GC. C/EBPα was able to decrease metastasis of GC by upregulating microRNA-100 (18). The results of the present study indicated that expressing C/EBPα is potentially useful in the targeted treatment of GC.

Previous studies have demonstrated that, during inflammation, C/EBPδ is recruited to the promoter region of cyclooxygenase-2, although its expression levels remained constant (19). Currently, to the best of our knowledge, no conclusive study of the expression levels of C/EBPδ in GC cells has been performed. In liver cancer, C/EBPδ acts as a tumor suppressor (20). In the present study, C/EBPδ was downregulated in GC cells, indicating that C/EBPδ acted as a tumor suppressor. However, C/EBPδ decreased the viability of MKN45 cells, but increased the viability of MKN74 cells. These results indicated that C/EBPδ may serve an ambiguous role as a tumor suppressor or promoter, but it was not possible to determine this conclusively. Further studies may assist in our understanding of the functional role of C/EBPδ in GC. In the present study, the expression levels of C/EBPβ were decreased in MKN45 cells. It was assumed that not all GC cells exhibited increased expression of C/EBPβ; however, it was not possible to determine the percentage of GC cells that did. The expression level of C/EBPβ in GC is generally increased compared with that in healthy gastric epithelium (21). In further studies, immunohistological and flow cytometric analysis of cell cycle

should be performed to improve our understanding of the expression of C/EBPα, C/EBPβ and C/EBPδ.

The expression levels of C/EBPα and C/EBPδ were decreased in GC cells compared with in healthy gastric epithelium. Expression of C/EBPα decreased the viability of GC cells, and, therefore, C/EBPα may potentially be used in novel GC therapies.

Acknowledgements

The present study was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (grant no. 15K09032).

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