c-myb activates CXCL12 transcription in T47D and MCF7 breast cancer cells

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Chemokine C-X-C motif ligand 12 (CXCL12) is a potent chemotactic and angiogenic factor that has been proposed to play a role in organ-specific metastasis and angiogenic activity in several malignancies. In this study, we found that the overexpression of c-myb could elevate CXCL12 mRNA level and CXCL12 promoter activity in human T47D and MCF-7 breast cancer cells. Chromatin immunoprecipitation assay demonstrated that c-myb could bind to the CXCL12 promoter in the cells transfected with c-myb expression vector. c-myb siRNA attenuated CXCL12 promoter activity and the binding of c-myb to the CXCL12 promoter in T47D and MCF-7 cells. These results indicated that c-myb could activate CXCL12 promoter transcription.

Keywords c-myb; CXCL12 promoter; transcription regulation

Introduction

Tumor angiogenesis, the sprouting of new capillaries from the preexisting vascular network that involves in proliferation of capillary endothelial cells and their migration toward the angiogenic stimulus, is an absolute requirement for the growth, progression, and metastasis of solid tumors [1,2]. Chemokine C-X-C motif ligand 12 (CXCL12) is a chemokine of the CXC family. This chemokine potently plays a role in organ-specific metastasis and angiogenic activity in several malignancies [3,4]. The anomalous elevation of CXCL12 is related to the specialized microenvironments created by the persistent growth of blood vessels [5]. Elevated CXCL12 not only promotes the growth of tumor cells, but also enhances the tumor angiogenesis and metastasis [6]. Consequently, the inhibition of CXCL12 signaling abrogates the development of a wide variety of tumors. Expression of CXCL12 was silenced by promoter hypermethylation in non-small cell lung cancer, breast cancer cell lines, and primary mammary tumors [7,8]. Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells [9]. CXCL12 gene expression is directly induced by hypoxia-inducible factor-1 (HIF-1) in direct proportion to reduced oxygen tension [10,11]. Some transcription factors can influence CXCL12 transcription. It is reported that the effects of AP1 on CXCL12 expression in human cancer cells involves in a Sp1 motif located between −57 and −39 upstream of the main transcription start site [12]. The proto-oncogene c-myb is also a transcription factor, and so far there is no report as to whether c-myb can activate CXCL12 transcription.

The aim of the present study was to explore the activity of c-myb on CXCL12 transcription and to reveal the role of c-myb involved in CXCL12-mediated angiogenesis. Our results showed that c-myb played an important role in inducing CXCL12 promoter activity by directly binding to the CXCL12 promoter, which is helpful to understand the angiogenic mechanism. The inhibition of CXCL12 signaling may provide potential targets for antiangiogenic therapy in several malignancies.

Materials and Methods

Cell lines, cell culture, plasmids, and cell transfection

Human breast cancer cell lines, T47D and MCF-7 were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were checked routinely and found to be free of contamination by Mycoplasma or fungi.

CXCL12 promoter/luciferase gene construct pCMVLUC-SDF1010 (−1010 to +122) and its negative control plasmid pDPROMLUC were kindly provided by Dr Antonio Caruz (Immunogenetics Unit, Faculty of Sciences, University of Jaen, Campus Las Lagunillas SN, Jaen, Spain) [12]. c-myb expression vector (pCMV-c-myb) was purchased from Origene Company (Rockville, USA).

Transfections were conducted by Lipofectamine method. Briefly, for transient transfection, cells were seeded in
6-well plates at a density of $4 \times 10^5$ cells/well. The following day, cells were transfected with 4 μg of c-myb expression vector or pcDNA3 using Lipofectamine 2000 (Gibco BRL, Carlsbad, USA). Following transfection, cells were maintained in RPMI 1640 containing 10% FBS and cultured for 48 h.

**Reverse transcription-PCR**

Total RNA was extracted from cells with Trizol reagent (Invitrogen, Carlsbad, USA) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen) in a final volume of 20 μl containing 5 μg of total RNA, 200 ng of random hexamers, 1 × reverse transcription buffer, 2.5 mM MgCl2, 1 mM deoxy-nucleoside triphosphate mixture, 10 μM DTT, RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), 50 units of superscript reverse transcriptase, and diethylpyrocarbonate-treated water. After incubation at 42°C for 50 min, the reverse transcription reaction was terminated by heating at 85°C for 5 min. The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 2 μl of cDNA template, 1.5 mM MgCl2, 2.5 U of Tag polymerase, and 0.5 μM of CXCL12 primer (5'-AGAGCCAAGC-TCAAGCATTCTC-3', 5'-CGCTTGGCCTTTCATCTC-3'), or c-myb primer (5'-GCCAATTATCTCCGAATCGA-3', 5'-ACCAACGTTCTCAGCGTA-3'), or GAPDH primer (5'-GCCAAAAGGGTCACTCTC-3', 5'-GTAGAGGCCA- GGATGGATGTTC-3'). GAPDH was used as an internal control. PCR conditions were: 94°C for 3 min, then 33 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, followed by 72°C for 10 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays were carried out according to the manufacturer’s protocol (Active Motif, Carlsbad, USA). Briefly, cells in 150 mm tissue culture dishes were fixed with 1% formaldehyde and incubated for 10 min at 37°C. The cells were then washed twice with ice-cold phosphate-buffered saline (PBS), harvested and re-suspended in ice-cold TNT lysis buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 1% aprotinin). The lysates were sonicated to shear the DNA to fragments of 200–600 bp, and subjected to immunoprecipitation with the following antibodies, respectively, c-myb or IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, USA). Antibodies (3 μg) were used for each immunoprecipitation. The antibody/protein complexes were collected by Protein G beads and washed three times with ChIP washing buffer (5% SDS, 1 mM EDTA, 0.5% bovine serum albumin, 40 mM NaHPO4, pH 7.2). The immune complexes were eluted with 1% SDS and 1 M NaHCO3, and the cross links were reversed by incubation at 65°C for 4 h in the presence of 200 mM NaCl and RNase A. The samples were then treated with proteinase K for 2 h, and then DNA was purified by mini-column, ethanol precipitation, and re-suspended in 100 ml of H2O. The primer corresponding to the CXCL12 promoter region (−395 to −213; sense: 5'-TCAGTTCCGCGATCGAAAGG-3', antisense: 5'-CT-CGGCCTTGTGACCCTTCTGAG-3') was used for PCR to detect the presence of the CXCL12 promoter DNA.

**Small-interfering RNA preparation and transfection**

c-myb small-interfering RNA (siRNA) is a target-specific 19 nt siRNA (5'-UGUUUUGCGAAGCUAAAA-3' and 5'-UAAGUGCUUUGCGCAUACGAGA-3') designed to knock down c-myb expression. The siRNA was synthesized by Shanghai GeneChem (Shanghai, China). The cells in the exponential phase of growth were seeded in 6-well plates at a concentration of $5 \times 10^5$ cells/well. After incubation for 24 h, the cells were transfected with siRNA specific for c-myb and non-targeting siRNA at a final concentration of 100 nM using oligofectamine and OPTI-MEMI reduced serum medium (Invitrogen), according to the manufacturer’s protocol. Silencing was examined 48 h after transfection.

**Western blot analysis**

Cells were washed twice with PBS containing 1 mM phenylmethylsulphonyl fluoride, lysed in mammalian protein extraction buffer (Pierce, Rockford, USA). The lysates were transferred to Eppendorf tubes and clarified by centrifugation at 12,000 g for 40 min at 4°C. Equal amounts (50 μg of protein) of cell lysates were resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membranes. Membranes were incubated in blocking solution consisting of 5% powered milk in PBST (PBS plus 0.1% Tween-20) at room temperature for 1 h, then immunoblotted with anti-c-myb antibody (Santa Cruz Biotechnology, Inc.) (1:1000) or anti-tubulin antibody (Sigma-Aldrich, St. Louis, USA) (1:5000), respectively. Detection by enzyme-linked chemiluminescence (Amersham Pharmacia Biotech, Piscataway, USA) was performed according to the manufacturer’s protocol.

**CXCL12 ELISA**

Cells were seeded in 6-well plates. Then cells were transfected with c-myb expression vector. After 48 h of growth, 1.5 ml of medium was collected from each well to evaluate CXCL12 levels by ELISA. The supernatants of 4 wells from each time point were collected and analyzed for CXCL12 expression using a commercially available ELISA.
kit (R&D Systems Inc., Minneapolis, USA) according to the manufacturer’s instructions. The plates were read at 450 nm. CXCL12 concentrations in conditioned media were calculated from a standard curve generated by adding recombinant CXCL12 to the specific unconditioned media.

**Mutagenesis**

CXCL12 promoter/luciferase gene construct pCMVLUC-SDF1010 was used as template. Methylated plasmid DNA with DNA methylase at 37°C for 1 h. Amplify the plasmid in a mutagenesis reaction with two overlapping primers, one of which contained the target mutation. The product was linear, double-stranded DNA containing the mutation. Transform the mutagenesis mixture into wild-type E. coli. The host cells circularized the linear mutated DNA, and McrBC endonuclease in the host cells digested the methylated template DNA, leaving only unmethylated and mutated product. For individual mutations, the sequence of c-myb-binding sites TTCAGTTTC was converted to TTCATATC.

**Luciferase reporter gene assay**

T47D or MCF-7 cells were seeded in 6-well plates at a density of 1–2 × 10^5 cells/well and cultured for 24 h. Cells were then co-transfected wild-type (pCMVLUC-SDF1010) or c-myb mutant (pCMVLUC-SDF1010mut) and CXCL12 reporter construct (0.5 µg/well), or co-transfected with 0.5 µg of pcDNA3.0 or c-myb expression vector together with 20 ng of control Renilla luciferase reporter gene construct, pRL-TK (Promega, Madison, USA). The total amount of DNA per well was adjusted to 1.5 µg by the addition of sonicated salmon sperm DNA. Luciferase assays were performed as recommended by the manufacturer (Promega) and normalized relative to protein concentration determined by bicinchoninic acid protein assay (Pierce).

**Results**

**Overexpression of c-myb induced CXCL12 expression in T47D and MCF-7 cells**

To explore the role of c-myb in regulating CXCL12 transcription, the c-myb expression vector or pcDNA3 was transfected into T47D cells and MCF-7 cells for 48 h, then CXCL12 mRNA and protein levels were detected. Figure 1(A,B) showed that the level of CXCL12 mRNA in the cells transfected with c-myb expression vector increased as determined by RT-PCR, compared with control cells transfected with pcDNA3. Figure 1(C,D) showed that as compared with control cells transfected with pcDNA3, the level of CXCL12 secretion in the cells transfected with c-myb expression vector increased as determined by ELISA. In this experiment, exogenous c-myb could induce CXCL12 expression, indicating c-myb played a role in regulating CXCL12 transcription.

**c-myb activated CXCL12 promoter activity in T47D and MCF-7 cells**

To identify the role of c-myb in regulating CXCL12 transcription, we co-transfected the CXCL12 promoter/luciferase gene construct with c-myb expression vector or pcDNA3 in T47D and MCF-7 cells and detected CXCL12 promoter activity. Figure 2(A,B) showed that the luciferase activity was enhanced by c-myb both in T47D and in MCF-7 cells, respectively, further indicating that c-myb could activate CXCL12 promoter activity. In this experiment, exogenous c-myb could activate CXCL12 promoter activity, suggesting that c-myb played a role in regulating CXCL12 transcription.

**c-myb bounds to the CXCL12 promoter in c-myb-overexpressed T47D and MCF-7 cells**

To investigate if c-myb bounds to the CXCL12 promoter in the cells transfected with c-myb expression vector, we performed ChIP experiments. The results showed that c-myb...
could bind to the *CXCL12* promoter both in T47D and in MCF-7 cells transfected with c-myb expression vector (Fig. 3). In this experiment, c-myb could bind to the *CXCL12* promoter in c-myb-overexpressed T47D or MCF-7 cells, indicating that c-myb activated *CXCL12* transcription by binding directly to the *CXCL12* promoter.

### c-myb siRNA inhibited c-myb mRNA, *CXCL12* mRNA and Protein in T47D and MCF-7 Cells

To further identify the role of c-myb in regulating *CXCL12* transcription, we knocked down the expression of c-myb with a gene-specific siRNA and measured c-myb mRNA. As shown in Fig. 4(A, B), c-myb siRNA inhibited c-myb mRNA significantly in T47D and MCF-7 cells after transfection with c-myb siRNA for 48 h, respectively. And it also inhibited *CXCL12* mRNA in the T47D and MCF-7 cells. Also c-myb siRNA inhibited *CXCL12* protein secretion significantly in T47D and MCF-7 cells after transfection with c-myb siRNA for 48 h [Fig. 4(C, D)]. This experiment indicated that c-myb siRNA could knock down c-myb expression efficiently and decrease *CXCL12* protein secretion significantly.

### c-myb siRNA repressed *CXCL12* promoter activity in T47D and MCF-7 cells

To determine if the decrease of c-myb would reduce *CXCL12* gene transcription, we knocked down the expression of c-myb and measured *CXCL12* promoter activity. As shown in Fig. 5, c-myb siRNA attenuated *CXCL12* promoter activity in T47D and MCF-7 cells (without incubating any
siRNA) after transfection with c-myb siRNA for 48 h. This experiment indicated that when endogenous c-myb was knocked down by siRNA, the promoter activity of endogenous CXCL12 also decreased.

c-myb siRNA attenuated the binding of c-myb to the CXCL12 promoter
To determine if the decrease of c-myb would influence the binding of c-myb on the CXCL12 promoter, we knocked down the expression of c-myb and measured the binding status of c-myb to the CXCL12 promoter. As shown in Fig. 6, c-myb siRNA attenuated the binding of c-myb to the CXCL12 promoter in T47D and MCF-7 cells after transfection with c-myb siRNA for 48 h. Result showed that when endogenous c-myb was knocked down by siRNA, the binding of c-myb to the CXCL12 promoter was decreased, and it also indicated that c-myb regulated CXCL12 transcription by binding directly to the CXCL12 promoter.

Discussion
The proto-oncogene c-myb is the cellular homolog of the v-myb oncogene carried by the avian myeloblastosis viruses [13]. It encodes a 75-kDa transcriptional factor protein c-myb [14]. c-myb function can be regulated by many factors including different RNA splicing [15], C/EBP [16], cyclin D1 [17], and so on [18,19]. The c-myb protein plays an essential role in the regulation of cell growth, survival, and differentiation of hematopoietic cells [20]. These results suggested that the c-myb protein is required for proliferation of cells. In fact, proliferation is often associated with cancer progression. Although c-myb expression was initially thought to be related to the hematopoietic cells, it has subsequently been reported in non-hematopoietic tissues and cell lines including the breast, colon, lung carcinomas, neuroblastomas, and so on [21]. Ramsay et al. [22] reported c-myb could activate COX-2 transcription in colorectal cancer. McHale et al. [23] found that c-myb protein was increased in invasive breast cancers compared with normal tissues via immunohistochemistry evaluation. c-myb affected estrogen/ER signaling pathway, functioned as a STAT5a co-activator and potentiated STAT5a-driven gene expression in human breast cancer [24,25]. These data at least can explain the potential role of the c-myb protein in myeloid leukaemias and in solid tumors. Disrupting the
function of c-myb may be an effective target for cancer treatment. All these studies indicate that c-myb is related to cancer progression and invasion, but there are no reports about the relationship between c-myb and CXCL12 transcription in breast cancer.

In our studies, overexpression of c-myb could increase CXCL12 mRNA and protein levels in T47D and MCF-7 cells. In order to analyze the putative effects of c-myb on CXCL12 transcription, we performed luciferase assay. Our results demonstrated that c-myb activated CXCL12 promoter activity. ChIP assay demonstrated that c-myb could bind to the CXCL12 promoter in the c-myb-overexpressed cells. Bioinformatic analysis of the 5′-flanking region of the human CXCL12 gene showed that there existed a c-myb-binding site (TTACAGTTC) in the CXCL12 promoter region from −396 to −389. It has been reported that c-myb could bind to the c-myb-binding site (CAGTTC) in GSTP1 promoter [26]. In our study, point mutant of c-myb-binding site in the CXCL12 promoter construct abrogated the activation effect of c-myb on CXCL12 promoter activity, indicating that c-myb activated CXCL12 promoter activity by directly binding to the c-myb-binding site of CXCL12 promoter.

In the following experiments, we found that c-myb siRNA attenuated CXCL12 secretion and CXCL12 promoter activity in T47D and MCF-7 cells; at the same time, c-myb siRNA attenuated the binding of c-myb to the CXCL12 promoter. These results further showed that c-myb could affect CXCL12 promoter activity by binding to the CXCL12 promoter.

We conclude that c-myb plays an important role in inducing CXCL12 promoter activity by directly binding to the CXCL12 promoter. These investigations are important and offer potential for defining angiogenic mechanism regulated by CXCL12 and c-myb. With this information it will be possible to demarcate potential targets and define appropriate reagents, such as antisense or small molecule antagonists, for inhibiting or preventing cancer progression and metastasis.

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