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Cellular nucleic acid binding protein suppresses tumor cell metastasis and induces tumor cell death by downregulating heterogeneous ribonucleoprotein K in fibrosarcoma cells

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ABSTRACT

Background: Cellular nucleic acid binding protein (CNBP) has been implicated in vertebrate craniofacial development and in myotonic dystrophy type 2 (DM2) and sporadic inclusion body myositis (sIBM) human diseases by controlling cell proliferation and survival to mediate neural crest expansion. CNBP has been found to bind single-stranded nucleic acid and promote rearrangements of nucleic acid secondary structure in an ATP-independent manner, acting as a nucleic acid chaperone.
Methods: A variety of methods were used, including cell viability assays, wound-scratch assays, chemotaxis assays, invasion assays, circular dichroic (CD) spectroscopy, NMR spectroscopy, chromatin immunoprecipitation, expression and purification of recombinant human CNBP, electrophoretic mobility shift assay (EMSA), surface plasmon resonance (SPR), fluorescence resonance energy transfer (FRET) analyses, luciferase reporter assay, Western blotting, and isothermal titration calorimetry (ITC).
Results: Up-regulation of CNBP induced human fibrosarcoma cell death and suppressed fibrosarcoma cell motility and invasiveness. It was found that CNBP transcriptionally down-regulated the expression of heterogeneous ribonucleoprotein K (hnRNP K) through its conversion of a G-rich sequence into G-quadruplex in the promoter of hnRNP K. G-quadruplex stabilizing ligand tetra-(*N*-methyl-4-pyridyl) porphyrin (TMPyP4) could interact with and stabilize the G-quadruplex, resulting in downregulation of hnRNP K transcription.
Conclusions: CNBP overexpression caused increase of cell death and suppression of cell metastasis through its induction of G-quadruplex formation in the promoter of hnRNP K resulting in hnRNP K down-regulation.
General significance: The present result provided a new solution for controlling hnRNP K expression, which should shed light on new anticancer drug design and development.

1. Introduction

CNBP is a single-stranded nucleic-acid-binding protein containing strikingly conserved two remarkable structural features: seven tandem CCHC-type zinc knuckle domains (C–Φ–X–C–G–X_3–H–X_4–C, where Φ = aromatic amino acid and X = variable amino acid) and a peculiar arginine/glycine-rich region between the first and the second Zn knuckles, which is highly similar to the arginine–glycine–glycine (RGG) box [1]. CNBP has been initially described as a DNA-binding protein acting as a negative transcriptional regulator in the coordinated control of cholesterol metabolism [2]. As a transcriptional regulator, CNBP has been reported to control the expression of the early promoter–enhancer of the JC virus [3] and the β-myosin heavy chain gene [4]. Conversely, CNBP has also been shown to up-regulate the expression of macrophage colony-stimulating factor (CSF-1) in fibroblasts [5] and c-Myc proto-oncogene [6]. Likely, CNBP may possess nucleic acid chaperone activity to catalyze the rearrangement of nucleic acids via ATP-independent repeated cycles of binding and release, and thus facilitating the folding of its thermodynamically stable three-dimensional structure required for nucleic acid biological function [7–9]. Various reports have implicated the effect of CNBP in vertebrate craniofacial development and in myotonic dystrophy type 2 (DM2) and sporadic inclusion body myositis (sIBM) human diseases [10–13]. Both apparently unrelated biological processes have progressed significantly,

Abbreviations: CD, circular dichroism; ChIP, chromatin immunoprecipitation; CNBP, cellular nucleic-acid-binding protein; EMSA, electrophoretic mobility-shift assay; FAM, 6-carboxyfluorescein; FRET, fluorescence resonance energy transfer; hnRNP K, heterogeneous ribonucleoprotein K; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; TAMRA, tetramethylrhodamine; TMPyP4, tetra-(*N*-methyl-4-pyridyl) porphyrin

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however, a complete understanding of CNBP function has not been achieved.

hnRNP K is a RNA-binding protein found in the nucleus, cytoplasm, mitochondria, and plasma membrane, and belongs to the family of heterogeneous nuclear ribonucleo-protein (hnRNP) complex [14–16]. hnRNP K has been implicated to function in transcription [17–19], pre-mRNA splicing [20], RNA processing [21], stability [22], and translation [23,24]. Besides, hnRNP K plays key roles in coordinating transcriptional responses to DNA damage [25]. The expression of hnRNP K is increased in several human malignancies: chronic myelogenous leukemia and solid tumors, including cancers of the esophagus, lung, nasopharynx and colorectum [26–30]. Some studies have shown that hnRNP K is indispensable for metastasis and cell death [31,32]. Overall, hnRNP K has been implicated as a potential key player in carcinogenesis, making it an attractive target for anticancer therapies. Although hnRNP K has been the subject of numerous studies, the mechanisms regulating its expression are still largely unknown.

In the present study, we found a new G-rich sequence in the promoter of hnRNP K gene, which can form stable G-quadruplex resulting in downregulation of hnRNP K. We found that elevated CNBP expression down-regulated the transcription and expression of hnRNP K through its conversion of this G-rich sequence into G-quadruplex, resulting in enhanced tumor cell death and suppressed tumor cell metastasis.

2. Materials and methods

2.1. Materials

Human fibrosarcoma cell HT1080 was obtained from the American Type Culture Collection (ATCC) and preserved in our lab. The cell culture was maintained in complete DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under humidified atmosphere with 5% CO_2. Cell line was tested negative for mycoplasma contamination prior to and after experimentation. All oligomers/primers (sequences shown in Table S1) used in this study were purchased from Sangon Biotech (China). All DNA oligomers were HPLC-purified and dissolved in double-distilled water before use, except for primers and CD experiments, which were purified by using PAGE (Table S1). All oligonucleotides were first heated at 95 °C for 5 min, and then slowly cooled to room temperature to form G-quadruplex structure. All antibodies were commercially available: β-actin rabbit mAb (#4970S, Cell Signaling), hnRNP K rabbit mAb (#9081S, Cell Signaling), CNBP goat polyclonal antibody (sc-51052X, Santa Cruz), nucleolin mouse mAb (ab13541, Abcam), SP1 rabbit mAb (sc-14027,Santa Cruz), NM23-H2 rabbit mAb (5969-1, Epitomics), Anti-rabbit IgG-HRP (#7074S, Cell Signaling), Anti-mouse IgG-HRP (#7076, cell signaling) and Donkey anti-goat IgG-HRP (sc-2020, Santa Cruz).

2.2. Plasmids

The pET28a-CNBP plasmid was generated by inserting the CNBP cDNA into the pET28a plasmid (Promega) using the Nde I and Xho I restriction sites. The cDNA was obtained by using PCR with the primers: CNBP-pET28a-F and CNBP-pET28a-R (Table S1). The EGFP–CNBP plasmid was generated by inserting the CNBP cDNA into the pEGFP-N3 plasmid (Promega) using the Nde I and BamH I restriction sites. The cDNA was obtained by using PCR with the primers: CNBP–GFP-F and CNBP–GFP-R (Table S1). The wild type psiCheck2-hnRNP K luciferase plasmid was generated by inserting the hnRNP K promoter sequence (from −998 to +50) into the psiCheck-2 reporter vector (Promega) using the Kpn I and Nhe I restriction sites. The promoter oligonucleotide was obtained by using PCR with the primers hnRNP K-WT-F and hnRNP K-WT-R. The mutated-1 (Mut1) psiCheck2-hnRNP K luciferase plasmid was generated by multi-point site-directed mutagenesis from wild type psiCheck2-hnRNP K (WT) luciferase plasmid. All reconstructed plasmids were sequenced and proved to be correct by BLAST.

2.3. Cell viability assays

Cell viability was measured by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) assay. After 24 h or 48 h transfection of pEGFP–CNBP, the medium was removed and the MTT solution (0.5 mg/mL in medium) was added. After 4 h, the MTT solution was removed and tetrazolium salt was dissolved in 100 μL of DMSO/ethanol solution (1:2). Colorimetric measurement was performed at 570 nm by using an ELISA reader.

2.4. Wound-scratch assays

Confluent monolayer HT1080 cells were maintained in serum-containing growth medium for 12 h, and then in serum-free medium for 12 h. A 200 μL plastic pipette tip was used to scratch the monolayers. The wounded cells were then cultured in serum-free medium for an additional 12 h and photographed. Three different points were marked on each plate. The experiment was repeated for three times.

2.5. Chemotaxis assays

Cell migration assays were performed by using 8-μm-pore Transwell inserts (Millipore). HT1080 cells were transfected with pEGFP–CNBP at ~75% confluency in 10-cm dishes. Chemotaxis assays were carried out as that previously described by Inoue et al. [33].

2.6. Invasion assays

Invasion assays were performed with a Cell Invasion Assay Kit (Millipore). HT1080 cells were transfected with pEGFP–CNBP at ~75% confluency in 10-cm dishes. Twenty-four hours after transfection, cells were collected, washed twice with PBS, and suspended in DMEM medium. For invasion assay, 5.0×10^4 cells were seeded in each well and the assay was performed following the manufacturer's instructions. After 24 h of incubation, Matrigel was swabbed off with cotton bud, and cells that invaded across the Matrigel were stained with Cell Stain (Millipore).

2.7. Circular dichroic (CD) spectroscopy and CD-melting experiments

CD measurements were performed on a Chirascan circular dichroism spectrophotometer (Applied Photophysics) at room temperature. CD experiments were carried out with oligonucleotides (5 μM) annealed in 10 mM Tris–HCl, 100 mM KCl, and pH 7.4. CD spectra were collected from 220 to 340 nm with a 0.2 cm path length cylindrical quartz cuvette. A buffer blank correction was made for all spectra. For CD melting temperature experiments, samples were annealed at first, G-quadruplex formation was induced and molar ellipticity at 263 nm was measured over a temperature range of 25–95 °C.

2.8. NMR spectroscopy

Sample for NMR study was dialyzed successively against 60 mM KCl solution and against water. The strand concentration of the NMR samples was 1 mM, and the solutions contained 100 mM KCl and 20 mM potassium phosphate (pH 7.0). NMR experiments were performed on 600 MHz Bruker spectrometers at 25 °C. The imino protons were observed using the 11-echo pulse sequence [34] with the excitation maximum adjusted to the center of the Hoogsteen imino region. Five millimeter NMR tubes were used for extract measurements with a sample volume of 500 μL. Samples analyzed via CD were also used for NMR experiments.