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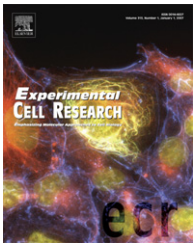

Research Article

Characterization of ZNF23, a KRAB-containing protein that is downregulated in human cancers and inhibits cell cycle progression

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ARTICLE INFORMATION

Article Chronology:
Received 16 June 2006
Revised version received
21 September 2006
Accepted 9 October 2006
Available online 14 October 2006

Keywords:
ZNF23
KRAB
Cell cycle
G_1 phase
$p27^{kip-1}$
p53

ABSTRACT

The Krupple-associated box-containing zinc-finger proteins (KRAB–ZFPs) make up one of the largest family of transcription factors. Several members of the KRAB–ZFPs modulate cell growth, survival and are implicated in malignant disorders. However, most members are not well characterized and their functions are largely unknown. Here we report that ZNF23, a member of KRAB–ZFPs, inhibits cell cycle progression. ZNF23 protein localized to the nucleus and was ubiquitously expressed in all tested normal tissues. However, the expression levels of ZNF23 protein were lost or greatly reduced in human cancer. Ectopic expression of ZNF23 led to enhancement of $p27^{kip-1}$ expression, growth inhibition and cell cycle arrest in G_1 phase. Downregulation of $p27^{kip-1}$ by siRNA against $p27^{kip-1}$ reversed growth inhibition induced by ZNF23. Furthermore, the growth-inhibitory effect of ZNF23 was p53-independent. Deletion analysis revealed that the effect of ZNF23 did not rely on its KRAB domain, but on the C-terminal zinc fingers. Thus, we have identified a new member of KRAB–ZNF superfamily with growth-inhibitory ability and its downregulation may contribute to carcinogenesis.

Introduction

The KRAB–ZFPs, a group of the most widely distributed transcriptional repression proteins in mammals [1,2] make up approximately one-third of 300 to 800 human C_2H_2 zinc-finger proteins [3,4]. A KRAB–ZFP is composed of a KRAB domain at its N-terminus and tandem C_2H_2 class zinc fingers at its C-terminus [4]. The KRAB domain divided into A and B boxes is required for repressing transcription by recruiting other proteins, such as KAP-/TIF1-complex [5,6]. The tandem zinc finger motifs number from 3 to upwards of 40 in a protein and presumably participate in DNA recognition [7].

It has been reported that KRAB–ZFPs play critical roles in regulation of development, cell differentiation, proliferation and apoptosis. For example, ZBRK1 is identified as a BRCA1-dependent transcriptional repressor and functions in cell growth control and survival [8]. Altered expression of ZBRK1 gene has been observed in human breast carcinomas [9]. In

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doi:10.1016/j.yexcr.2006.10.009

addition, many KRAB-containing genes are mapped to the chromosome regions commonly deleted in solid tumors or implicated in recurrent chromosomal rearrangements in hematological malignancies, suggesting that they are potential genes related with malignancies [10–12]. However, the functions of these genes remain largely unknown.

Using bioinformatics approach, we have identified ZNF23, a human KRAP–ZFP. This gene is located in 16q22 [13,14], a region containing tumor suppressor genes and frequently altered in solid tumors, such as ovarian cancers and endometrial carcinomas [10–12,15,16]. However, its function and exact role in human cancer are largely unknown. In this study, we provided evidence to support a notion that ZNF23 is a novel member of 16q22 genes with growth-inhibitory potential and may be implicated in tumorigenesis.

Materials and methods

Materials

The Texas Red-conjugated anti-mouse and Alexa Fluor 488-conjugated anti-rabbit secondary antibodies were from Molecular Probes (Eugene, OR). All other reagents used were from Sigma.

Cell culture, transfection and tissue samples

The SK-OV-3 and ES-2 cells were maintained in RPMI1640 supplemented with 10% (v/v) calf serum (Invitrogen, CA). Other cell lines were incubated in Dulbecco's modified Eagle medium containing 10% (v/v) fetal bovine serum. The cells were transfected using Lipofectamine™ 2000 according to the manufacturer's protocol (Invitrogen, CA). Clinical specimens were collected with consent from the patients registered at the Medical Center of Fudan University (Shanghai, China).

Plasmids

Human ZNF23 cDNAs were amplified by PCR using a human adult brain cDNA library (Invitrogen, CA) as template and cloned into T-easy vectors (Clontech). The full-length ZNF23 and deletion mutants were amplified and cloned into pcDNA3 or pEGFP-C1 with the myc or GFP tag sequence. GAL4–ZNF23–KRAB or GAL4–KS1–KRAB was generated by amplifying the DNA fragments coding for KRAB domain of ZNF23 (amino acids 1–43) or for KRAB domain of KS1 (amino acids 7–78). The two fragments were cloned in-frame downstream from GAL4 DBD (amino acids 1–147) in pcDNA3. The full length of ZBP-89 was produced and inserted into pcDNA3 with the myc tag. The primers used for amplification were listed in the Table 1. These expression constructs were verified by sequencing. The pGL2–GAL4–TK plasmid contains GAL4 UAS upstream from a minimal herpes simplex virus TK promoter to drive the expression of a luciferase gene. The p53-dependent luciferase reporter plasmid contains five copies of the p53 consensus binding site in tandem to drive expression of a luciferase gene.

siRNA

The sense strand sequences of siRNA duplexes used for p27 were 5′-AAGTACGAGTGGCAAGAGGTG dTdT-3′. The stand corresponded to nucleotides 217 to 238 of the human $p27^{Kip1}$ coding region. The siRNA duplexes have been reported to efficiently silence $p27^{kip-1}$ expression [17]. The siRNA for luciferase used as a negative control was 5′-CUUACGCUGAGUACUUCGATT-3′. Transfection of siRNA was conducted using Lipofectamine™ 2000.

Generation of the antibody and Western blotting

A fragment of ZNF23 protein (amino acids 79–234) was used to immunize rabbits according to the protocol as described previously [18]. The antiserum was affinity-purified on a nickel–nitrilotriacetic acid column attached with the antigenic protein as described previously [19]. For Western blotting, protein extracts were prepared using RIPA buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM sodium pyrophosphate, 40 μM sodium orthovanadate, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride]. Protein concentration was determined by BioRad assay. Proteins were separated on SDS–10% PAGE, transferred to polyvinylidene difluoride membranes (Amersham) and probed with indicated primary antibodies. Protein bands were detected by the Amersham ECL system. In some cases, the protein bands were scanned and their densities were determined by ImageQuant 5.2 software (Amersham, USA).

Luciferase assay

The GAL4-base KRAB repression assay was performed as described previously [20]. Briefly, HEK293 cells were transiently cotransfected with 200 ng of luciferase reporter plasmid (pGL2–GAL4–TK), 200 ng of pSV40-β-galactosidase expression plasmid (Promega) and different amounts of GAL4–ZNF23–KRAB or GAL4–KS1–KRAB. For p53-dependent transcriptional activation assay, HEK293 cells were transiently cotransfected with 100 ng

Table 1 – Primers used for PCR analysis

Construct	Forward primer	Reverse primer
ZNF23	5′GGGCTCGAGTGATGCTGGAGAATTATGGGAATGTG3′	5′GGCGGATCCACGGATTTTCCTTCACTATGGAC3′
△1–195	5′GGGCTCGAGTGATGCCTTATCAGTGTTCGGAGTGT3′	5′GGCGGATCCACGGATTTTCCTTCACTATGGAC3′
△532–643	5′GGGCTCGAGTGATGCTGGAGAATTATGGGAATGTG3′	5′GGCGGATCCACAGGTTTCTCCCCAGTATGTAT3′
△1–419	5′GGGCTCGAGTGATGCTGGAGAATTATGGGAATGTG3′	5′GGCGGATCCACTTTCTCGCCTGTGTGTTCTCT3′
△1–531	5′GGGCTCGAGTGATGCTGGAGAATTATGGGAATGTG3′	5′GGCGGATCCACTTTCTCCCCAGTATGTATCCT3′
ZBP-89	GGGCTCGAGATGAACATTGACGACAAACTGGAA	GGCAAGCTTGCCAAAAGTCTGGCCAGTTGTGGCA