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A real-time PCR-based quantitative assay for 3-methylcytosine demethylase activity of ALKBH3

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ABSTRACT

Human AlkB homolog 3 (ALKBH3), a homolog of the *Escherichia coli* protein AlkB, demethylates 1-methyladenine and 3-methylcytosine (3-meC) in single-stranded DNA and RNA by oxidative demethylation. Immunohistochemical analyses on clinical cancer specimens and knockdown experiments using RNA interference *in vitro* and *in vivo* indicate that ALKBH3 is a promising molecular target for the treatment of prostate, pancreatic, and non-small cell lung cancer. Therefore, an inhibitor for ALKBH3 demethylase is expected to be a first-in-class molecular-targeted drug for cancer treatment. Here, we report the development of a novel, quantitative real-time PCR-based assay for ALKBH3 demethylase activity against 3-meC by highly active recombinant ALKBH3 protein using a silkworm expression system. This assay enables us to screen for inhibitors of ALKBH3 demethylase, which may result in the development of a novel molecular-targeted drug for cancer therapy.

1. Introduction

AlkB is an *Escherichia coli* enzyme that demethylates 1-methyladenine (1-meA) and 3-methylcytosine (3-meC) in single-stranded (ss)DNA and double-stranded (ds)DNA through its 2-oxoglutarate- (2OG) and Fe(II)-dependent oxygenase domain [1]. In humans, there are nine AlkB homologs (ALKBH) with the 2OG and Fe(II)-dependent oxygenase domain, which include ALKBH1 to ALKBH8 [2,3] and fat mass and obesity-associated (FTO) [4], also known as ALKBH9. Of the ALKBH family proteins, ALKBH3 preferentially demethylates 1-meA and 3-meC in ssDNA and RNA in a 2OG and Fe(II)-dependent manner [5,6].

ALKBH3, originally cloned as prostate cancer antigen-1 (*PCA-1*), is overexpressed in prostate cancer tissues compared with non-cancerous tissues [7]. Higher expression of PCA-1 was immunohistochemically confirmed in human prostate cancer tissues but not in normal adjacent prostate tissues or benign prostatic hyperplasia. Prostate cancer is the second leading cause of cancer-related deaths in Western countries. Hormone therapy is effective for patients with prostate cancer; however, many patients develop castrate resistant prostate cancer (CRPC) within a few years. At present, effective therapies are limited for prostate cancers resistant to hormone therapy. Higher expression of ALKBH3 in prostate cancer specimens is correlated with the development of CRPC. Higher expression of ALKBH3 is also observed in pancreatic cancer [8], non-small cell lung cancer (NSCLC) [9], as well as renal cancer [10] specimens. In patients with these intractable cancers and high ALKBH3 expression also have a poorer prognosis.

In CRPC, pancreatic cancer, and NSCLC, the development of effective molecular-targeted drugs is highly desired. The knockdown of *ALKBH3* by RNA interference induced apoptotic cell death *in vitro* and suppressed tumor formation in *in vivo* xenograft models [8,11,12]. These results strongly indicate that ALKBH3 is a promising molecular target for cancer therapy. However, to develop a molecular-targeted drug for ALKBH3, it is necessary to establish an assay to measure enzymatic activity, which could be used to screen for low molecular weight compound inhibitors. Few assays to measure the demethylase activity of ALKBH3 have been described [13–15]. These assays, although sensitive and fast, require special equipment or reagents such as capillary electrophoresis with laser-based fluorescence detection or mass spectrometer and radiolabeled substrates. Therefore, they are

Abbreviations: ALKBH, AlkB homolog; 1-meA, 1-methyladenine; 3-meC, 3-methylcytosine; 2OG, 2-oxoglutarate; FTO, fat mass and obesity-associated; ss, single-stranded; ds, double-stranded; CRPC, castrate resistant prostate cancer; NACLC, non-small cell lung cancer; LC-MS/MS, liquid chromatography-tandem mass spectrometry

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unsuitable for a high-throughput screening of low molecular weight compounds. In the present study, we obtained highly active recombinant ALKBH3 protein using a silkworm expression system. The production of recombinant ALKBH3 from silkworms enabled us to develop a real-time PCR-based quantitative assay of its demethylation activity against 3-meC in ssDNA.

2. Materials and methods

2.1. Expression and purification of recombinant ALKBH3 from silkworms

A silkworm vector, pMONFT21, for expression of human ALKBH3 (NM_139178.3) cDNA encoding the FLAG-His-tagged ALKBH3 (FLAG-His-ALKBH3) recombinant protein was constructed by Link Genomics (Tokyo, Japan). The expression vector was co-transfected with ABv baculovirus DNA into BmN cells to obtain the recombinant virus (Sysmex, Hyogo, Japan). The silkworm pupae were infected with the recombinant virus on the first day after pupation. Six days after infection, ten silkworm pupae expressing FLAG-His-ALKBH3 were homogenized at 10,000 rpm for 5 min in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The homogenates were then solubilized by incubating with 1% Triton X-100 at 4 °C for 1 h. The lysate was centrifuged at 4 °C for 1 h at 100,000*g*. The protein purification was performed in two steps in order to achieve higher purity. First, the supernatant was purified by a 5-mL, 1.6 × 2.5-cm HisTrap HP affinity column (GE Healthcare) at 4 °C, using AKTA Prime Plus (GE Healthcare). The fractions containing recombinant FLAG-His-ALKBH3 were eluted with a linear gradient of 60–280 mM imidazole in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100. Subsequently, the eluate fractions containing recombinant ALKBH3 were diluted five-fold with 20 mM Tris-HCl (pH 8.0), 10% glycerol, and 0.1% Triton X-100 (binding buffer), and applied to a 0.5 × 5.0-cm Mono Q 5/50 GL anion ion exchange column (GE Healthcare) at 4 °C, using AKTA Prime Plus (GE Healthcare). After extensive washing with binding buffer containing 50 mM NaCl, recombinant FLAG-His-ALKBH3 was eluted with a linear gradient of 500 mM NaCl in the binding buffer, followed by dialysis in 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50% glycerol, and 0.1% Triton X-100. Purity was confirmed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel (Bio-Rad) stained with SimplyBlue SafeStain (Life Technologies).

2.2. Western blot analysis with anti-ALKBH3 antibody

Purified recombinant FLAG-His-ALKBH3 was resolved on a 10% denaturing acrylamide gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 3% bovine serum albumin at room temperature for 1 h. The membrane was incubated with anti-ALKBH3 antibody (1:5000 dilution, Millipore #09-882) overnight at 4 °C and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology). Bound horseradish peroxidase conjugates were visualized using enhanced chemiluminescence reagent (GE Healthcare) under ImageQuant LAS 4000 (GE Healthcare).

2.3. Quantitative assay of ALKBH3 3-meC demethylase activity by real-time PCR

The reaction solution of the ALKBH3 demethylation assay comprised purified recombinant FLAG-His-ALKBH3 from silkworms (4 ng), 2 mM ascorbic acid, 100 μM 2-oxoglutarate, 40 μM $Fe(SO)_4 \cdot 7H_2O$, and 80 fmol oligo ssDNA containing 3-meC (GGAAACAGCTATGACCATGATTACTAGACATTGCCATTCTCGATAGGA TCCGGTCAAACCTAGACGAATTCCGGT-3-meC-GTGACTGGGAAAACC CTGGCG, Gene Design) as a substrate in 50 mM Tris-HCl (pH 8.0). The reaction mixture was incubated for 1 h at 37 °C, and then diluted 20-fold with distilled water for quantitative real-time PCR (qRT-PCR) analysis. The non-methylated ssDNA was used as a standard template. The qRT-PCR analysis was performed using a CFX96 real-time PCR system (Bio-Rad) in a total reaction volume of 20 μL containing 2 μL of the diluted reaction mixture, and 18 μL of Bio-Rad iQ SYBR Green Supermix with 200 nmol of each primer (sense: GGAAACAGCTATGACCATGATTAC, antisense: CGCCAGGGT TTTCCCAGTCGTG). Thermal cycling conditions were as follows: an initial incubation at 95 °C for 10 s to activate the polymerase followed by 40 cycles of 95 °C for 5 s, 61 °C for 30 s and 72 °C for 15 s. The level of the amplified products was calculated from a standard curve as the demethylation activity of ALKBH3 and ALKBH2. Recombinant His-tagged ALKBH2 (His-ALKBH2) and His-tagged ALKBH3 (His-ALKBH3) from *E. coli* were purchased from Abcam.

2.4. Digestion of ssDNA oligo containing 3-meC demethylated by ALKBH3 to nucleosides for LC-MS/MS analysis

After incubation with or without silkworm recombinant ALKBH3, the ssDNA oligo containing 3-meC was purified by ethanol-precipitation. The precipitate was suspended in 45 μL of H_2O, after which 5 μL of 0.1 m ammonium acetate (pH 5.3) and 0.5 units of nuclease P1 (Wako) were added. After incubating for 2 h at 45 °C, 5.5 μL of 1 m ammonium bicarbonate and 0.002 units of venom phosphodiesterase II (Wako) were added to the mixture, followed by additional incubation for 2 h at 25 °C. Thereafter, the mixture was incubated for 1 h at 37 °C with 0.5 units alkaline phosphatase (NEW ENGLAND BioLabs). HCl (1.3 μL, 0.1 N), H_2O (50 μL) and chloroform (20 μL) were then added. The sample was vortexed and the resulting suspension was centrifuged for 5 min at 5000*g*. The aqueous layer was collected and evaporated to dryness. The nucleosides left behind were solubilized in Milli-Q water.

2.5. LC-MS/MS confirmation of 3-meC demethylation by ALKBH3

LC-ESI-MS/MS analyses were performed on a Waters ACQUITY UPLC system (Waters) coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Waters). For DNA samples, LC separations were carried out at 50 °C with an ACQUITY UPLC BEH C18 column, 1.7 μm, 2.1 × 100 mm (Waters). Mobile-phase A was 5 mM ammonium formate and 0.2% (v/v) formic acid, and mobile-phase B was acetonitrile. The analyte was eluted with mixed mobile-phase A and B (98/2, v/v) at a flow rate of 0.3 mL/min. The data for one run was acquired for 4 min. The mass spectrometer was operated using an electrospray ionization (ESI) source in the positive mode. ESI-MS/MS was conducted in the negative ion mode. The ionization parameters were capillary voltage, 3.0 kV; extractor voltage, 2 V; source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h. Selected reaction monitoring (SRM) transitions (*m*/*z* of precursor ion/*m*/*z* of product ion) and parameters (cone voltage and collision energy) for the deoxynucleosides are listed in Supplementary Table 1. Inter-channel delay and inter-scan delay were set at 0.01 and 0.05 s, respectively. The dwell time for each SRM was set at 50 ms.

Statistics. Differences between values were statistically analyzed using a Student's *t*-test. A p-value < 0.05 was considered statistically significant.