A Novel Human AlkB Homologue, ALKBH8, Contributes to Human Bladder Cancer Progression

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Abstract

We recently identified a novel human AlkB homologue, ALKBH8, which is expressed in various types of human cancers including human urothelial carcinomas. In examining the role and function of ALKBH8 in human bladder cancer development in vitro, we found that silencing of ALKBH8 through small interfering RNA transfection reduced reactive oxygen species (ROS) production via down-regulation of NAD(P)H oxidase-1 (NOX-1) and induced apoptosis through subsequent activation of c-jun NH2-terminal kinase (JNK) and p38. However, we also found that JNK and p38 activation resulted in phosphorylation of H2AX (γH2AX), a variant of histone H2AX, which contributes to the apoptosis induced by silencing ALKBH8 and NOX-1. Silencing of ALKBH8 significantly suppressed invasion, angiogenesis, and growth of bladder cancers in vivo as assessed both in the chorioallantoic membrane assay and in an orthotopic mouse model using green fluorescent protein–labeled KU7 human urothelial carcinoma cells. Immunohistochemical examination showed high expression of ALKBH8 and NOX-1 proteins in high-grade, superficially and deeply invasive carcinomas (pT2 and pT3) as well as in carcinoma in situ, but not in low-grade and noninvasive phenotypes (pTa). These findings indicate an essential role for ALKBH8 in urothelial carcinoma cell survival mediated by NOX-1-dependent ROS signals, further suggesting new therapeutic strategies in human bladder cancer by inducing JNK/p38/γH2AX–mediated cell death by silencing of ALKBH8. [Cancer Res 2009;69(7):3157–64]

Introduction

In E. coli, AlkB protein is involved with repair of methylation-induced DNA damage by oxidative demethylation; in various types of mammalian cells, AlkB is implicated in preventing mutation and carcinogenesis (1, 2). Human homologues of the AlkB family include ALKBH1, ALKBH2, and ALKBH3, and our laboratory has recently identified some additional members, ALKBH4, ALKBH5, ALKBH6, ALKBH7, and ALKBH8, and determined their expression in human tissues (3). Immunohistochemical analysis has shown that ALKBH8 is highly expressed in a variety of human cancers including bladder cancer; however, the physiologic role of ALKBH8 in cancer progression remains unclear.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Reactive oxygen species (ROS) are implicated in both stimulation and inhibition of cell proliferation, apoptosis, and cell senescence (4, 5). ROS trigger genetic programs associated with transformation, resulting in alteration of genes by manipulating the cell cycle and signal transduction (6). Similarly, oxygen radical generation is increased in Ras-transformed fibroblasts (7) and antioxidants can block DNA synthesis, suggesting that ROS are involved in mitogenic signaling in the course of neoplastic transformation. NADPH oxidase (NOX) is one of the major sources for cellular ROS (8, 9). NOX enzymes are the structural homologues of phagocytic NOX (gp91phox/NOX2) and consist of both single (NOX-1–NOX-5) and dual oxidases (DuOX1 and DuOX2; ref. 10). Emerging evidence continues to accumulate that implicates low levels of ROS generated by NOX enzymes as mediators in inflammation, apoptosis, cell growth, and angiogenesis in various types of human cancers. Among NOX family members, the function of NOX-1, in particular, has been focused on in human cancer. NOX-1 potentially stimulates branching morphogenesis in the nontubulogenic endothelial cell line NP 31 (11). de Carvalho and colleagues (12) reported that 12-lipoxygenase signals downstream NOX-1 to control cell proliferation in human colon cancer cells, whereas another group showed that ROS induced by NOX-1 overexpression can modulate the c-fos–mediated growth factors, interleukin-8 and Cav-1, which participate specifically in prostate cancer cell proliferation (13). It would thus seem that NOX and downstream targets, including ROS, are necessary for tumor growth, angiogenesis, and potentially metastasis and, therefore, are attractive targets for therapeutic intervention in cancer development.

In the present study, we found ALKBH8 to be an upstream target of NOX-1 and to be involved in intracellular ROS generation, and we further determined that ALKBH8/NOX-1 signals function mainly in the acquisition of the aggressive human urothelial carcinoma phenotype. In addition, our results clearly showed that transurethral injection of ALKBH8 small interfering RNA (siRNA) offers promise as a new therapeutic strategy for bladder cancer.

Materials and Methods

Bladder cancer cell line and transfection. We purchased the human urothelial carcinoma cell line UMUC2 from American Type Culture Collection. KU7 was derived from human papillary bladder cancer (14). We generated stable green fluorescent protein (GFP) clones as previously described (15).

Chemicals, antibodies, and preparation of antisera. The c-jun NH2-terminal kinase (JNK) inhibitor SP600125, JNK inhibitor cell-permeable peptide, and the p38 inhibitor SB203580 were obtained from Calbiochem.
Anti-poly(ADP-ribose) polymerase (PARP), anti–phosphorylated JNK, anti–phosphorylated p38, and anti–basic fibroblast growth factor (bFGF) antibodies were purchased from Cell Signaling; anti-actin, anti-JNK, anti-p38, and anti–vascular endothelial growth factor (VEGF) antibodies were purchased Santa Cruz Biotechnology, Inc. Antibodies to NOX-1, platelet-derived growth factor (PDGF)-BB, and H2AX were supplied by Abcam, whereas anti–phosphorylated H2AX (s313) was purchased from Upstate Biotechnology. Anti-ALKBH8 antisera were raised against the synthetic peptide of ALKBH8 (NKQKSKYLRGNRNS) as an antigen. The peptides (0.5 mg) were emulsified in an equal volume of Freund's complete adjuvant and then injected i.c. at several sites in each rabbit. Antiserum was prepared and we evaluated the relative activity of the antisera against the synthetic peptide by ELISA, and those antisera with high titer were affinity purified using SulfoLink (Pierce Biotech).

Preparation of cell lysates and Western blotting analysis. We resolved the cell lysates in SDS-polyacrylamide gels and transferred them onto polyvinylidene difluoride membranes (Millipore, Ltd.), which were then blocked in 5% skim milk at room temperature for 1 h. The membranes were incubated with the indicated primary antibody for 1 h, and then incubated with horseradish peroxidase–conjugated antiserum or antirabbit IgG (Amersham Pharmacia Biotech). We detected peroxidase activity on X-ray films using an enhanced chemiluminescence detection system.

Reverse transcription-PCR. Using the OneStep RT-PCR kit (Qiagen), we extracted total RNA using Trizol reagent and subjected it to reverse transcription-PCR (RT-PCR). PCR conditions were 95°C for 30 s, 55 to 60°C for 30 s, and 72°C for 1 min through a total of 30 cycles. The PCR primer sequences for ALKBH8 were 5'-AGATGATCTGTTCCCTGGC-3' (sense) and 5'-CCTCAGGAACATCTGAGTAG-3'(antisense). The primers for Bcl-2 were 5'-TTGGGGCCTTTTCTGATGC-3' (sense) and 5'-TCTTTCCAGACAGCCAGGAG-3'(antisense). NOX-1 primers were 5'-CTGCCATCTCCCTCCG-3' (sense) and 5'-CACCCTGAGGAGACGCAAG-3'(antisense). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the primers used were 5'-ACAGCTTCAATGCATCA-3' (sense) and 5'-TCTCACACACCTGTTCTGTA-3'(antisense).

Measurement of H2O2 production. We assessed the production of intracellular H2O2 with 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA; Wako) using a method modified from Bae and colleagues (16). Briefly, KU7 and UMUC2 cells were plated on chambered slides, transfected with either control RNA or ALKBH8 siRNA, and incubated for 60 h, or were treated with 1,500 units/mL of catalase for 30 min. Then, we incubated them with a 5 mol/L CM-H2DCF-DA solution for 20 minutes at room temperature. H2O2 formation in the cells was visualized using a Leica TCR6000 photomicroscope and analyzed by measuring the fluorescence intensity of 50 to 100 randomly selected cells (3).

siRNA transfection of ALKBH8, NOX-1, and H2AX. For our transfection experiments, 106 cells from each bladder cancer cell line were seeded in 6-cm dish plates and transfected either with control RNA or ALKBH8 siRNA, and incubated for 60 h, or were treated with 1,500 units/mL of catalase for 30 min. Then, we incubated them with a 5 mol/L CM-H2DCF-DA solution for 60 min at 37°C. Exosomes, H2AX, and ALKBH8 were considered significant if P < 0.05.

Cell cycle analysis. We performed cell cycle analyses by flow cytometry as previously described (19); in cell cycle analysis, fragmented apoptotic nuclei as well as necrotic cells or cell debris are recognized by their subdiploid (sub-G1) DNA content. All experiments were done at least three in duplicate.

Chorioallantoic membrane assay. Cells (106) from each cell line transfected with pEGFP (Clontech) were seeded on the chorioallantoic membranes (CAM) of 11-d-old chick embryos. After a 24-h cultivation, we added control RNA or 10 μmol/L ALKBH8 siRNA combined with atelocollagen (Atelogenex, Koken Co., Ltd.) according to the manufacturer's protocol. Five days post-implantation, CAM samples were collected and fixed, then stained with anti-GFP to allow the numbers of invading GFP-positive cells to be quantified in three or more randomly selected fields. The depth of invasion from the CAM surface was defined as the leading front of three or more invading cells in five randomly selected fields. We assessed angiogenesis as the number of visible blood vessel branch points within a defined area of the sample (20). At least three CAMs were used for each experiment.

Orthotopic tumor implantation and intravesical treatment. The method we used for the orthotopic instillation of tumor cells has been described (15); briefly, we anesthetized 8-wk-old female athymic nude mice and inserted a 24-gauge catheter transurethrally into the bladder. Then, 1 × 107 GFP-tagged KU7 cells suspended in 100 μL medium were instilled into the bladder lumen and the urethra was ligated for 2 to 3 h. Fourteen days after the cell instillation, we transurethrally injected either control RNA (n = 5) or 10 μmol/L of the ALKBH8 siRNA + atelocollagen mixture (n = 4) previously described (Koken Co., Ltd.) into the bladder (retain the RNA for 1 h).

In vivo fluorescence and image analyses. Twenty-eight days after the instillation of tumor cells, digital images were captured under both fluorescent and incandescent light. Image analysis was done with Image J public domain software available through the NIH. All images were spatially calibrated for area measurements. The mice were then sacrificed, and the bladders were excised and fixed for histologic examination by H&E and immunohistochemistry.

Gelatin zymography. The activity and expression of matrix metalloproteinase (MMP)-9 and MMP2 were analyzed by gelatin zymography. Equal amounts of concentrated condition medium were mixed with sample buffer [0.25 mol/L Tris-HCl (pH 6.8), 0.4% SDS, 40% glycerol, and bromophenol blue] and loaded onto 7.5% SDS-PAGE containing 1 mg/mL gelatin (Wako Pure Chemical Industries, Ltd.). After electrophoresis, the gel was stained with Coomassie blue solution.

Statistical analysis. Data were statistically analyzed using the Student t test or, for nonparametric analysis, the Kruskal-Wallis test (18, 21). Results were considered significant if P < 0.05.

Results

Silencing of ALKBH8 induced apoptosis in human urothelial carcinoma. Two human urothelial carcinoma cell lines, KU7 and UMUC2, were selected because these cells express ALKBH8 mRNA and protein, exhibiting high cell growth, invasion, and angiogenesis as shown in CAM assay. Both mRNA and protein expressions were

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strongly reduced in both human urothelial carcinoma cell lines, KU7 and UMUC2, when transfected with 100 nmol/L of ALKBH8 siRNA for up to 72 hours (Fig. 1A). As can be seen in Fig. 1B and C, the TUNEL and flow cytometric analyses showed a significant increase in the percentage of TUNEL-positive cells with down-regulation of ALKBH8 (KU7: 3.8% versus 35.3%; UMUC2: 2.8% versus 41.8%; *P* < 0.01). In addition, the number of cells in the sub-G1 fraction was also significantly increased by silencing ALKBH8. ALKBH8 silencing also resulted in PARP cleavage (Fig. 1D).

**JNK and p38 activation contributes to apoptosis induced by ALKBH8 down-regulation.** Because mitogen-activated protein (MAP) kinases, including JNK and p38, are known to play essential roles in apoptosis induced by various extrinsic stimuli in cancer cells (22), we examined whether MAP kinase activation contributes to cytotoxicity induced by silencing ALKBH8. As assessed by TUNEL assay and flow cytometric analyses shown in Fig. 2A, p38 and JNK were activated in both KU7 and UMUC2 cells in a time-dependent manner in response to transfection with ALKBH8 siRNA, an effect significantly blocked by treatment with specific inhibitors for JNK and p38, such as JNK inhibitory peptide (JNKi), SP500125, and SB203580 (Fig. 2B and C).

**NOX-1–dependent ROS generation is a downstream effect of ALKBH8 silencing.** As assessed by fluorescence microscopy using CM-H2DCF-DA, silencing ALKBH8 significantly suppressed intracellular levels of ROS in both KU7 and UMUC2 cells to a degree almost similar to that seen with catalase treatment (Fig. 3A and B). RT-PCR indicated that NOX-1, ubiquitously expressed in both cell lines, was also decreased by ALKBH8 down-regulation, and protein expression was similarly reduced (Fig. 3C). Other isoforms of NOX gene were not significantly modified by ALKBH8 silencing (data not shown). Transfection with 100 nmol/L NOX-1 siRNA induced JNK and p38 activation and strongly suppressed not only NOX-1 expression but also intracellular ROS levels in KU7 and UMUC2 cells; apoptosis induced by NOX-1 silencing was inhibited by treatments with JNK/p38–specific inhibitors (Fig. 3D; Supplementary Fig. S1). The percentages of apoptotic cells detected as a result of NOX-1 silencing and the degree of suppression of apoptosis by JNK/p38 inhibitors were similar to those resulting from silencing ALKBH8, indicating that NOX-1–mediated signals downstream of ALKBH8 are key to ROS generation and survival of urothelial carcinoma. JNK-dependent phosphorylation of H2AX has recently been shown...
ALKBH-8 siRNA

KU7 UMUC2

0 24 48 72 (h)

p-p38 p38

UMUC2

KU7

Cont.

ALKBH-8 siRNA

JNKI SP SB

ALKBH-8 siRNA

JNKI SP SB

ALKBH-8 siRNA

JNKI SP SB

ALKBH-8 siRNA

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ALKBH-8 siRNA

JNKI SP SB

Figure 2. Both JNK and p38 were activated by ALKBH8 gene silencing. A, KU7 and UMUC2 cells were collected after transfection with ALKBH8 siRNA, and the expression of phosphorylated p38 or JNK was examined by Western blotting. B and C, KU7 and UMUC2 cells were treated with 25 μmol/L of SP600125 (SP), 1 μmol/L of JNK inhibitory peptide (JNKI), or 25 μmol/L of SB202190 (SB) for 1 h before transfection with either control RNA (Cont.) or ALKBH8 siRNA and subjected to TUNEL staining. The percentages of TUNEL-positive cells and those in sub-G1 phase were determined by fluorescence microscopy and flow cytometric analysis, respectively. Columns, mean; bars, SE.

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Invasion and angiogenesis are suppressed by ALKBH8 down-regulation in urothelial carcinoma cells. Figure 4 shows the significant suppression of angiogenesis and of both the numbers of invasive cells and the depth of invasion by transfection of ALKBH8 siRNA in KU7 and UMUC2 cells in the CAM assay. The efficacy of this suppression was similar to the growth inhibitory effect induced by down-regulating ALKBH8, suggesting that ALKBH8 may enhance cancer invasion and angiogenesis by promoting cell survival signals. To investigate this line of reasoning, we examined several molecules involved in the process of invasion and angiogenesis, including MMPs, PDGF, bFGF, and VEGF; however, in our urothelial carcinoma cells, we found that expression of these molecules was not significantly modified by ALKBH8 silencing (Supplementary Fig. S3). The data indicate that ALKBH8 silencing resulted in suppression of invasion and angiogenesis mainly through apoptosis induction.

In vivo growth of urothelial carcinoma is suppressed by ALKBH8 siRNA transfection in the orthotopic implant mouse model. Figure 5A illustrates the urinary catheterization procedure used in the murine orthotopic implant model. Using a KU7 cell line stably overexpressing GFP, we have recently established GFP image analysis in this system in which tumor growth and treatment response can be quickly measured by image analysis in lieu of slower histologic methods. As shown in Fig. 5B and C, ALKBH8 down-regulation produced an ~6-fold decrease in tumor area. Conventional histologic evaluation showed the same results: Control mice showed large tumors with cancer cells deeply infiltrating into the bladder wall, whereas only a few small foci of cancer cells were observed in mice administered ALKBH8 siRNA in the presence of atelocollagen. Western blotting further confirmed that ALKBH8 and NOX-1 expression was strongly decreased; in contrast, phosphorylated JNK, p38, and cleaved PARP were increased in vivo by this single injection of ALKBH8 siRNA versus control RNA, using GFP expression as a positive internal control (Fig. 5D). This model also proves the efficacy of atelocollagen to successfully deliver ALKBH8 siRNA to orthotopically implanted bladder cancer cells.
**ALKBH8 and NOX-1 are expressed in human urothelial carcinoma of the urinary bladder.** We performed immunohistochemical analysis of ALKBH8 and NOX-1 in human bladder cancer specimens to examine the correlation of expression to tumor grade, invasiveness, and degree of angiogenesis. To ensure the specificity of antibodies used in this study, we used Western blotting on surgical specimens of low-grade/noninvasive, high-grade/invasive, and carcinoma in situ (CIS), all of which were diagnosed by two urological pathologists. ALKBH8 and NOX-1 were observed as a single band, indicating that the antibodies selected were of high specificity (Fig. 6B). The expression pattern of these molecules was consistent with the immunohistochemical analysis: As shown in Fig. 6A and B, the percentages of cells immunopositive for ALKBH8 and NOX-1 were much higher in high-grade (G3) urothelial carcinomas, including CIS, with both minimal and wide invasion (pT1) than in low-grade (G1 and G2) lesions with a noninvasive phenotype (pTa; Supplementary Table S2).

**Discussion**

We have shown here for the first time that ALKBH8, a novel member of the human AlkB family, plays important roles in the survival and progression of human urothelial carcinoma cells both in vitro and in vivo. Because ALKBH8 is a member of DNA repair molecules, we speculated that ALKBH8 knockdown-induced apoptosis might be mediated by DNA damage. It is well known that JNK, p38, or γH2AX is activated in response to DNA damage and closely associated with cell death induction. The present results clearly showed that JNK, p38, and γH2AX were significantly activated following ALKBH8 gene silencing. Then, we examined whether ALKBH8 knockdown affected endogenous stress including free radicals, resulting in apoptosis induction. Gene silencing experiments in human bladder cancer cells by means of siRNA transfection definitively identified NOX-1 as a downstream target of ALKBH8 involved mainly in intracellular ROS generation and in resistance to apoptosis. ROS generation induced through NOX-1 genes is known to be essential for oncogenesis and tumor progression (23, 24). Several other reports, however, suggest that NOX genes may regulate signal transductions far more than ROS generation. We found that down-regulation of NOX-1 strongly reduced generation of ROS, but treating urothelial carcinoma cells with catalase did not induce MAP kinase activation and apoptosis. This indicates the presence of a ROS-dependent and a ROS-independent transduction pathway operating in NOX-mediated cancer progression.

There have been conflicting reports about the function of ROS in cancer cells. Overproduction of ROS contributes to cytotoxicity by...
such extracellular stimuli as anticancer drugs, for instance, the enhanced bystander effects on paclitaxel cytotoxicity mediated by ROS, demonstrated by Alexandre and colleagues (25). The key point in these discrepancies is the effect of differences in intracellular ROS levels: Low endogenous levels of ROS generated mainly by NOX are linked to increased aggressiveness of cancer cells, whereas the explosive production of ROS stimulated by cytotoxic drugs promotes cell death.

It is widely accepted that the phosphoinositide 3-kinase–related protein kinase ataxia-telangiectasia mutated rapidly phosphor-
ylates the COOH-terminal SQE motif of the mammalian histone variant, H2A (H2AX), to generate ’’g-H2AX’’ over large chromatin domains flanking double-strand breaks induced by DNA damage. The resulting repair of oxidative DNA damage suppresses genomic instability (26). Moreover, H2AX confers cellular protection against alkylation-induced DNA damage (27). Lu and colleagues (28) also reported that, at the sites of DNA double-strand breaks induced by ionizing radiation, H2AX is phosphor-
ylated by JNK and plays an important role in the induction of apoptosis, suggesting that H2AX phosphorylation has biphasic functions on cell growth. Our results suggest that the level of expression (i.e., the degree of ALKBH8 regulation) determines whether γH2AX promotes cell death or cell survival. We have seen that mild but persistent activation of JNK promotes malignant potential (18), whereas strong transient activation resulted in cell death, as shown in this present study. This “bipolar” characteristic of JNK is similar to that of p53; p53 participates in DNA repair following various mild DNA damage yet mediates apoptosis induction with severe damage (29).

Both our in vitro and immunohistochemical analyses implicate the ALKBH8 gene as a potential therapeutic target in human bladder cancer. Silencing of ALKBH8 resulted in strong suppression of cancer invasion and angiogenesis in the CAM assay. This is probably due to induction of apoptosis because the apoptotic index in vitro was similar to that resulting from ALKBH8 down-regulation; further, the malignant potential of cancer cells was suppressed without significant modification of the representative molecules associated with migration and angiogenesis (Supple-
mentary Fig. S3). Therefore, induction of cell death, particularly apoptosis, might be a key mechanism by which ALKBH8 silencing suppressed the malignant potential of urothelial carcinoma. However, we do not deny any possibilities that there are other critical mechanisms than apoptosis induction because the effects of suppression by ALKBH8 down-regulation on invasion/angiogenesis were more prominent than on the percentages of cells undergoing apoptosis.

In our orthotopic mouse model of implanted bladder cancer, designed to assess the growth and response of GFP-labeled human
bladder cancer cells to intravesical treatment, we found exciting that a single injection of ALKBH8 siRNA, in the presence of atelocollagen, reduced both ALKBH8 and NOX-1 protein expression and resulted in significant reduction of tumor volume. Nogawa and colleagues also showed that intravesical injection of siRNA/liposomes to silence PLK-1 successfully inhibited bladder cancer growth. Intravesical injection of siRNA for target genes in the presence of atelocollagen or liposome instillation could thus be a useful therapeutic tool. The immunohistochemical analyses we performed on human bladder cancer samples suggest the contribution of signal activation to early development of urothelial carcinoma in that increased expression of ALKBH8/NOX-1 is linked growth. Intravesical injection of siRNA for target genes in the presence of atelocollagen or liposome instillation could thus be a useful therapeutic tool. The immunohistochemical analyses we performed on human bladder cancer samples suggest the contribution of signal activation to early development of urothelial carcinoma in that increased expression of ALKBH8/NOX-1 is linked.
to the change from a noninvasive to an invasive bladder cancer. Other studies posit that immunoprofiling of ALKBH8 and NOX-1, as well as of TP53, will provide more reliable information for bladder cancer prognosis (30).

In summary, a novel member of the human AlkB family, ALKBH8, plays an important role in cell survival by regulating NOX-1–dependent signals and enhances cancer progression in urothelial carcinoma of human bladder. Amplification of ALKBH8/NOX-1 expression is closely associated with transformation from a noninvasive to an invasive phenotype as determined by immunohistochemical analysis. Silencing ALKBH8 by siRNA transfection strongly suppressed malignant potential in vitro and in vivo; moreover, intravesical injection of ALKBH8 siRNA combined with atelocollagen may be a good delivery system to achieve successful down-regulation of the gene and provide a new therapy in the treatment of human bladder cancer (Supplementary Fig. S4).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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