Molecular and Cellular Pathobiology

Cell-Permeable Peptide DEPDC1-ZNF224 Interferes with Transcriptional Repression and Oncogenicity in Bladder Cancer Cells

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Abstract

Bladder cancer is the second most common genitourinary cancer worldwide, yet its oncogenic origins remain poorly understood. The cancer-testis antigen DEPDC1 was shown recently to contribute to bladder cancer oncogenesis. In this study, we examined the biological functions of DEPDC1 and defined a potential therapeutic strategy to target this molecule. Coimmunoprecipitation and immunocytochemistry revealed that DEPDC1 interacted and colocalized with zinc finger transcription factor ZNF224, a known transcriptional repressor. Inhibiting this interaction with a cell-permeable peptide corresponding to the ZNF224-interacting domain in DEPDC1 induced apoptosis of bladder cancer cells in vitro and in vivo. By inhibiting DEPDC1-ZNF224 complex formation, this peptide triggered transcriptional activation of A20, a potent inhibitor of the NF-κB signaling pathway. Our findings indicate that the DEPDC1-ZNF224 complex is likely to play a critical role in bladder carcinogenesis. Cancer Res; 70(14): 5829–39. ©2010 AACR.

Introduction

Bladder cancer is the second most common genitourinary cancer worldwide, and approximately 357,000 patients are diagnosed to have bladder cancer every year (1). Among them, approximately one third are suspected to be at an invasive stage or have metastasis at the time of diagnosis (2, 3). Radical cystectomy is the standard treatment for patients with localized invasive bladder cancer, but nearly 50% of such patients develop metastases within 2 years after the surgical treatment and subsequently die of the disease. In the last 2 decades, combined chemotherapy regimens, such as CMV (cisplatin, methotrexate, and vinblastine), M-VAC (methotrexate, vinblastine, doxorubicin, and cisplatin), and GC (gemcitabine and cisplatin), have shown their effectiveness in advanced or metastatic bladder cancer patients (4–6). However, the overall prognosis still remains very poor and adverse reactions caused by these combination chemotherapies are serious issues to be solved (2, 3, 6). Hence, development of novel molecular-targeted drugs with higher efficacy and minimum risk of adverse reactions for bladder cancer is essential to improve clinical management.

To develop such therapeutic modalities, we had analyzed the genome-wide gene expression profile of bladder cancers (7) and found upregulation of the DEPDC1 (DEP domain containing 1) gene in the great majority of bladder cancers (8). We also showed that downregulation of DEPDC1 by an RNA interference resulted in remarkable suppression of bladder cancer cell growth, indicating the critical role of DEPDC1 on bladder cancer cell growth (8). However, the precise mechanism through which DEPDC1 contributes to bladder carcinogenesis remains unclear.

Here, we describe a possible molecular mechanism of the DEPDC1 signaling in bladder cancer cells. We show that inhibition of the interaction of DEPDC1 and a transcriptional repressor, ZNF224, by the cell-permeable dominant-negative peptide causes significant growth suppression of bladder cancer cells in vitro and in vivo. Our findings indicate that a DEPDC1-ZNF224 complex is likely to play a critical role in bladder carcinogenesis and that inhibition of their complex formation would lead to potential strategies for treatment of bladder cancer.

Materials and Methods

Cell lines and clinical samples

The following cell lines were obtained from the American Type Culture Collection in 2001 to 2003: UM-UC-3, J82, HT1197, HT1376, HEK293, and COS7. Human mammary epithelial cell line (HMEC), normal small airway epithelial cell line (SAEC), and normal human dermal fibroblast cell line
(NHDF) were purchased from Lonza in 2007. All cells were cultured under conditions recommended by their respective depositors. We monitored the cell morphology of these cell lines by microscopy and confirmed to maintain their morphologic images in comparison with the original morphologic images. No Mycoplasma contamination was confirmed in cultures of all of these cell lines using a Mycoplasma Detection kit (Roche) in 2009. Tissue samples from surgically resected invasive or superficial bladder cancers and their corresponding clinical information were obtained from three hospitals—Kochi Medical School, Nagoya City University Graduate School of Medical Sciences, and Iwate Medical University—with written informed consent in 2005. This study, as well as the use of all clinical materials described above, was approved by individual institutional ethical committees.

Screening of DEPDC1-interacting proteins
Cell extracts from UM-UC-3 cells (1 × 10^6) were incubated at 4°C for 1 hour with 50 μL of protein G–agarose beads with immunoprecipitation buffer (0.5% NP40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) with protease inhibitor cocktail set III (Calbiochem). After centrifugation, the supernatant was incubated at 4°C with rabbit anti-DEPDC1 polyclonal antibody (8) or normal rabbit IgG. After SDS-PAGE, the gels were stained with silver. Protein bands that were specifically immunoprecipitated with anti-DEPDC1 antibody (8) were excised and served for matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF-MS) analysis (AXIMA-CFR plus; Shimazu Biotech Corp.).

Semiquantitative reverse transcription-PCR
Total RNA extraction, reverse transcription-PCR (RT-PCR), and T7-based RNA amplification for minimal RNAs from microdissected clinical bladder cancer cells were described previously (7, 8). The sequences of each set of primers were shown in Supplementary Table S1.

Construction of expression vectors
Full-length ZNF224 cDNA and a part of coding sequences of DEPDC1 were amplified by PCR using primers as shown in Supplementary Table S2. The PCR products were cloned into pCAGGSFLAG and pCAGGSchHA vectors, respectively. All of the constructs were confirmed by DNA sequencing with ABI 3700 DNA sequencer (Applied Biosystems). The entire coding region of DEPDC1 construct (pCAGGS-DEPDC1-V1-HA) was generated as described previously (8).

Western blot and immunoprecipitation analyses
Western blot and immunoprecipitation analyses were performed as described previously (8). After SDS-PAGE, membranes blotted with proteins were incubated with anti-DEPDC1 polyclonal (1:100; ref. 8), anti-ZNF224 polyclonal (1:100; Abcam), anti-A20 monoclonal (1:50; 59A426; Abcam), or anti-18S-β–actin (ACTB; Sigma) using standard procedures.

Immunocytochemical staining analysis
The immunocytochemical staining was performed as described previously (8). Briefly, UM-UC-3 cells (1 × 10^6) were transfected with ZNF224-FLAG construct and incubated with anti-DEPDC1 polyclonal (1:40) or anti-FLAG M2 monoclonal antibodies (1:1,000; Sigma). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). For detection of NF-κB expression in the nucleus after treatment with dominant-negative peptide (see below), UM-UC-3 cells (1 × 10^5) were treated with each peptide for 12 hours and incubated with anti–NF-κB (p65) monoclonal antibody (1:200; Santa Cruz Biotechnology). For the image analysis, the nuclear signal intensities of NF-κB (p65) were measured by observing nuclei of 50 cells treated with the 11R-DEP:611-628 or scramble peptides (see below).

RNA interference
To knock down endogenous DEPDC1 and ZNF224 proteins in bladder cancer cells, we used the synthetic oligo duplex for small interfering RNA (siRNA; Sigma-Aldrich Japan) against the specific target genes as follows: EGFP (enhanced green fluorescent protein gene) (as a control), 5′-GAGCAGCAGACGCUUCUC-3′; SCR (chloroplast Euglena gracilis gene coding for 5S and 16S rRNAs) (as a control), 5′-GGGCGCGUUGTAGGAUU-3′; si-ZNF224-1, 5′-CCGAAUUGGAUGAAGAAGA-3′; and si-ZNF224-2, 5′-CCGCGGAACACAUAGAAGA-3′, siRNA (10 nmol/L) was transfected into UM-UC-3 cells using RNAiMAX (Life Technologies) according to the supplier’s recommendations. To evaluate the knockdown effect of each siRNA by semiquantitative RT-PCR with specific primers (see below), total RNAs were extracted from the transfected cells after 4 days of incubation. The specific primer set of ZNF224 for RT-PCR is 5′-GAGCAGCACATGGGAAGAACAT-3′ and 5′-TGACGCTCTGACTAAAGCACA-3′. The transfected UM-UC-3 cells were cultured for 7 days, and the numbers of colonies were counted by Giemsa staining. Viability of UM-UC-3 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 7 days with Cell Counting Kit-8 (Wako). Absorbance at 570-nm wavelength was measured with Microplate Reader 550 (Bio-Rad).

Cell proliferation assay
To examine the effect of DEPDC1 and ZNF224 on cell growth, UM-UC-3 cells (1 × 10^6) were transfected with 8 μg each of DEPDC1-HA or ZNF224-FLAG or both constructs using FuGENE6 (Roche). After 24 hours, cells were cultured with Eagle’s MEM containing 1 mg/mL neomycin (Geneticin; Life Technologies) for 5 more days. Viability of UM-UC-3 cells was evaluated by MTT assays 7 days after the treatment of neomycin with Cell Counting Kit-8.

Identification of the ZNF224-binding regions in DEPDC1 protein
Cell extracts from each COS7 cell that was transfected with a plasmid expressing one of the six partial DEPDC1 proteins (see “Construction of expression vectors”) were precleared with 20 μL of protein G–agarose beads. After
centrifugation, the supernatants were incubated at 4°C with anti-HA rat antibody. After SDS-PAGE, membranes blotted with proteins were incubated with an anti-HA rat and anti-FLAG M2 monoclonal antibodies (Sigma-Aldrich), respectively.

**Dominant-negative peptide**

Four 18–amino acid peptides derived from the minimum ZNF224-binding domain of DEPDC1 (codons 598–653) were covalently linked at its NH₂ terminus to a membrane-transducing 11 polyarginine sequence (11R; refs. 9, 10). The sequences of each peptide are shown in Supplementary Table S3. Scramble peptide derived from 11R-DEP:611-628 was synthesized as a control. To examine the effect of these 11R-linked peptides on inhibition of the DEPDC1-ZNF224 complex formation, we transfected UM-UC-3 cells with ZNF224-FLAG construct. After 3 hours of transfection, cells were incubated in the media containing 11R-DEP:611-628 or scramble peptides at a concentration of 3 μmol/L for 12 hours.

**In vitro tumor growth inhibition**

UM-UC-3 or NHDF cells were incubated in the media supplemented with 2% fetal bovine serum containing the 11R-DEP:611-628 or scramble peptides at a concentration of 0, 1, 2, or 3 μmol/L for 5 days, respectively. Each peptide was added every 24 hours at the appropriate concentrations, and the viability of cells was evaluated by MTT assay every day from day 2 (day 0; before treatment).

**In vitro and in vivo terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay**

For *in vitro* terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, UM-UC-3 cells were incubated for 12 hours after treatment with 11R-DEP:611-628 or scramble peptides and then evaluated using an Apoptosis *in situ* Detection kit according to the supplier’s recommendation (Wako). The apoptotic cells were observed with a TCS SP2 AOBS microscope. The TUNEL positivity was determined by observing 200 cells at random for each experiment. For *in vivo* TUNEL assay, tumor-bearing mice were administered with intratumoral injections of 24.7 μg of the 11R-DEP:611-628 peptide, scramble peptide, or PBS every 3 days (see Supplementary Materials and Methods). After the fourth injection every third day, tumors were taken and the respective sections were prepared for TUNEL assay as described above.

**Quantitative real-time PCR**

Quantitative real-time PCR was conducted with the SYBR Green I Master on a LightCycler 480 (Roche) using the amplified RNAs from UM-UC-3 cells used for cDNA microarray experiments (see Supplementary Materials and Methods). Each gene-specific primer is 5′-TGGACACTGTGGTTTACGAG-3′ and 5′-ACGCTTGAGGACGTCACTTC-3′ for *A20* and 5′-TCTCTCTGCTGCCTGAGAG-3′ and 5′-AAATGTGAGTGGGTAAAACC-3′ for *β2-microglobulin* (*β2-MG* control).

**Luciferase reporter assay**

The fragment of the *A20* promoter (position −330 to −35) was amplified by PCR using the primers 5′-GATCCACGCGTTAGCCCCAGAGAGTACG-3′ and 5′-GATCTCTGAGGAGGACGTCACTTC-3′ (underlines indicate *Mlu* and *Xho* sites, respectively) and cloned into the pGL3-enhancer luciferase reporter vector (Promega). NHDF cells were cotransfected with either 1 μg of pGL3-enhancer-A20 promoter or mock vector in combination with 0.5 μg of the pRL-TK-promoter vector using Human Dermal Fibroblast Nucleofector kit (Lonza). After 24 hours, luciferase activity was measured using Dual-Luciferase Reporter Assay kit (Toyo Ink) as described previously (11).

**Chromatin immunoprecipitation assay**

HEK293 (4 × 10⁶) cells were cotransfected with HA-DEPDC1 and FLA-ZNF224 constructs using FuGENE 6. After 24 hours, chromatin immunoprecipitation (ChIP) assay was performed using ChIP assay kit (Upstate). The recovered DNA was analyzed using the primers 5′-AGCCGAGACGGAGGTCACGT-3′ and 5′-CTTTCTGGAAGGCGCAGTCT-3′ that covered the *A20* promoter region (position −330 to −35).

**Statistical analysis**

Statistical significance was determined by Student’s *t* test using StatView 5.0 software (SAS Institute). *P* < 0.05 was considered to be statistically significant.

**Results**

**Identification of interacting protein(s) with DEPDC1**

To elucidate biological functions of DEPDC1 in bladder cancer cells, we searched for a protein interacting with DEPDC1 by immunoprecipitation with anti-DEPDC1 polyclonal antibody (8) and MALDI-TOF-MS analyses. Cells extracts from UM-UC-3 cells were immunoprecipitated with anti-DEPDC1 polyclonal antibody (8) and silver stained on SDS-PAGE gels. An ∼93-kilodalton protein, which was seen in immunoprecipitates by rabbit anti-DEPDC1 polyclonal antibody, but not in those by control rabbit IgG, was extracted and analyzed by MALDI-TOF-MS (Supplementary Fig. S1). This approach identified it to be ZNF224 (zinc finger protein 224), a Kruppel-associated box-containing zinc finger protein (12, 13). To validate their interaction, we constructed a plasmid designed to express FLAG-tagged ZNF224 (ZNF224-FLAG) and performed immunoprecipitation experiments. Cell lysates from UM-UC-3 cells that were transfected with the ZNF224-FLAG plasmid were used for immunoprecipitation experiments with anti-DEPDC1 polyclonal antibody. Immunoblotting of the precipitates with anti-FLAG M2 monoclonal antibody revealed that endogenous DEPDC1 was cocoprecipitated with ZNF224-FLAG protein (Fig. 1A). To further examine subcellular localization of DEPDC1 and ZNF224, we transfected ZNF224-FLAG construct into UM-UC-3 cells. Figure 1B shows that endogenous DEPDC1 was clearly colocalized with exogenous ZNF224 at the nucleus. Additionally, semiquantitative RT-PCR detected co-upregulation of ZNF224 and DEPDC1.
in seven of nine bladder cancer cases (Supplementary Fig. S2).

**Oncogenic activity of the DEPDC1-ZNF224 complex**

To investigate the biological significance of ZNF224 in bladder carcinogenesis, we introduced the oligo duplex siRNAs against ZNF224 (si-ZNF224-1 and si-ZNF224-2) into UM-UC-3 cells, in which ZNF224 was highly expressed (Supplementary Fig. S3). We found that introduction of either si-ZNF224-1 or si-ZNF224-2 into UM-UC-3 cells resulted in significant reduction of ZNF224 expression that was accompanied by suppression of cell proliferation, whereas no change was observed in the cells transfected with si-EGFP or si-SCR (negative controls; Fig. 1C). These results clearly indicated that ZNF224 is likely to have crucial roles on the growth of bladder cancer cells.

To further examine the growth-promoting effect of the DEPDC1-ZNF224 complex, we transfected DEPDC1-HA, ZNF224-FLAG, or both into UM-UC-3 cells and then investigated cell viability by MTT assay. We confirmed the expression of exogenous DEPDC1-HA and ZNF224-FLAG proteins by Western blot analysis using anti-HA and anti-FLAG antibodies (Fig. 1D, bottom). Double-transfected UM-UC-3 cells showed significantly higher cell proliferation than those transfected with DEPDC1 or ZNF224 alone \( (P < 0.05) \), in comparison with those transfected with mock plasmid (Fig. 1D, top). Taken together, our results imply that the DEPDC1-ZNF224 complex is likely to play crucial roles on bladder carcinogenesis.

**Identification of the ZNF224-binding region in DEPDC1**

To determine the domain in DEPDC1 that is required for the interaction with ZNF224, we cotransfected either of six partial constructs of HA-tagged DEPDC1 (Fig. 2A) and ZNF224-FLAG into COS7 cells. Immunoprecipitation with anti-HA monoclonal antibody indicated that DEPDC1(300–669) and DEPDC1(587–740) were able to interact with ZNF224-FLAG as well as the full-length DEPDC1, but...
DEPDC1(1–147), DEPDC1(1–300), DEPDC1(177–597), or DEPDC1(654–811) could not (Fig. 2B). These results suggested that the 56-amino acid polypeptide [DEPDC1(598–653)] in DEPDC1 should be essential for the interaction with the ZNF224 protein.

**Growth inhibitory effects of the 11R-DEP:611-628 peptide in vitro and in vivo**

To examine a possibility of the dominant-negative effect of the cell-permeable peptides on inhibition of the interaction between DEPDC1 and ZNF224, we synthesized four 18-amino acid polypeptides that could cover DEPDC1(598–653), the ZNF224-binding domain, with a membrane-permeable 11 residues of arginine (11R) at its NH2 terminus. We investigated the effect of these peptides on the protein-protein interaction as well as bladder cancer cell growth. First, we cotransfected DEPDC1-HA and ZNF224-FLAG constructs into COS7 cells and then treated cells with each of the four peptides at 6 hours after the transfection. After 24 hours, we performed the immunoprecipitation experiments with anti-HA antibody, followed by Western blot with anti-FLAG antibody. We showed that treatment of the 11R-DEP:611-628 peptide clearly inhibited the interaction of DEPDC1-HA and ZNF224-FLAG in COS7 cells, and that of 11R-DEP:598-615 revealed moderate effect, whereas other peptides showed no effect on their interaction (Supplementary Fig. S4). Moreover, we confirmed that 11R-DEP:611-628 effectively inhibited a complex formation of endogenous DEPDC1 and exogenous ZNF224-FLAG proteins in UM-UC-3 cells, whereas the scramble sequence peptide of 11R-DEP:611-628 (scramble) showed no effect on their complex formation (Fig. 2C).

Furthermore, addition of the 11R-DEP:611-628 peptide caused significant decreases in cell viability of UM-UC-3 cells in a dose-dependent manner, as measured by MTT assay (Fig. 3A, left), although its scramble peptide revealed no effect (Fig. 3A, right). We also observed its similar effect on another bladder cancer cell line, J82 (Supplementary Fig. S5), in which DEPDC1 and ZNF224 were co-upregulated (Supplementary Fig. S6). In contrast, 11R-DEP:611-628 peptide revealed no significant effect on cell viability of NHDF cells (Fig. 3B), in which DEPDC1 and ZNF224 expressions were hardly

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**Figure 2. Identification of the ZNF224-binding region in DEPDC1.** A, schematic representation of six HA-DEPDC1 partial clones lacking either or both of the terminal regions. B, identification of the region in DEPDC1(598–653) that binds to ZNF224 by coimmunoprecipitation. C, reduction of the complex formation detected by immunoprecipitation between endogenous DEPDC1 and exogenous ZNF224-FLAG in UM-UC-3 cells that were treated with the 11R-DEP:611-628 peptide.
detectable (Supplementary Fig. S3). To further clarify the mechanism of growth suppression by the 11R-DEP:611-628 peptide, we performed TUNEL assay using the bladder cancer cells treated with this peptide in vitro (Fig. 3C). The results showed that treatment of the 11R-DEP:611-628 peptide led to a significant increase in TUNEL-positive cells compared with the treatment of scramble peptide as well as PBS as a control. Additionally, we confirmed that the 11R-DEP:611-628 peptide revealed a significant increase in sub-G1 population by flow cytometric analysis (Supplementary Fig. S7), implying that treatment of the 11R-DEP:611-628 peptide caused apoptotic cell death of bladder cancer cells. Collectively, these data suggest that 11R-DEP:611-628 peptide could specifically inhibit a functional complex formation of DEPDC1-ZNF224, and led to significant growth suppression, whereas it showed no toxic effect on normal human cells that did not express these proteins.

For in vivo imaging using fluorescence, tumor-bearing mice were administered with intratumoral injections of Atto647N-labeled 11R-DEP:611-628 peptide, and distribution of each of the peptides was visualized using the IVIS system. Accumulation of fluorescent signal was reached at the maximum level at ~6 hours after the injection and could be detectable at the tumor location until 72 hours after the injection (Supplementary Fig. S8). We then examined the antitumor activity of the 11R-DEP:611-628 peptide in vivo. UM-UC-3 cells were injected subcutaneously in the flanks of BALB/c nude mice. When tumors were fully established, 24.7 μg of the 11R-DEP:611-628 peptide, scramble peptide, or PBS were administered into the tumors every 3 days.
Figure 4. Identification of A20 as a candidate downstream gene of the DEPDC1-ZNF224 complex. A, expression of A20 gene in response to knockdown of DEPDC1 or ZNF224 by siRNA. Quantitative RT-PCR was performed using RNA from UM-UC-3 cells transfected with si-DEPDC1, si-ZNF224, or si-EGFP as a control. Top, results are given as ratio against the cells transfected with si-EGFP. *, P < 0.05, unpaired t test. Bottom, knockdown effects of ZNF224 and DEPDC1 expressions were confirmed by semiquantitative RT-PCR. B, effect of the DEPDC1-ZNF224 complex on the luciferase activity of reporter plasmids containing the promoter region of A20 gene in NHDF cells. Luciferase activity is indicated relative to the activity of pRL-TK-promoter vector without A20 promoter region. Significant differences were determined by unpaired t test. Bottom, Western blot analysis showed the DEPDC1-HA and ZNF224-FLAG protein expressions, respectively. C, association of the DEPDC1-ZNF224 complex with DNA fragment containing A20 promoter region, detected by ChIP assay. DNA from HEK293 cells was immunoprecipitated with indicated antibodies and served for PCR.
because this peptide was considered to be stable for at least 72 hours as shown in Supplementary Fig. S8. The growth of bladder cancer xenografts in nude mice was rapidly attenuated by treatment with the 11R-DEP:611-628 peptide compared with the tumors treated with scramble peptide or PBS (Supplementary Fig. S9). We then performed in vivo TUNEL assay to clarify the mechanism involved in the antitumor activity of the 11R-DEP:611-628 peptide. Clusters of apoptotic cells were observed in the tumor specimens treated with the 11R-DEP:611-628 peptide (Fig. 3D), whereas such clusters were not observed in the tumor specimens treated with scramble peptide or PBS (Fig. 3D).

Identification of a gene regulated by the DEPDC1-ZNF224 complex

ZNF224, a member of the Kruppel-like zinc finger protein family, was reported to function as a transcriptional repressor (12–14). Therefore, we hypothesized that DEPDC1 may play some roles as a cotranscriptional repressor through its interaction with ZNF224. We first attempted to identify downstream genes regulated by the DEPDC1-ZNF224 complex in bladder cancer cells. siRNA-DEPDC1, siRNA-ZNF224, or siRNA-EGFP (control siRNA) was transfected into UM-UC-3 cells, and alterations in gene expression at two time points were monitored using cDNA microarray analysis (see Supplementary Materials and Methods). We identified a dozen genes that were commonly upregulated by knockdown of either of the two genes and also were commonly downregulated in clinical bladder cancers in our cDNA microarray data reported previously (Supplementary Table S4; ref. 7). Because disruption of the DEPDC1-ZNF224 complex formation by the dominant-negative peptide resulted in induction of apoptosis (Fig. 3C and D; Supplementary Fig. S7), we focused on one gene, A20 (also known as TNFAIP3), which is reported to function as a negative regulator in the NF-κB antiapoptotic pathway (15, 16) and as a candidate downstream gene. The cDNA microarray profiles of bladder cancer specimens showed that the A20 gene was downregulated (>0.5-fold more than normal bladder cells) in 21 of the 41 informative bladder cancer cases (7). Quantitative RT-PCR analysis confirmed the time-dependent increase of A20 expression in UM-UC-3 cells transfected with either si-DEPDC1 or si-ZNF224 compared with the cells transfected with si-EGFP (control; Fig. 4A).

To examine the potential promoter-specific repression of A20 transcription by the DEPDC1-ZNF224 complex, we first searched the promoter region of A20 gene by the computer prediction program WWW Promoter Scan (17). Then, we cotransfected UM-UC-3 cells with the reporter plasmid containing a ∼300-bp fragment corresponding to a promoter region of A20 gene that was fused to a luciferase reporter gene as well as either of two plasmid clones...
designed to express DEPDC1 or ZNF224 or both clones. In the luciferase reporter assay using the A20 reporter plasmid, NHDF cells cotransfected with both DEPDC1-HA and ZNF224-FLAG constructs showed significant reduction of luciferase reporter activity compared with those transfected with DEPDC1 alone, ZNF224 alone, or mock plasmid (Fig. 4B).

To further investigate whether the DEPDC1-ZNF224 complex could bind to the A20 promoter region, we performed ChIP assay using the cell extracts from HEK293 cells transfected with both DEPDC1-HA and ZNF224-FLAG constructs. The 296-bp genomic fragment (position −330 to −35) of A20 specifically detected a DEPDC1-ZNF224-DNA complex in immunoprecipitation products with anti-HA antibody, suggesting that ZNF224 directly bound to the A20 gene promoter region, but DEPDC1 did not (Fig. 4C).

Together, our findings strongly suggest that the DEPDC1-ZNF224 complex might function as a transcriptional repressor and repress transactivation of A20 gene in bladder cancer cells.

**Inhibition of DEPDC1-ZNF224-mediated antiapoptosis by 11R-DEP:611-628 peptide**

A20 was initially identified as a cytoplasmic zinc finger protein that was rapidly induced after stimulation of tumor necrosis factor-α and functioned as a negative regulator of the NF-κB canonical pathway (18, 19). Therefore, we focused on the effect of A20 in the NF-κB signaling pathway. Western blot analysis showed that expression of A20 was elevated 6 hours after the treatment of the 11R-DEP:611-628 peptide that inhibited the DEPDC1-ZNF224 complex formation (Fig. 5A).

A20 was indicated to inhibit the phosphorylation of IκB-α (inhibitor of NF-κB) and subsequently block its ubiquitination and proteasomal degradation (20, 21). Therefore, we examined the effects on the IκB-α protein level by treatment of the 11R-DEP:611-628 peptide and found that IκB-α protein level was elevated at 12 hours after the treatment of the 11R-DEP:611-628 peptide (Fig. 5A), whereas its mRNA level was unchanged (Fig. 5A). Moreover, we showed that the 11R-DEP:611-628 peptide clearly diminished the nuclear staining of NF-κB (p65) protein, although the treatment with the scramble peptide revealed no effect by immunocytochemistry (Fig. 5B and C), implying inhibition of nuclear transport of NF-κB (p65) protein by the 11R-DEP:611-628 peptide.

Taken together, our results strongly suggest that the 11R-DEP:611-628 peptide inhibited the DEPDC1-ZNF224 complex formation, activated the transcription of its downstream gene A20, and resulted in apoptosis induction through inactivation of the NF-κB pathway.
Discussion

Significant advances in development of molecular targeting drugs for cancer therapy have been achieved in the last 2 decades. However, the proportion of patients showing good response to presently available treatments is still very limited and some proportion of the patients suffer from severe adverse reactions without any clinical benefit (3). Therefore, we have established our effective screening system to identify therapeutic targets as well as biological function analysis of their functionally relevant partners toward development of agents that have specific and efficient anticancer effects with lower risk of adverse reactions. Through the gene expression profile analysis of various kinds of cancer, we have identified several cancer-specific molecules and characterized them for possible application to development of cancer therapy (8, 22–25). Among them, we here report characterization of DEPDC1 as a druggable target by showing its critical roles in bladder carcinogenesis.

We previously reported the upregulation of DEPDC1 protein in invasive and superficial bladder cancers and showed its critical roles in cancer cell growth (8). In this report, we first examined its cell migration and invasion potential but its critical roles in cancer cell growth (8). In this report, we have established our effective screening system to identify DEPDC1 overexpression (Supplementary Fig. S10). To clarify its pathophysiologic function (28–30), and is constitutively activated in various human tumors including bladder cancers (30–32). Moreover, it was recently reported that dysregulation of NF-κB signaling caused by loss of tumor-suppressive function of A20 protein is involved in the pathogenesis of a subset of B-cell lymphomas, implying that A20 has the tumor-suppressive function (33, 34). Our data presented here indicate that the DEPDC1-ZNF224 complex represses the transcription of the A20 gene and leads to transport of NF-κB protein into the nucleus, resulting in suppression of apoptosis of bladder cancer cells (Fig. 6A). Furthermore, we designed the cell-permeable dominant-negative peptides (11R-DEP611-628 peptide) that corresponded to the binding domain to ZNF224 protein and could inhibit DEPDC1 with ZNF224 complex formation (Fig. 6B). Blocking their interaction by 11R-DEP611-628 peptide clearly resulted in induction of apoptotic cell death of bladder cancer cells in vitro and in vivo.

Particularly, we have shown here that the growth of bladder cancer xenografts in nude mice was rapidly attenuated by intratumoral injections of the 11R-DEP611-628 peptide. Therefore, because intravesical instillation therapy of bacillus Calmette-Guerin has currently become a standard treatment for patients with carcinoma in situ of the urinary bladder, this peptide inhibitor may also be expected to be used for patients with bladder cancer.

In conclusion, we successfully generated a peptide inhibitor, which specifically inhibits their complex formation and led to growth-suppressive effects. We are confident that inhibition of the DEPDC1-ZNF224 complex formation could be a promising therapeutic target for the treatment of bladder cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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