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SHORT COMMUNICATION

Involvement of upregulation of *DEPDC1* (DEP domain containing 1) in bladder carcinogenesis

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In an attempt to disclose mechanisms of bladder carcinogenesis and discover novel target molecules for development of treatment, we applied a cDNA microarray to screen genes that were significantly transactivated in bladder cancer cells. Among the upregulated genes, we here focused on a novel gene, (*DEPDC1*) DEP domain containing 1, whose overexpression was confirmed by northern blot and immunohistochemical analyses. Immunocytochemical staining analysis detected strong staining of endogenous DEPDC1 protein in the nucleus of bladder cancer cells. Since *DEPDC1* expression was hardly detectable in any of 24 normal human tissues we examined except the testis, we considered this gene-product to be a novel cancer/testis antigen. Suppression of *DEPDC1* expression with small-interfering RNA significantly inhibited growth of bladder cancer cells. Taken together, these findings suggest that *DEPDC1* might play an essential role in the growth of bladder cancer cells, and would be a promising molecular-target for novel therapeutic drugs or cancer peptide-vaccine to bladder cancers.
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Bladder cancer is the second most common genitourinary tumor in human populations, having an incidence of approximately 357 000 new cases each year worldwide and 145 000 die of the disease (Parkin *et al*., 2005). Although radical cystectomy is considered as the 'gold standard' for treatment of patients with localized but muscle-invasive bladder cancer, about 50% of such patients develop metastases within 2 years after cystectomy (Sternberg, 1995). Additional studies directed toward understanding of the factors involved in the progression of bladder cancer will facilitate development of molecular-based diagnostic and therapeutic approaches. In the last two decades, cisplatin-based combination chemotherapy regimens, such as CMV (cisplatin, methotrexate and vinblastine) or M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin) have been mainly applied to patients with advanced bladder cancers (Lehmann *et al*., 2002; Ardavanis *et al*., 2005; Rosenberg *et al*., 2005; Theodore *et al*., 2005). However, the overall prognosis remains still very poor and adverse reactions caused by these combination chemotherapies are significantly severe (Vaughn, 1999). Therefore, development of a new molecular target drug(s) against bladder cancer is desired earnestly.

Gene-expression profiles obtained by cDNA microarray analysis have been proven to provide detailed characterization of individual cancers and such information should contribute to improve clinical strategies for neoplastic diseases through development of novel drugs as well as providing the basis of personalized treatment (Petricoin *et al*., 2002). Through the genome-wide expression analysis, we have isolated a number of genes that functioned as oncogenes in the process of development and/or progression of breast cancers (Park *et al*., 2006; Shimo *et al*., 2007), hepatocellular carcinomas (Hamamoto *et al*., 2004), pancreatic cancer (Taniuchi *et al*., 2005a, b), prostate cancers (Anazawa *et al*., 2005; Ashida *et al*., 2005), synovial sarcomas (Nagayama *et al*., 2004, 2005) and renal cell carcinomas (Togashi *et al*., 2005). Such molecules are considered to be good candidate targets for development of new therapeutic modalities.

To attempt to identify novel molecular targets for bladder cancer therapy, we previously analysed a detailed gene-expression profiles of bladder cancers using a genome-wide cDNA microarray consisting of 27 648 genes or expressed sequence tags (ESTs) after purification of cancer cells by laser microbeam microdissection (Takata *et al*., 2005). In this study, we report the identification and characterization of a novel gene, (*DEPDC1*) DEP domain containing 1 that was significantly overexpressed in a great majority of bladder cancer cases. Proteins containing the DEP (Dishevelled, EGL-10, Pleckstrin) domain have been reported to

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regulate a broad range of cellular functions including a large number of signaling proteins; *Drosophila melanogaster* dishevelled is known to be an adaptor in the Wnt signaling. EGL-10, a regulator of G-protein signaling (RGS) protein, negatively regulates signaling by G-protein coupled receptors in *Caenorhabditis elegans*. Pleckstrin modulates signaling in platelets and neutrophils (Kharrat *et al*., 1998). Although DEPDC1 contains a highly conserved DEP domain, its pathophysiologic roles in growth of human cancer cells have not been investigated.

To verify *DEPDC1* expression though microarray data, we performed semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis, and confirmed elevated *DEPDC1* expression in 17 of 21 bladder cancer cases (Figure 1a). Subsequent northern blot analysis found that a 4.6-kb transcript of *DEPDC1* was abundantly and specifically expressed in four bladder cancer cell lines, while a 5.3-kb transcript was expressed in bladder cancer cell lines as well as the testis among 24 normal tissues examined (Figure 1b and c).

To characterize these two *DEPDC1* transcriptional variants, we performed exon-connection, 5′ RACE and 3′ RACE experiments using cDNAs prepared from a bladder cancer cell line, J82, as a template, and isolated the full-length cDNA sequences corresponding to the two transcriptional variants, denoted as *DEPDC1* isoform 1 (*DEPDC1-V1*: GeneBank Accession AB281187) and *DEPDC1* isoform 2 (*DEPDC1-V2:* GeneBank Accession AB281274) consisting of 5318 and 4466 nucleotides that encode 811 and 527 amino-acid peptides. These two variants consist of 12 and 11 exons, respectively; the V2 variant lacked of exon 8 (852 nucleotides), and the other exons were common to the two variants (Figure 1d), spanning an approximately 23-kb genomic region on the chromosomal band 1p31.2. The Simple Modular Architecture Research Tool (SMART) computer program suggests that the predicted proteins contain the DEP (Dishevelled, EGL-10, Pleckstrin) domain in their N-terminal regions.

We developed the two kinds of polyclonal antibodies; anti-DEPDC1 antibody can recognize a common region of the two variant proteins and anti-DEPDC1-V1 antibody can recognize a region specific to the DEPDC1-V1 protein (see legend of Figure 2). To investigate the specificity of these antibodies, we performed western blotting analysis, and demonstrated that these DEPDC1 antibodies could specifically recognize exogenously expressed DEPDC1-V1 and -V2 proteins without producing any nonspecific bands, respectively, while no band was detected in mock-transfected cells (Supplementary Figure 1). We performed immunohistochemical staining using those antibodies, and observed strong staining in bladder cancer cells of both superficial and invasive bladder cancer cases, while no staining was observed in the corresponding normal bladder cells (Figure 2a and b). Moreover, in concordance with the results of northern blotting analysis, expression of DEPDC1-V1 protein was detected in testis, but expression of neither of the variant proteins was observed in heart, liver, kidney and lung (Figure 2c).

To investigate expression of endogenous DEPDC1 proteins, we performed western blotting analysis with anti-DEPDC1 antibody using cell lysates from six bladder cancer cell lines. As shown in Figure 3a, we detected two bands, one corresponding to the predicted size of V1 protein (93.1 kDa) and the other corresponding to the predicted size of V2 protein (61.5 kDa), in all of the six bladder cancer cell lines examined, indicating that both DEPDC1-V1 and -V2 proteins were expressed in bladder cancer cells.

Furthermore, we performed immunocytochemical staining analysis with anti-DEPDC1 and -V1 polyclonal antibodies using UM-UC-3 cells that expressed both DEPDC1-V1 and -V2 proteins, respectively. The results showed that both antibodies could detect strong signals in nucleus of bladder cancer cells (Figure 3b). Moreover, we verified the specificity of signals in immunocytochemical analysis using COS7 cells transfected with DEPDC1-V1, DEPDC1-V2 or mock vector. We observed the specific nuclear staining in DEPDC1-V1- and DEPDC1-V2-transcfected cells, but no staining in mock-transfected cells (Supplementary Figure 2a, b). Together, these results suggest that DEPDC1-V1 and -V2 proteins could be colocalized in nucleus of bladder cancer cells.

Subsequently, we examined a possibility of interaction and hetero-dimer formation of V1 and V2 variants in bladder cancer cells by co-immunoprecipitation analysis and obtained the data indicating hetero-dimerization of the two variant proteins (Figure 3c). In addition, we could detect homo-dimerization of each of V1 and V2 proteins by co-immunoprecipitation analysis (data not shown).

To assess whether DEPDC1 plays a role in growth or survival of bladder cancer cells, we knocked down the expression of endogenous DEPDC1-V1 and -V2 in bladder cancer cell lines, J82 and UM-UC-3, in which overexpression of DEPDC1 was observed, by means of the mammalian vector-based RNA interference (RNAi) technique (see legend of Figure 4). We initially designed a DEPDC1-specific small-interfering RNA (siRNA) construct that targeted to the region common to the two variants. Semiquantitative RT–PCR and western blotting analyses showed that the DEPDC1-specific siRNA (si-DEPDC1) significantly suppressed the expression of DEPDC1 at both mRNA and protein levels, as compared with si-EGFP as a control (Figure 4a). We then performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony-formation assays (Figure 4b and c), and found that introduction of si-DEPDC1 construct remarkably suppressed growth of both J82 and UM-UC-3 cells (J82: si-DEPDC1, $P<0.001$; UM-UC-3: si-DEPDC1, $P<0.001$; student *t*-test), in concordance with the results of the knock-down effect. To exclude a possibility of off-target effect by si-DEPDC1, we generated a mismatched-siRNA of which contained 3-bp replacement in si-DEPDC1 (si-DEPDC1 mismatch), and found that this mismatched construct had no suppressive effect on DEPDC1 expression (Figure 4a) as well as growth of J82 and UM-UC-3 cells (Figure 4b and c). Moreover, we demonstrated that DEPDC1V1-specific siRNAs-V1

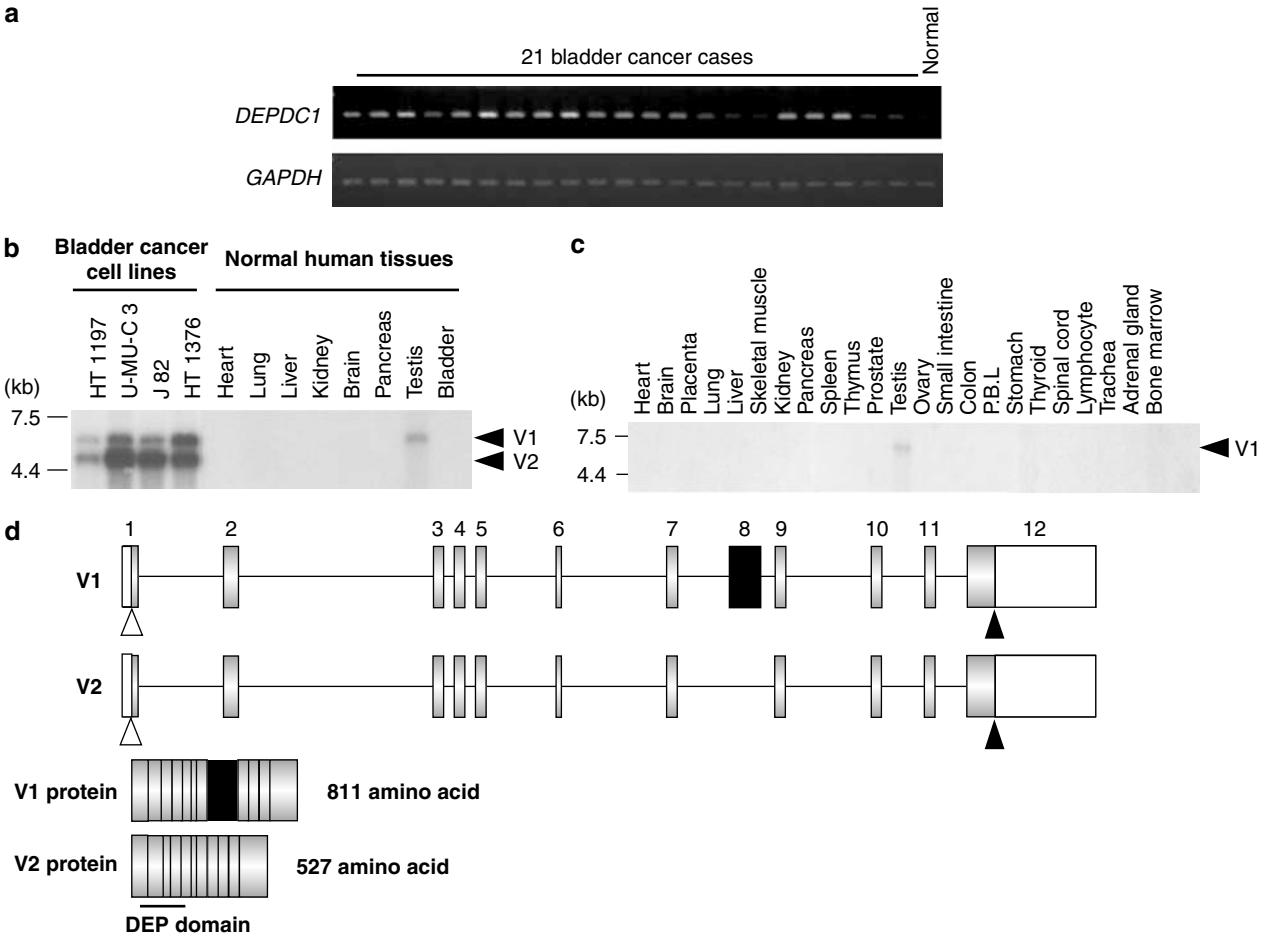

Figure 1 Expression of *DEPDC1* in bladder cancers and normal tissues. (**a**) Expression of *DEPDC1* in tumor cells from 21 microdissected bladder cancer samples (Nos. 1001, 1009, 1010, 1012, 1013, 1014, 1015, 1016, 1017, 1018, 1019, 1020, 1021, 1022, 1023, 1024, 2003, 2014, 3001, 5001 and 5002) and microdissected normal human bladder epithelial cells (Normal) examined by semiquantitative RT–PCR. Microdissection of bladder cancer cells was performed as described previously (Takata *et al*., 2005). We prepared total RNA extracted from each of microdissected bladder clinical samples and normal human bladder epithelial cells using RNeasy Micro Kits (Qiagen, Valencia, CA, USA). Subsequently, we performed T7-based amplification and RT as described previously (Takata *et al*., 2005). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR by monitoring an amount of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a quantitative control. The sequences of each set of primer were as follows; *DEPDC1*, 5′-GCTACAAGTAAAGAGGGGATGG-3′ and 5′-GGACAGAAAGGTAAGTCAGTGGG-3′ (the common region for both DEPCD1-V1 and -V2 transcripts); *GAPDH*, 5′-CGACCACTTTGTCAAGCTCA-3′ and 5′-GGTTGAGCACAGGG TACTT TATT-3′. (**b**) Northern blot analysis of the *DEPDC1* transcripts in four bladder cancer cell lines (HT1197, UM-UC-3, J82 and HT1376) and normal human organs (heart, lung, liver, kidney, brain, pancreas, testis and bladder). Human bladder cancer cell lines, HT-1197, UM-UC-3, J82, HT-1376, T-24 and 5637 were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). All of bladder cancer cell lines were grown in monolayer in appropriate medium; that is EMEM (Sigma, St Louis, MO, USA) with 0.1 mM essential amino acid (Roche, Basel, Switzerland), 1 mM sodium pyruvate (Roche) for HT-1197, UM-UC-3, J82 and HT-1376; McCoy's 5a (Sigma) for T-24; RPMI-1640 (Sigma) for 5637. Each medium was supplemented with 10% fetal bovine serum (Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma). Cells were maintained at 37°C in atmosphere with 5% CO_2. (**c**) Northern blot analysis of the *DEPDC1* transcript in various human tissues. PBL indicates peripheral blood leukocyte. Northern blot using bladder cancer cell lines was prepared according to the procedures of our previous report (Park *et al*., 2006). Bladder cancer northern blots and human multiple-tissue blots (Takara Clontech, Kyoto, Japan) were hybridized with [α^{32}P]-dCTP-labeled PCR product of *DEPDC1* (GeneBank Accession AB281274) prepared by RT–PCR using the following primer sets; 5′-AGGCAGGCAACTTTCATTTG-3′ and 5′-GGACAGAAAGGTAAGTCAGTGGG-3′. Prehybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at −80°C for 14 days. (d) Genomic and protein structures of two variants of *DEPDC1* (*DEPDC1-V1* and *-V2*). Gray boxes indicate coding regions, and white boxes indicate non-coding regions. White triangles indicate positions of initiation codons and black triangles indicate stop codons. The number above each box indicates the exon number. Black box indicates *DEPDC1-V1*-specific exon 8 (852 bp). DEP domain is indicated by underline (16–104 amino acids).

no. 1 moderately and -V1 no. 2 remarkably suppressed the amount of the V1 transcript, and resulted in growth suppression of UM-UC-3 cells, concordantly to their knockdown effect (Figure 4d). Although we were unable to knockdown the V2 transcript alone, our results clearly indicated that *DEPDC1* has a significant function in the cell growth of bladder cancer cells. Furthermore, to investigate the involvement of apoptosis

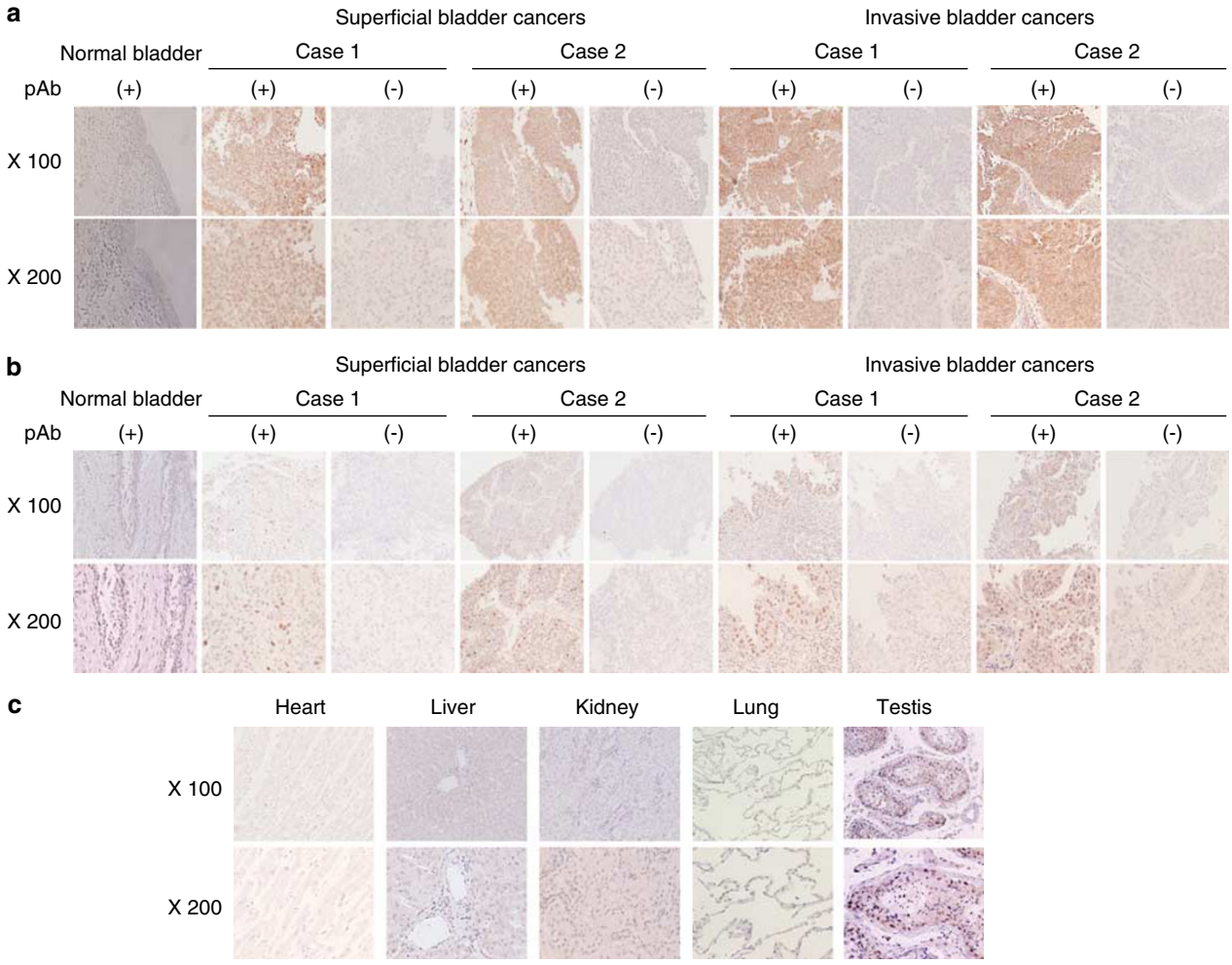

Figure 2 Expression of DEPDC1 in bladder cancer and normal human tissue sections. Expression of DEPDC1-V1 protein in surgically resected bladder cancer tissues (two superficial bladder cancer and two invasive bladder cancer cases) and normal bladder tissue sections by immunohistochemical staining using (**a**) affinity-purified anti-DEPDC1-V1 polyclonal antibody (+) or no primary antibody (–) and (**b**) affinity-purified anti-DEPDC1 polyclonal antibody (+) or no primary antibody (–) (upper panels, × 100; lower panels, × 200). (**c**) Expression of DEPDC1 protein that was detected by affinity-purified anti-DEPDC1 polyclonal antibody in normal human tissue sections (heart, liver, kidney, lung and testis) (upper panels, × 100; lower panels, × 200). To generate the recombinant proteins as immunogens, the plasmids expressing a common region of two variants of DEPDC1 (621–811 amino acids of DEPDC1-V1) as well as a DEPDC1-V1-specific fragment (303–588 amino acids of DEPDC1-V1), both of which contained His-tagged epitopes at their COOH-terminus, were cloned into pET21 vector (Novagen, Madison, WI, USA), respectively. These recombinant peptides were expressed in *Escherichia coli*, BL21 codon-plus (Stratagene, La Jolla, CA, USA), and purified using Ni-NTA resin (Qiagen, Valencia, CA, USA) according to the supplier's protocol. To remove *E. coli*'s proteins as contaminations, each DEPDC1 fragment protein was cut from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, and extracted by using electro-eluter (Bio-Rad, Hercules, CA, USA). The extracted proteins were inoculated into rabbits, and subsequently the immune sera were purified on antigen affinity columns using Affigel 15 gel (Bio-Rad), according to the supplier's instructions.

in DEPDC1-depleted cells, we performed fluorescence-activated cell sorting (FACS) analysis using UM-UC-3 cells to measure the proportions of apoptotic cell population. The results showed the significant increase in the population of apoptotic (sub-G1) cells by DEPDC1-siRNA (31.48%), compared with siEGFP (18.27%) and si-DEPDC1 mismatch (15.68%) as controls, indicating the inhibition of DEPDC1 expression induced apoptosis (Figure 4e).

Gene-expression profile analysis obtained by means of cDNA microarray has shown its great potential to effectively generate comprehensive information for screening of novel molecular targets that are applicable for development of novel therapeutic drugs (Sternberg, 1995). In conventional drug screening approaches, the great majority of compounds that enter into clinical trials fail in development due to the adverse reactions or the insufficient efficacy. To reduce the failure risk during drug-development processes, selection of target molecules that are applicable for screening of small molecular compounds, therapeutic antibodies or peptide vaccines are critically important. In this regard, we have established the genome-wide gene expression profiles of cancer cells as well as 29 normal human tissues, and have been taking a strategy to select genes that are frequently overexpressed in cancer cells, but not

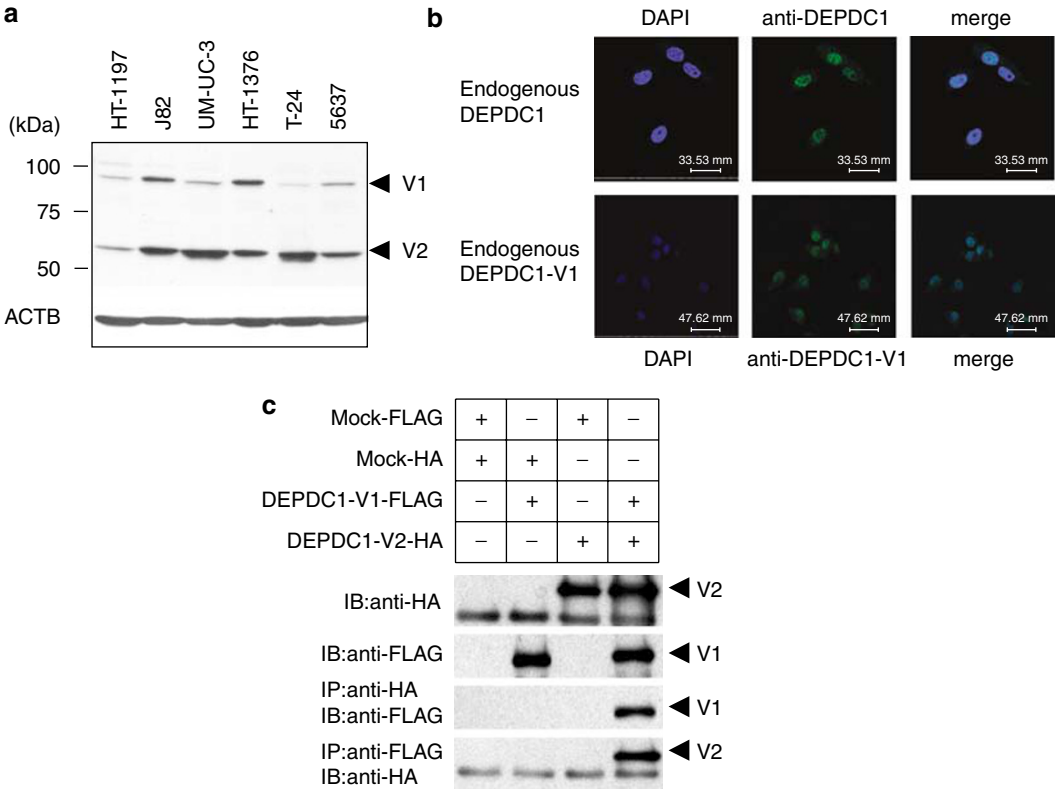

Figure 3 Subcellular localization of DEPDC1 protein in bladder cancer cells. (**a**) Expression of endogenous DEPDC1 protein in six bladder cancer cell lines (HT-1197, J82, UM-UC-3, HT1376, T-24 and 5637) by western blotting analysis using anti-DEPDC1 antibody, which can recognize both variant proteins. Cells was extracted with lysis buffer (50 mM Tris-HCL (pH 8.0)/150 mM NaCL/0.5% NP-40) including 0.1 % protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). The amount of total protein was estimated by protein assay kit (Bio-Rad), and then proteins were mixed with SDS-sample buffer and boiled for 3 min before loading at 10% SDS–PAGE gel. After electrophoresis, the proteins were blotted onto nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). After blocking with 4% BlockAce (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan), membranes were incubated with anti-DEPDC1 polyclonal antibody for detection of the endogenous DEPDC1 protein. Finally, the membrane was incubated with HRP conjugated-secondary antibody, and bands were visualized by ECL detection reagents (GE Healthcare). Expression of ACTB served as a quantity control. (**b**) UM-UC-3 cells were immunohistochemically stained with the affinity-purified anti-DEPDC1 (upper panels) or DEPDC1-V1 (lower panels) polyclonal antibody (green). Twenty-four hours after seeding, cells were fixed with phosphate-buffered saline (PBS) (–) containing 4% paraformaldehyde, and then rendered permeable with PBS (–) containing 0.1% Triton X-100 for 2 min at room temperature. Subsequently, the cells were covered with 3% BSA in PBS (–) for 12 h at 4°C to block nonspecific hybridization. Then, cells were incubated with affinity-purified anti-DEPDC1 or DEPDC1-V1-specific polyclonal antibodies diluted 1:100 in the blocking solution. After washing with PBS (–), the cells were stained by an Alexa488-conjugated anti-rabbit secondary antibody (Molecular Probe, Eugene, OR, USA) at 1:1000 dilutions. Nuclei were counter-stained with 4′, 6′-diamidine-2′-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained under a TCS SP2 AOBS microscope (Leica, Tokyo, Japan). (**c**) Heterodimerization of two variants of DEPDC1 protein. COS-7 cells were transiently co-transfected with FLAG-tagged DEPDC1-V1 (DEPDC1-V1-FLAG) and HA-tagged DEPDC1-V2 (DEPDC1-V2-HA) or both together. The cells were harvested 48 h after the transfection, and then were lysed in lysis buffer (50 mM Tris-HCL (pH 8.0), 150 mM NaCL, 0.5% NP-40 and Protease Inhibitor Cocktail Set III (Calbiochem). Equal amounts of total proteins were incubated at 4°C for 1 h with either a rat anti-HA antibody (Roche) or a mouse anti-FLAG antibody (Santa Cruz, CA, USA). Immunocomplexes were incubated with protein G-Sepharose (Zymed Laboratories, South San Francisco, CA, USA) for 1 h at 4°C. After washing three times with lysis buffer, co-precipitated proteins were separated by SDS–PAGE, and immunoblotted using either a rat anti-HA antibody or a mouse anti-FLAG antibody.

expressed in normal organs. Through the genome-wide expression profile analysis of clinical invasive bladder cancer tissues and normal human tissues (Saito-Hisaminato *et al*., 2002; Takata *et al*., 2005), we identified a novel gene, *DEPDC1* that was highly overexpressed in the majority of clinical bladder cancer samples, but was not expressed in any of normal human organs except the testis. Immunohistochemical staining analysis using anti-DEPDC1 and -V1 polyclonal antibodies clearly indicated that upregulation of DEPDC1-V1 and -V2 expression in bladder cancer cells, but no expression in surrounding normal bladder cells or in normal organs in concordance with the results of northern blot analyses (Figure 2). In addition, we demonstrated that knock-down of endogenous DEPDC1 expression by siRNA resulted in remarkable growth suppression of bladder cancer cell lines (Figure 4). However, introduction of both DEPDC1-V1 and -V2 variants together into NIH3T3 cells could not enhance the growth of the cells (data not shown), we assume that *DEPDC1* is essential for the survival of bladder cancer cells, but *DEPDC1* alone does not have the transforming activity.

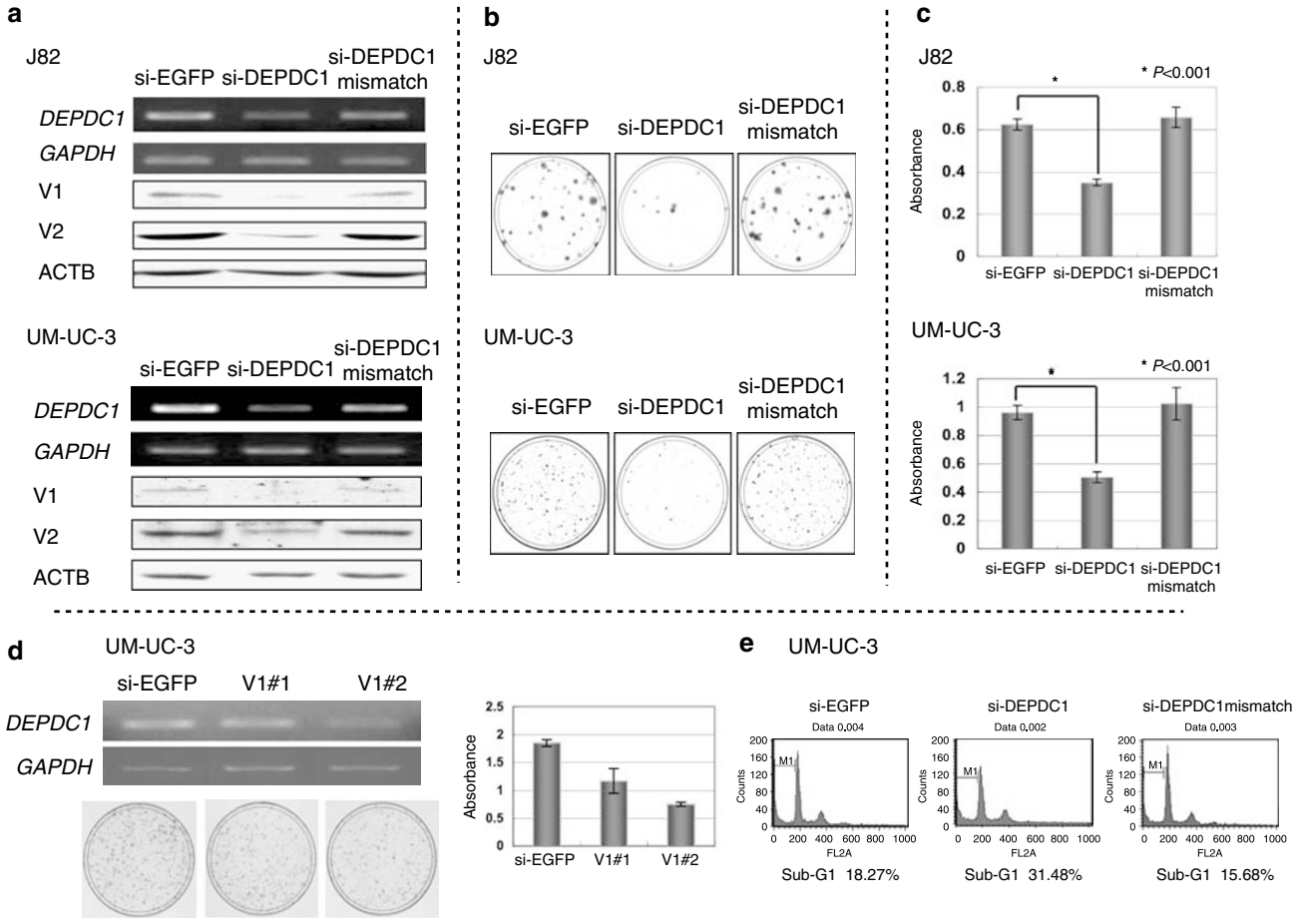

Figure 4 Growth-inhibitory effects of DEPDC1 siRNAs to bladder cancer cells. The plasmids designed to express siRNA to DEPDC1 (common to two variants) or specific to *DEPDC1-V1* were prepared by cloning of double-stranded oligonucleotides into the *Bbs*I site of psiU6BX vectors as described previously (Shimokawa *et al*., 2003). The target sequences of the synthetic oligonucleotides for RNAi were as follows: *EGFP* (enhanced green fluorescent protein gene, a mutant of *Aequorea Victoria* green fluorescent protein) as a control, 5′-GAAGCAGCACGACTTCTTC-3′; si-DEPDC1, 5′-CCAAAGTTCCGTAGTCTAA-3′ for the common sequence of V1 and V2; si-DEPDC1 mismatch, 5′-TCATAGTTCCGTAGTCTAG-3′ (underlines indicate mismatched sequences in si-DEPDC1); si-DEPDC1-V1 no. 1 (V1 no. 1), 5′-CTGAGTGCCTTCTTCTCAG-3′; si-DEPDC1-V1 no. 2 (V1 no. 2), 5′-GGAATTGTAG ATGTGC CAG-3′ for the *DEPDC1-V1*-specific sequence. DNA sequences of all constructs were confirmed by DNA sequencing. Eight-microgram each of siRNA-expression vector were transfected into J82 or UM-UC-3 cells (1×10^6 cells/10 cm-dish), respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or FuGENE6 transfection reagent (Roche) according to the supplier's recommendations. (**a**) Semi-quantitative RT–PCR (first and second panels) and western blot analyses (third, fourth and fifth panels) showing suppression of endogenous expression of *DEPDC1* by DEPDC1-specific siRNAs (si-DEPDC1) in bladder cancer cell lines, J82 (upper panels) and UM-UC-3 (lower panels) cells. Total RNAs and total cell lysates were extracted from the transfected cells after 4-day and 6-day incubation with neomycin (Geneticin, Invitrogen), respectively. The specific primer sets for RT–PCR are as follows; 5′-GCTACAAGTAAAGAGGGGATGG-3′ and 5′-GGACAGAAAGGTAAGTCAGTGGG-3′ for detection of both *DEPDC-V1* and *-V2* expression; 5′-AGCACAAGTGTGCAAACAGC-3′ and 5′-TTTGGTGGGGGAAGTAACAA-3′ for detection of V1 specific expression; 5′-CGACCACTTTGTCAAGCTCA-3′ and 5′-GGTTGAGCACAGGGTACTTTATT-3′ for *GAPDH* as a quantitative control. Expression of *GAPDH* and ACTB served as quantity controls at transcriptional and protein levels, respectively. (**b**) Colony-formation assays of J82 (upper panels) and UM-UC-3 (lower panels) cells transfected with plasmids expressing a control siRNA (si-EGFP), si-DEPDC1 or si-DEPDC1 mismatch. The transfected J82 or UM-UC-3 cells were cultured for 28 or 21 days in the presence of 0.6 or 1.0 mg/ml of neomycin, and the number of colonies were counted by Giemsa staining. These experiments were performed in triplicate. (**c**) Viability of J82 (left panels) and UM-UC-3 (right panels) cells evaluated by MTT assays at 28 or 14 days after the treatment of neomycin with cell counting kit-8 according to the manufacturer's recommendation (Wako, Osaka, Japan). Absorbance at 570 nm wavelength was measured with a Microplate Reader 550 (Bio-Rad). These experiments were performed in triplicate. Statistical significance was determined by Student's *t*-test, using Statview 5.0 software (SAS Institute, Cary, NC, USA). A difference of $P < 0.05$ was considered to be statistically significant. (**d**) DEPDC1-V1-specific-siRNAs (V1 no. 1 and V1 no. 2) designed to knock down expression of *DEPDC1-V1* at transcriptional level in UM-UC-3 cells by semi quantitative RT–PCR analysis. Viability of UM-UC-3 cells evaluated by colony-formation and MTT assays. (**e**) FACS analysis showing an increase of the apoptotic-cell population (represented by sub-G1 percentage) through the suppression of endogenous expression of DEPDC1 in UM-UC-3 bladder cancer cells. UM-UC-3 cells were harvested after 4-day incubation in selective media containing 1.0 mg/ml of neomycin. Cells were collected and fixed with 70% ethanol, and maintained at 4°C before use. Cells were incubated with 10 mg/ml RNaseI in PBS (–) at 37°C for 30 min and stained with 50 μg of propidium iodide (PI) at room temperature for 30 min. Cell suspensions were analysed for DNA content by flow cytometer (FACS calibur; Becton Dickinson, San Diego, CA, USA). The data were analysed by CELLQuest software (BD Biosciences, San Jose, CA, USA). Assays were performed in triplicate independently.

A number of proteins containing the DEP domain have been identified in many organisms ranging from yeast to humans. The DEP domain is indicated to mediate intracellular signaling through regulation of protein stability (Ponting and Bork, 1996; Martemyanov *et al*., 2003). Mammalian RGSs also contain this domain, and regulate signal transduction by increasing the GTPase activity of G-protein alpha subunits, thereby driving them into their inactive guanosine diphosphate (GDP)-bound form. Recently, it was reported that DEP domain appeared to be involved in membrane association (Boutros *et al*., 1998; Li *et al*., 1999). However, we confirmed by immunocytochemical-staining experiments the localization of DEPDC1-V1 and -V2 to be in nucleus of bladder cancer cells. Hence, although DEPDC1 contains the DEP domain, it may have unique cellular functions different from other DEP-domain molecules reported previously (Ponting and Bork, 1996; Martemyanov *et al*., 2003).

In conclusion, our findings clearly suggested that DEPDC1-V1 and -V2 were overexpressed in bladder cancer cells, and that downregulation of DEPDC1-V1 and -V2 by siRNA treatment significantly suppressed the growth of bladder cancer cells, indicating its crucial role in cell growth of bladder cancer cells. Although further analysis of the function of DEPDC1 will be necessary, the data provided should contribute to more profound understanding of bladder cancer carcinogenesis and to development of novel therapies for bladder cancers.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).