Oncogenic Role of MPHOSPH1, a Cancer-Testis Antigen Specific to Human Bladder Cancer

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Abstract
To disclose the molecular mechanism of bladder cancer, the second most common genitourinary tumor, we had previously done genome-wide expression profile analysis of 26 bladder cancers by means of cDNA microarray representing 27,648 genes. Among genes that were significantly up-regulated in the majority of bladder cancers, we here report identification of M-phase phosphoprotein 1 (MPHOSPH1) as a candidate molecule for drug development for bladder cancer. Northern blot analyses using mRNAs of normal human organs and cancer cell lines indicated this molecule to be a novel cancer-testis antigen. Introduction of MPHOSPH1 into NIH3T3 cells significantly enhanced cell growth at in vitro and in vivo conditions. We subsequently found an interaction between MPHOSPH1 and protein regulator of cytokinesis 1 (PRC1), which was also up-regulated in bladder cancer cells. Immunocytochemical analysis revealed colocalization of endogenous MPHOSPH1 and PRC1 proteins in bladder cancer cells. Interestingly, knockdown of either MPHOSPH1 or PRC1 expression with specific small interfering RNAs caused a significant increase of multinuclear cells and subsequent cell death of bladder cancer cells. Our results imply that the MPHOSPH1/PRC1 complex is likely to play a crucial role in bladder carcinogenesis and that inhibition of the MPHOSPH1/PRC1 expression or their interaction should be novel therapeutic targets for bladder cancers. [Cancer Res 2007;67(7):3276–85]

Introduction
Bladder cancer is the second most common genitourinary tumor, having an incidence of ~357,000 new cases each year worldwide (1). Approximately one third of them are suspected to be invasive or metastatic disease at the time of diagnosis (1–3). Although radical cystectomy is considered as the gold standard for treatment of patients with localized but muscle-invasive bladder cancer, nearly half of such patients develop metastases within 2 years after cystectomy and subsequently die of the disease.

Neoadjuvant chemotherapy is usually applied to patients with muscle-invasive bladder cancer for management of micrometastasis and improvement of their prognosis (4, 5). The MVAC regimen, a combination of methotrexate, vinblastine, doxorubicin, and cisplatin, before radical cystectomy has been shown to improve prognosis of patients, compared with cystectomy alone (6). Downstaging by neoadjuvant chemotherapy was indicated to have significant survival benefits (7, 8), and a small subset of patients may have better quality of life due to preservation of bladder function. However, because the overall prognosis still remains very poor (9), development of a new molecular-target drug(s) for bladder cancer is earnestly desired.

Gene expression profiles obtained by cDNA microarray analysis have been proved 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 provide the basis of personalized treatment (10). Through 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 (11), hepatocellular carcinomas (12), pancreatic cancer (13, 14), prostate cancers (15, 16), synovial sarcomas (17, 18), and renal cell carcinomas (19). Such molecules are considered to be good candidate targets for development of new therapeutic modalities.

Toward identification of molecular targets for drug development, we had analyzed the gene expression profiles of 26 bladder cancers and 29 normal human tissues (20, 21). Among the up-regulated genes in bladder cancers, we focused on M-phase phosphoprotein 1 (MPHOSPH1), which was highly overexpressed in the great majority of bladder cancer cells examined but not expressed in normal human organs except testis. Furthermore, we identified the interaction of MPHOSPH1 with protein regulator of cytokinesis 1 (PRC1), the expression of which was also up-regulated in bladder cancers. We also found that suppression of either of MPHOSPH1 or PRC1 expression using their specific small interfering RNAs (siRNA) leads to the formation of multinuclear cells and subsequent cell death. These findings suggest that the MPHOSPH1/PRC1 pathway is involved in bladder carcinogenesis. Taken together, we here propose that MPHOSPH1 may be a promising molecular target for bladder cancer therapy through inhibition of the MPHOSPH1/PRC1 expression or their interaction and/or cancer vaccine-mediated immunotherapy.

Materials and Methods
Bladder cancer cell lines and tissue samples. Human bladder cancer cell lines, HT-1197, UM-UC-3, J82, HT-1376, SW780, and RT4, were purchased from American Type Culture Collection (Rockville, MD). All of bladder cancer cell lines, as well as COS-7 and NIH 3T3 cells, were grown in monolayer in appropriate medium [i.e., EMEM (Sigma, St. Louis, MO) with 0.1 mmol/L essential amino acid (Roche, Basel, Switzerland), 1 mmol/L sodium pyruvate (Roche), for HT-1197, UM-UC-3, J82, and HT-1376; L-15 for SW780; McCoy’s 5A (Sigma) for RT4; and DMEM (Invitrogen, Carlsbad, CA) for COS-7 and NIH3T3]. Each medium was supplemented with 10% fetal...
bovine serum (Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma). SW780 was maintained at 37°C in atmosphere without CO₂ although the remaining cells were maintained at 37°C in atmosphere with 5% CO₂. Tissue samples from surgically resected invasive or superficial bladder cancers and their corresponding clinical information were obtained from five hospitals (Kochi Medical School, Kyoto Prefectural University of Medicine, Nagoya City University Graduate School of Medical Sciences, Kanazawa University Graduate School of Medical Sciences, and Iwate Medical University) with written informed consent.

**Semiquantitative reverse transcription-PCR.** Microdissection of bladder cancer cells was done as previously described (20). We extracted total RNA from each of microdissected bladder clinical samples and cultured cells from Takara Clontech (Kyoto, Japan). Subsequently, we did T7-based amplification and reverse transcription as previously described (22). 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 primers were as follows: MPHOSPH1, 5′-CC-GGGAAGTAAACTGACTCAC-3′ and 5′-TCTTAGCTCCATCAACAAAATC-CT-3′; PRC1, 5′-GTGGTCTCAGGAGACTTTGGTTTT-3′ and 5′-TACATCGATA-CCCCAAACA-3′; and GAPDH, 5′-CGACCACCTCTGCAAGGCTCA-3′ and 5′-GGTGA GGCGGGGTACTTTATT-3′.

**Northern blot analysis.** Northern blot for bladder cancer cell lines was prepared according to the procedures of our previous report (11). Human multiple-tissue blots (Takara Clontech) and bladder cancer Northern blots were hybridized with [α-32P]dCTP–labeled MPHOSPH1 PCR products (542 bp) of MPHOSPH1 cDNA (GenBank accession no. NM_016195) prepared by reverse transcription-PCR (RT-PCR) using the following primer set: 5′-TGCTGGTTCAGAACGAACTATG-3′ and 5′-TCCTCGTGGCTAATGAAAGC-3′. Prehybridization, hybridization, and washing were done according to the supplier’s recommendations. The blots were autoradiographed with intensifying screens at ~80°C for 14 days.

**Construction of the expression vectors.** We obtained an open reading frame sequence of MPHOSPH1 by RT-PCR using human testis mRNA as a template.

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![Figure 1](https://example.com/figure1.png)

**Figure 1.** Expression of MPHOSPH1 in bladder cancer and normal tissues. A, expression of MPHOSPH1 in tumor cells from 10 bladder cancer cases and normal human tissues (Bladder; microdissected normal bladder transitional cells, heart, lung, liver, and kidney) examined by semiquantitative RT-PCR. Expression of GAPDH served as a quantity control. B, Northern blot analysis of the MPHOSPH1 transcript in bladder cancer cell lines (HT-1197, UM-UC-3, J82, HT-1376, SW780, and RT4) and normal human organs (heart, lung, liver, kidney, brain, pancreas, testis, and bladder). Arrowhead, MPHOSPH1 transcript. C, Northern blot analysis of the MPHOSPH1 transcript in various normal human tissues. MPHOSPH1 was exclusively expressed in testis. P.B.L., peripheral blood leukocytes. Arrowhead, MPHOSPH1 transcript. D, specificity of purified anti-MPHOSPH1 polyclonal antibody, indicated by expression of exogenous HA-tagged and endogenous MPHOSPH1 in COS-7 cells and bladder cancer cell line UM-UC-3, respectively. The exogenous HA-tagged MPHOSPH1 lane contains lysate of cells transfected with pCAGGS-MPHOSPH1-HA (MPHOSPH1-HA). Arrow, an ~220-kDa MPHOSPH1 protein. E, expression of MPHOSPH1 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 with affinity-purified anti-MPHOSPH1 polyclonal antibody. These immunohistochemical staining results were well correlated with those of RT-PCR (A). Original magnifications, ×100 (top) and ×200 (bottom).
template and KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) with the primer set as follows: forward, 5'-ATAAAGATGCGGCGCAATGGAATCT-AATTTTAACTCAAGAGG-3'; reverse, 5'-ATAAAGATGCGGCGCTTTGATGC-GTCATTTTTGCTAGA-3' (underlines indicate NotI restriction enzyme site). The PCR product was cloned into the NotI site of pCAGGSnhc expression vector in frame with hemagglutinin (HA) tag at the COOH terminus.

For construction of the PRC1 expression vector, the entire coding region of PRC1 cDNA (GenBank accession no. NM_003981) was gene- rated by RT-PCR using a human testis mRNA as the template and the following set of primers: forward, 5'-CCGGAAATTCCTCCGCCATGAGGAGAGAAGTGA-3' (underline indicates EcoRI site); reverse, 5'-TTGCCGGCTCGAGGGACTGGATTTGTGTGCTAA-3' (underline indicates XhoI site). The PCR product was cloned into the EcoRI and XhoI sites of pCAGGSnhc vector in frame with NH2-terminal FLAG tag. DNA sequences of all constructs were confirmed by DNA sequencing (ABI3700, PE Applied Biosystems, Foster, CA).

Anti-MPHOSPH1 specific polyclonal antibodies. Plasmid designed to express a part of MPHOSPH1 (1,612–1,780 amino acids) with His tag at its COOH terminus was prepared using pET21 vector (Merck, Novagen, Madison, WI). The recombinant peptide was expressed in Escherichia coli, Bl21 codon-plus strain (Stratagene, La Jolla, CA), and purified using both Ni-NTA resin agarose (Qiagen) and TALON (Takara Clontech) according to the supplier's protocols. Subsequently, the immune sera were purified on antigen affinity columns using Affigel-15 gel (Bio-Rad, Hercules, CA) according to the supplier's instructions. We confirmed that this antibody could specifically recognize endogenous MPHOSPH1 protein in UM-UC-3 bladder cancer cells by Western blot analysis. Affinity-purified anti-MPHOSPH1 antibodies were used for Western blot, immunoprecipitation, immunohistochemical analysis, and immunocytochemical analysis as described below.

Immunohistochemical staining analysis. Conventional paraffin-embedded tissue sections from bladder cancers were obtained from surgical specimens. To investigate the expression of MPHOSPH1 protein in clinical materials, tissue sections were processed for antigen retrieval by autoclave (108°C, 15 min) in antigen retrieval solution, high pH (DakoCyto- mation, Carpinteria, CA), and treated with peroxidase blocking reagent (DakoCyto- mation). Tissue sections were incubated with anti-MPHOSPH1 polyclonal antibody at 1:80 dilution, followed by horseradish peroxidase–conjugated secondary antibody (DakoCyto- mation). Antigen was visualized with substrate–chromogen (Dako liquid 3,3-diaminobenzidine chromogen, DakoCyto- mation). Finally, tissue sections were stained with hematoxylin to discriminate nucleus from cytoplasm.

Immunocytochemical staining analysis. UM-UC-3 cells were seeded at 1 × 10^4 per well (Lab-Tek II chamber slide, Nalgen Nunc, International, Naperville, IL). After 24 h, cells were fixed with 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% bovine serum albumin in PBS(−) for 12 h at 4°C to block nonspecific hybridization followed by incubation with affinity-purified anti-MPHOSPH1 polyclonal antibody diluted 1:100 in blocking solution. After washing with PBS(−), the cells were stained with an Alexa 488–conjugated antirabbit secondary antibody (Molecular Probes) at 1:1,000 dilution. Nuclei were counterstained with 4'6-diamidino-2-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained under a TCS SP2 AOBS microscope (Leica, Tokyo, Japan).

Gene silencing effect by siRNA. We had previously established a vector-based RNA interference system that was designed to synthesize siRNAs in mammalian cells (23). Plasmids designed to express siRNA were prepared by cloning of double-stranded oligonucleotides into the BbsI site of psiU6BX vectors. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: si-EGFP as a control, 5'-GTGGTCCTAGGAGACTTGGTTTT-3' and 5'-TACATGCATACCCCA-CAA-3'; and, as a quantitative control, GAPDH, 5'-CGACCTATTTTG-CAAGCTCA-3' and 5'-GGTTGAGCAGGTTACTTATT-3'. 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 neomycin, and the numbers of colonies were counted by Giemsa staining. Viability of the j82 or UM-UC-3 cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 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 (model 550, Bio-Rad). These experiments were done in triplicate.

Establishment of NIH 3T3 cells stably expressing MPHOSPH1. HA-tagged MPHOSPH1 expression vector (pCAGGSnhc-MPHOSPH1) or mock
vector (pCAGGSnHC) was transfected into NIH3T3 cells using FuGENE6 transfection reagent (Roche). Transfected cells were incubated in the culture medium containing 0.9 mg/mL neomycin (Geneticin, Invitrogen). Three weeks later, 20 individual colonies were selected by limiting dilution and screened for clones stably expressing MPHOSPH1. The expression level of HA-tagged MPHOSPH1 was detected in each clone by Western blot and immunohistochemical staining analyses with anti-HA monoclonal antibody (Sigma). We established three independent clones and designated them as follows: NIH3T3-MPHOSPH1-1, -2, and -3, and NIH3T3-Mock-1, -2, and -3.

**Effect of MPHOSPH1 on cell growth in vitro and in vivo.** To investigate growth-promoting effect of MPHOSPH1 in vitro, we seeded each of three independent NIH 3T3-MPHOSPH1 cells (MPHOSPH1-1, -2, and -3) and three independent NIH 3T3-Mock cells (Mock-1, -2, and -3; 0.5 × 10⁴) and maintained them in media containing 10% FCS with 0.9 mg/mL neomycin for 24, 48, 72, 96, or 120 h. At each time point, cell numbers were evaluated by using cell counting kit-8 (Wako). These experiments were done in triplicate.

**In vivo** experiments were done in our animal facility in accordance with institutional guidelines. To further examine tumor growth effect of MPHOSPH1 in nude mice, cells transfected with MPHOSPH1-1 or Mock-1 (5 × 10⁶) were injected s.c. into the posterior mid-dorsum of 16 BALB/cA Jcl-nu mice (female, 7 weeks old). Tumor volumes were measured every 3 days for 3 weeks, and volumes were estimated by the following formula: \( V = 0.5 \times \text{(larger diameter)} \times \text{(smaller diameter)}^2 \), as previously described (18). Mean tumor volume ± SD was plotted. Subsequently, the mice were euthanized at 21 days, and the tumors were dissected. To confirm the expression of MPHOSPH1 protein in tumor, Western blot analysis with anti-MPHOSPH1 antibody was done.

**Statistical analysis.** Statistical significance was determined by Student’s \( t \) test using Statview 5.0 software (SAS Institute, Cary, NC). \( P < 0.05 \) was considered to be statistically significant.

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**Figure 3.** Growth inhibitory effects on bladder cancer cell lines J82 and UM-UC-3 by MPHOSPH1 siRNA. A, knockdown effect of MPHOSPH1 expression by siRNAs was confirmed by semiquantitative RT-PCR (top) and Western blot (bottom) analyses. Expression of GAPDH and ACTB served as a quantity control at transcriptional and protein levels, respectively. B, colony formation assay of J82 and UM-UC-3 cells transfected with plasmids expressing MPHOSPH1 siRNA (si-MPHOSPH1), a control siRNA (si-EGFP), or si-MPHOSPH1 mismatch. C, viability of J82 and UM-UC-3 cells evaluated by MTT assay in response to si-MPHOSPH1 in comparison with that to si-EGFP as a control. J82, \( P < 0.001 \); UM-UC-3, \( P < 0.001 \) (unpaired \( t \) test).
Coimmunoprecipitation and immunoblotting analyses. COS-7 cells were transiently transfected with HA-tagged MPHOSPH1 (pCAGGSnHC-MPHOSPH1), FLAG-tagged PRC1 (pCAGGSn3FC-PRC1), or both together. The cells were harvested 48 h after the transfection and then were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, and Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA)]. Equal amounts of total proteins were incubated at 4°C for 2 h with 2 μg of rat anti-HA (Roche) or mouse anti-FLAG (Sigma) antibodies. Immunocomplexes were incubated with protein G-Sepharose (Zymed Laboratories, South San Francisco, CA) for 2 h at 4°C. After washing with lysis buffer, coprecipitated proteins were separated by SDS-PAGE and immunoblotted with either mouse anti-FLAG (Sigma) or rat anti-HA (Roche) antibodies.

Immunocytochemical staining analysis of MPHOSPH1 and PRC1. UM-UC-3 cells, which endogenously express high level of MPHOSPH1 protein, were transiently transfected with FLAG-tagged PRC1 (pCAGGSn3FC-PRC1). Cultured cells were synchronized in their cell cycle by treatment with aphidicolin (2 μg/mL; Sigma-Aldrich) for 16 h and then washed five times with PBS (−); fresh culture medium was then added to release the cells from the cell cycle arrest. After the synchronized cells were fixed at different cell cycle points as described in a previous section (Immunocytochemical staining analysis), they were incubated with rabbit anti-MPHOSPH1 and mouse anti-FLAG M2 (Sigma) antibodies, washed with PBS (−), and stained with Alexa 594-conjugated antirabbit and Alexa 488-conjugated antirabbit secondary antibodies (Molecular Probes). Fluorescent images were obtained with a TCS SP2 AOBS microscope (Leica).


The knockdown effects of MPHOSPH1 and PRC1 on cell morphology. After transfection of UM-UC-3 cells with si-EGFP (negative control), si-MPHOSPH1, or si-PRC1 constructs as described above, their cellular morphologies were examined. The cells were stained immunocytochemically with Alexa 594 phalloidin (Molecular probes) and DAPI at 4 days after the transfection. Fluorescent images were obtained under a TCS SP2 AOBS microscope (Leica).

Results

Identification of MPHOSPH1 as an up-regulated gene in bladder cancers. To screen molecules that could be applicable as targets for development of novel therapeutic drugs, we previously carried out genome-wide expression profile analysis of 26 invasive bladder cancers by means of cDNA microarray representing 27,648 genes. The growth-promoting effect of exogenous MPHOSPH1 in NIH3T3 cells. A, Western blot analysis of cells expressing exogenous MPHOSPH1 at high level or those transfected with mock vector. Exogenous introduction of MPHOSPH1 expression was validated with anti–HA tag monoclonal antibody. ACTB served as a loading control. B, in vitro growth of NIH3T3-MPHOSPH1 cells. The growth measurement by MTT assay shows that three NIH3T3-MPHOSPH1 clones (MPHOSPH1-1, -2, and -3) grew faster than three of NIH3T3-Mock clones (Mock-1, -2, and -3). C, overexpression of MPHOSPH1 caused loss of contact inhibition. NIH3T3-MPHOSPH1 formed multilayer (top) whereas NIH3T3-Mock did not (bottom). D, in vivo tumor growth assay of NIH3T3-MPHOSPH1 cells. Tumor growth curves in nude mice after inoculation of NIH3T3 derivative cells transfected with MPHOSPH1-1 (NIH3T3-MPHOSPH1-1) or Mock-1 (NIH3T3-Mock-1). Points, mean of the tumor volumes in each stable clone–transplanted mouse (n = 16); bars, SD. E, confirmation of expression of MPHOSPH1 protein in NIH3T3-MPHOSPH1-1 tumor. The tumors were dissected from NIH3T3-MPHOSPH1-1 mice and Western blot analysis with anti-MPHOSPH1 antibody was done. Expression of ACTB served as a quantity control of protein.
genes or expressed sequence tags (20). Among up-regulated genes, we in this study focused on MPHOSPH1 gene whose expression was significantly increased in the majority of bladder cancers. Semiquantitative RT-PCR experiments confirmed elevated expression of MPHOSPH1 in 9 of 10 clinical bladder cancers (Fig. 1A). Subsequent Northern blot analysis confirmed that an ~7 kb of MPHOSPH1 transcript was up-regulated in all of six bladder cancer cell lines (Fig. 1B), although its expression was hardly detectable in any normal organs except testis (Fig. 1C). We then developed a polyclonal antibody against MPHOSPH1 to investigate expression of this protein in bladder cancer tissues (see Materials and Methods). We first confirmed that the purified MPHOSPH1-specific polyclonal antibody could recognize endogenous MPHOSPH1 (~220 kDa) protein in bladder cancer cell line UM-UC-3 without producing any nonspecific bands (Fig. 1D). Immunohistochemical analysis with affinity-purified anti-MPHOSPH1 polyclonal antibody revealed positive staining in bladder cancer cells of invasive bladder cancer tissue sections, whereas no staining was observed in normal bladder (Fig. 1E). We also found up-regulated expression of MPHOSPH1 in bladder cancers at an earlier stage (Fig. 1E, superficial bladder cancer). Finally, we observed strong staining in 17 of 20 invasive types and 21 of 25 superficial types of bladder cancer (data not shown), consistent with the results obtained by RT-PCR. However, no difference in the intensity or other relevant findings between superficial and invasive bladder cancer tissues was observed by the immunohistochemical staining.

**Subcellular localization of MPHOSPH1 in bladder cancer cells.** To further characterize the biological role of MPHOSPH1, we examined the subcellular localization of endogenous MPHOSPH1 in UM-UC-3 cells by immunocytochemical analysis with an anti-MPHOSPH1 antibody. The endogenous MPHOSPH1 was mainly localized in the nucleus of interphase cells but was observed diffusely within M-phase cells (data not shown). Because the immunocytochemical analysis suggested the cell cycle-dependent localization of MPHOSPH1 protein, we synchronized UM-UC-3 cells using aphidicolin and then examined localization of MPHOSPH1 at different cell cycle points. As shown in Fig. 2, endogenous MPHOSPH1 protein was localized in the nucleus at the interphase. After disappearance of nuclear membrane, it was present diffusely in cells at prophase, metaphase, and early anaphase. Subsequently, it accumulated at the midzone of cells in late anaphase and was finally concentrated at the contractile ring when cells were at telophase. These findings suggest an important role of MPHOSPH1 in cytokinesis in bladder cancer cells.

**Oncogenic activity of MPHOSPH1.** We knocked down the expression of endogenous MPHOSPH1 in bladder cancer cell lines J82 and UM-UC-3, which showed high level of MPHOSPH1 expression, by means of the mammalian vector–based RNA interference technique. We examined the expression levels of MPHOSPH1 by semiquantitative RT-PCR and Western blot analyses and found that a MPHOSPH1-specific siRNA (si-MPHOSPH1) significantly suppressed expression of MPHOSPH1 at both mRNA and protein levels as compared with a control siRNA construct (si-EGFP; Fig. 3A). As concordant to the knockdown effect, colony formation and MTT assays revealed that introduction of si-MPHOSPH1 significantly suppressed growth of both J82 and UM-UC-3 control cells (MTT assay: J82, P < 0.001; UM-UC-3, P < 0.001 (unpaired t test); Fig. 3B and C). To exclude a possibility of off-target effect by si-MPHOSPH1, we generated siRNA that contained a 3-bp replacement in si-MPHOSPH1 (si-MPHOSPH1 mismatch; see Materials and Methods) and found that this had no suppressive effect on the expression of MPHOSPH1 or the growth of bladder cancer cells (Fig. 3).

To further confirm the growth-promoting effect of MPHOSPH1, we established NIH3T3 derivative cells that stably expressed exogenous MPHOSPH1 (MPHOSPH1-1, -2, and -3). As shown by Western blot analysis with anti-HA-antibody, exogenous MPHOSPH1 protein was observed at high level in these three derivative clones (Fig. 4A). Subsequent MTT assays showed that the three MPHOSPH1-stable derivative cells (MPHOSPH1-1, -2, and -3) grew much faster than those transfected with mock plasmid (Mock-1, -2, and -3; Fig. 4B), suggesting a growth-enhancing effect of MPHOSPH1. In addition to the rapid growth, we observed multilayer growth of these three NIH3T3-MPHOSPH1 cells after they reached the confluence phase, indicating loss of the contact inhibition mechanism by MPHOSPH1 introduction into NIH3T3 cells (Fig. 4C).

To further investigate the oncogenic role of MPHOSPH1 in vivo, we s.c. transplanted either NIH3T3-MPHOSPH1-1 cells or NIH3T3-Mock-1 cells into BALB/c Jcl-nu mice (female, 7 weeks old). As shown in Fig. 4D, none of 16 transplantations of NIH3T3-Mock-1 cells formed tumors by 21 days. In contrast, all of 16 transplantations of NIH3T3-MPHOSPH1-1 formed tumors in which we confirmed overexpression of MPHOSPH1 protein by Western blot analysis (Fig. 4E). These findings imply an oncogenic role of MPHOSPH1 for development of bladder cancer in vivo and in vitro.

**Interaction of MPHOSPH1 with PRC1.** MPHOSPH1 was previously reported to interact with mitotic peptidyl-prolyl isomerase (Pin1) protein in Homo sapiens (24). However, because
the Pin1 expression was undetectable in bladder cancer cells in our microarray data and semiquantitative RT-PCR analysis (data not shown), we attempted to identify protein(s) interacting with MPHOSPH1 in bladder cancer cells. We focused on protein-regulating cytokinesis 1 (PRC1) protein as a possible candidate to interact with MPHOSPH1 because PRC1 is known to be involved to cytokinesis through the interaction with kinesin family proteins (25,26) and its expression pattern was similar to MPHOSPH1 in our expression profile analysis of bladder cancers (20).

We first investigated the expression levels of PRC1 in bladder cancers by semi quantitative RT-PCR analysis and found co-regulation of PRC1 and MPHOSPH1 in bladder cancer cases (Fig. 5A). Subsequently, to confirm the interaction between MPHOSPH1 and PRC1, we did coimmunoprecipitation experiments (see Materials and Methods). HA-tagged MPHOSPH1 (MPHOSPH1-HA) and FLAG-tagged PRC1 (PRC1-FLAG) constructs were cotransfected into COS-7 cells and the cell lysates were immunoprecipitated with anti-Flag or anti-HA antibodies. Immuno blotting of the precipitates with rabbit anti-FLAG and mouse anti-HA antibodies revealed that MPHOSPH1-HA had coprecipitated with PRC1-FLAG protein (Fig. 5B).

Furthermore, we did immunocytochemical analysis of MPHOSPH1 and PRC1 in UM-UC-3 bladder cancer cells. The PRC1-FLAG construct was transfected into UM-UC-3 cells according to the procedure described in Materials and Methods. The results showed that exogenous PRC1 colocalized with endogenous MPHOSPH1 in the cells at interphase, prophase, and late anaphase (Fig. 6A). However, interestingly, in the cells at telophase, MPHOSPH1 was likely to overlap with PRC1 but be located adjacent to PRC1 in the center of midbody (Fig. 6B).

Growth-inhibitory effects of PRC1-specific siRNAs in bladder cancer. To further validate the biological significance of PRC1 in bladder carcinogenesis, we constructed PRC1-specific siRNA expression vectors and examined the knockdown effect of each construct in J82 and UM-UC-3 bladder cancer cell lines, which overexpressed PRC1 and MPHOSPH1 (data not shown). Semi-quantitative RT-PCR showed that si-PRC1 had drastic knockdown effect on PRC1 expression, whereas a si-PRC1 mismatch construct that contained a 4-bp replacement to si-PRC1 or a negative control si-EGFP revealed no knockdown effect (Fig. 7A). Introduction of si-PRC1 into J82 and UM-UC-3 cells resulted in a significant decrease of the number of colonies and of cell viability, whereas control siRNA and a si-PRC1 mismatch had no or little effect on colony number or cell viability (Fig. 7B and C). These findings suggest that PRC1 is also likely to have crucial roles in the growth of bladder cancer cells.

Morphologic changes by treatment with MPHOSPH1 or PRC1 siRNA. Furthermore, we examined morphologic changes of the
UM-UC-3 cells transfected with either MPHOSPH1- or PRC1-specific siRNA that revealed the significant knockdown effect (see Materials and methods). We observed a significant increase of multinuclear cells by introduction of each siRNA (si-MPHOSPH1 and si-PRC1) at 4 days after the transfection (Fig. 7D, top). Immunocytochemical analysis using Alexa 594 phalloidin confirmed the presence of multiple nuclei in the cells transfected with si-MPHOSPH1 or si-PRC1 (Fig. 7D, bottom). These findings indicate that the absence of MPHOSPH1 or PRC1 caused the failure of cytokinesis, resulted in formation of multinucleated cells, and then induced cell death.

**Discussion**

Significant advances in development of molecular targeting drugs for cancer therapy have been achieved in the last two decades. However, the proportion of patients showing good response to
presently available treatments is still very limited and a subset of the patients suffer from severe adverse reactions without any benefit (3). Hence, it is urgent to develop new anticancer agents that are highly specific to malignant cells, with a minimum risk of adverse reactions. Through the detailed expression profile analysis of clinical invasive bladder cancers, we identified MPHOSPH1 to be significantly overexpressed in majority of invasive bladder cancer cases. We here reported the biological significance of MPHOSPH1 and its interacting partner, PRC1, in bladder carcinogenesis. Northern blot analysis revealed the hardly detectable level of MPHOSPH1 expression in any of normal human tissues examined except the testis. Moreover, immunohistochemical experiments with anti-MPHOSPH1 polyclonal antibody clearly indicated MPHOSPH1 expression in both invasive and superficial bladder cancer cells, indicating MPHOSPH1 to be a cancer-testis antigen. These results implied that MPHOSPH1 could serve as a valuable target for development of anticancer agents or cancer peptide vaccines for bladder cancer.

MPHOSPH1 was previously reported as one of proteins that were specifically phosphorylated at the G2-M transition and as a plus-end-directed kinesin-related protein with a crucial role in cytokinesis (24, 27). The MPHOSPH1 cDNA encodes a 1,780-amino-acid protein that was composed of three domains: an NH2-terminal kinesin motor domain, a central coiled-coil stalk domain, and a C-globular tail domain. Our immunocytochemical experiments indicated the localization of MPHOSPH1 to be in the nucleus in the bladder cancer cells at the interphase, in the midzone in late anaphase, and at the contractile ring in telophase. Furthermore, we showed that knockdown of endogenous MPHOSPH1 by siRNAs induced failure of cytokinesis in bladder cancer cells and resulted in accumulation of multinuclear cells and subsequent cell death. In addition, introduction of MPHOSPH1 into NIH3T3 cells significantly enhanced cell growth in vitro and in vivo. Hence, we further examined the biological roles of MPHOSPH1 in bladder cancer cells by identification of its interacting protein(s).

Due to the coexpression in bladder cancers and the similarity in subcellular protein localization, we focused on PRC1 as a candidate to interact with MPHOSPH1. We showed the in vivo interaction and colocalization of MPHOSPH1 and PRC1 during interphase to late anaphase in bladder cancer cells. Interestingly, as shown in Fig. 6B, MPHOSPH1 was observed in the lateral position of PRC1 in the midbody of telephase cells. PRC1 was reported to interact with kinesin family proteins, KIF4 or KIF14, which are also associated with mitotic events, especially with cytokinesis (28–30). It is considered to be a central spindle matrix protein that is essential to control many motor proteins associated with this structure (31). Particularly, KIF4 is known to be the motor protein that translocates PRC1 along the mitotic spindle during mitosis, especially telophase to cytokinesis (28). Moreover, Zhu and Jiang (29) reported that KIF4 interacts with PRC1 in vivo through the KIF4 COOH-terminal stalk plus tail domain, and PRC1 is a downstream cargo of KIF4 on the spindle during mitosis. However, because the expression of those kinesin family proteins in the bladder cancer cells was not detected in our microarray data (20) and MPHOSPH1 bound to PRC1 through its COOH-terminal stalk plus tail domain (data not shown), MPHOSPH1 might function as a motor protein that translocates PRC1 along the mitotic spindles during mitosis of bladder cancer cells.

To assess whether MPHOSPH1 or PRC1 plays a critical role in the growth or survival of bladder cancer cells, we knocked down the expression of endogenous MPHOSPH1 or PRC1 in bladder cancer cell lines, J82 or UM-UC-3, which revealed high expression levels of MPHOSPH1 and PRC1 using specific siRNAs. Each of the specific siRNAs significantly suppressed their expression and resulted in the significant growth suppression of these cells, indicating that both MPHOSPH1 and PRC1 are essential for the growth of bladder cancer cells. Moreover, knockdown of MPHOSPH1 or PRC1 expression in UM-UC-3 bladder cancer cells caused a significant increase of multinuclear cells (Fig. 7D). Because inhibition of their interaction may lead to cell death following the failure of cytokinesis in bladder cancer cells, the inhibitor for their interaction would be a possible valuable target to develop agents against bladder cancer. Although further analysis of the function of MPHOSPH1 will be necessary, the data provided should contribute to a more profound understanding of bladder cancer carcinogenesis and to development of novel therapies for bladder cancers.

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References

overexpressed in prostate cancers, promotes prostate cancer cell growth through phosphorylation of oncoprotein TAF-I/SET. Cancer Res 2005;65:4378–86.


Oncogenic Role of MPHOSPH1, a Cancer-Testis Antigen Specific to Human Bladder Cancer

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