

Ectonucleoside triphosphate diphosphohydrolase 6 expression in testis and testicular cancer and its implication in cisplatin resistance

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Abstract. The development of resistance to cisplatin during treatment of testicular cancer constitutes a major obstacle to the cure of testicular cancer. To resolve its mechanism will provide useful information for making clinical decisions. We found 4 and 15 gene expressions were, respectively, up-regulated and down-regulated in cisplatin-resistant testicular cancer NEC-8/DDP cells compared with their parental NEC-8 cells (about 2.5-fold) using cDNA microarray analysis. After screening, we selected the ENTPD6 among these 19 genes. ENTPD6 was less expressed in cancerous region compared with that in non-cancerous lesion. In addition, ENTPD6 expression in seminoma was higher than that in other testicular tumors. ENTPD6 expression was involved in cellular sensitivity to cisplatin through an interaction with E-cadherin. ENTPD6 is a promising molecular biomarker of cisplatin resistance in testicular cancer, and also a novel therapeutic target modulating cisplatin resistance in testicular cancer.

Introduction

Although testicular cancer is a relatively rare tumor, it is the most common cancer in young males (1). Cisplatin-based combination chemotherapy such as bleomycin, etoposide and cisplatin (BEP) plays an indispensable role in the treatment of testicular cancer (2,3). Concurrently, about 80% of metastatic testicular cancer patients can be cured by chemotherapy and appropriate surgery. However, for those not cured, a method to overcome resistance to anticancer agents is required.

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Ectonucleoside triphosphate diphosphohydrolase (ENTPD) is a family of enzymes that catalyze the hydrolysis of phosphate residues of nucleotides (4). ENTPD family constitutes eight members that localize in the plasma membrane and/or in intracellular organelles with one or two transmembrane domains, and are secreted to extracellular space (5,6). ENTPD family regulates nucleotide-mediated signaling by controlling the rate, amount and timing of nucleotide (e.g. ATP) degradation and ultimately, the nucleoside (e.g. adenosine) formation. Extracellular nucleotides/nucleosides are shown to play a role in several physiological functions including haemostasis vascular and cardiac function (7-9). ENTPD6, a member of ENTPD family shows an intracellular localization such as the endoplasmic reticulum and Golgi apparatus, and the plasma membrane localization and could be secreted by proteolytic cleavage (10-12). However, its characteristics and roles in testis and testicular cancer remain unknown.

In this study, we aimed to identify the diagnostic and therapeutic molecular target of cisplatin resistance in testicular cancer. Then, we performed cDNA microarray analysis using human testicular cancer NEC-8 cells and the corresponding cisplatin-resistant NEC-8/DDP cells, and selected *ENTPD6* as a candidate gene among several genes listed up by cDNA microarray analysis by semi-quantitative RT-PCR and quantitative real-time PCR. Furthermore, we characterized ENTPD6 expression in testis and testicular cancer, and elucidated the mechanism of involvement of ENTPD6 to cisplatin sensitivity.

Materials and methods

Cell culture. Human testicular cancer NEC-8 cells were cultured with RPMI-1640 and human bladder cancer T24 and KK47 cells were cultured with MEM (13). These media were purchased from Invitrogen (San Diego, CA, USA) and supplemented with 10% fetal bovine serum. Cisplatin-resistant T24/DDP10 and KK47/DDP20 cells were established and maintained as described previously (14,15). Cisplatin-resistant derivatives of NEC-8 cells (NEC-8/DDP cells) were established by long-term culture under medium containing gradually increasing concentrations of cisplatin and main-

tained under medium containing 4 $\mu\text{g}/\text{ml}$ of cisplatin. The cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Antibodies and plasmid. Antibodies against ENTPD6 (M03, clone 2D10) and E-cadherin were purchased from Abnova (Taipei, Taiwan) and BD Biosciences (San Jose, CA, USA), respectively. Anti- β -actin antibody was purchased from Sigma (St. Louis, MO, USA). The ENTPD6 expression plasmid (pCMV6-AC-ENTPD6) expressing ENTPD6 protein was purchased from OriGene (Rockville, MD, USA). The shENTPD6 plasmids expressing shRNA against ENTPD6 (shENTPD6 #1, pGFP-V-RS-shENTPD6 GI352789 and shENTPD6 #2, pGFP-V-RS-shENTPD6 GI352791) were purchased from OriGene.

Cytotoxicity analysis. Cytotoxicity analysis was performed as described previously (17). NEC-8 cells (2×10^3) transfected with pCMV6-AC or pCMV6-AC-ENTPD6 plasmid were seeded into 96-well plates. The following day, various concentration of cisplatin was added. After 48 h, the surviving cells were stained with the alamarBlue assay (TREK Diagnostic Systems, Cleveland, OH, USA) for 180 min at 37°C. The absorbances of the wells were measured using a plate reader (ARVO™ MX; Perkin Elmer Inc., Waltham, MA, USA).

cDNA microarray analysis. The procedure of cDNA microarray analysis was performed as described previously (18). Briefly, cDNA generation, hybridization, and data collection for cDNA microarray analyses were performed by the Incyte Corporation (Palo Alto, CA, USA). In brief, alterations in gene expression were evaluated by the reverse transcription of poly-(A) RNAs in the presence of Cy3 or Cy5 fluorescent-labeling dyes followed by hybridization to a UniGEM V 2.0 microarray chip. Each chip displays a total of 9182 elements, of which 8556 are unique gene/EST clusters. These unique gene/EST clusters can be further defined as 8412 annotated and 144 unannotated sequences. A 16-color log scale was used for visual representation and the differential expression (\log_2) was determined for Cy3 signal: the signal of parental cell lines NEC-8 cells vs. Cy5 signal: the signal of resistant cell lines NEC-8/DDP cells as viewed in the Incyte GEM™ Tools software (Incyte Corporation). The subsets of genes were selected for further study based on differential Cy3/Cy5 expression ratios that were ≥ 3 .

Tissues and clinical data. Thirty-one patients with testicular cancer who provided sufficient tissue for the gene expression analysis were included in the study. Written informed consent was obtained from all subjects. The study was approved by the Institutional Review Board of Kyushu University. Immunohistochemical analysis was performed using samples from patients with or without testicular cancer. They underwent orchiectomy at Kyushu University Hospital, Japan. All testicular cancer specimens were completely reviewed to establish histological classification.

RNA isolation and RT-PCR. Total RNA was prepared from cultured cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1.0 μg of total RNA using a Transcriptor First Strand cDNA Synthesis

Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol.

Semi-quantitative RT-PCR analysis. Semi-quantitative RT-PCR was performed as described previously (19). The ENTPD6 forward primer was 5'-ACTCCCACGTTAACCCACGA-3' and the reverse primer were 5'-AAACGCCTTCATCTGTTCC-3'. The reaction conditions had an initial denaturation step for 10 min at 95°C, 35 cycles of 95°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec, and a final extension step at 72°C x 10 min. The PCR products were separated by electrophoresis on 2% agarose gels, which were then stained with ethidium bromide. GAPDH was used as a control for RT-PCR. The GAPDH forward primer was 5'-ACCCAGAAGACTGTGGATGG-3' and the reverse primer was 5'-AGGGGTCTACATGGCAACTG-3'.

Quantitative real-time PCR. Quantitative real-time PCR was performed as described previously (17,20). The synthesized cDNA was diluted 1:2 and 2.0 μl of the diluted sample was used. Quantitative real-time PCR with TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) and TaqMan Gene Expression Master Mix (Applied Biosystems) was performed using an ABI 7900HT System. The expression level of each ENTPD6 gene was corrected by the corresponding GAPDH expression level. The results are representative of at least three independent experiments.

Western blot analysis. Whole-cell extracts were prepared as described previously (17,20). The protein concentrations were quantified using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Whole-cell extracts (30 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ, USA) using a semi-dry blotter. The blotted membranes were sequentially incubated with appropriate primary antibodies for 1 h, and peroxidase-conjugated secondary antibodies for 40 min at room temperature. The bound antibodies were visualized using an ECL Kit (GE Healthcare Bio-Science) and the membranes were exposed to X-OMAT film (Kodak, Tokyo, Japan).

Immunohistochemistry. Immunohistochemical analysis was performed as described previously (21). The primary antibodies used in this study were anti-ENTPD6 antibody (mouse monoclonal, 3 $\mu\text{g}/\text{ml}$ of concentration). Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 20 min.

Transfection. Transfection was performed as described previously (17,20). Briefly, NEC-8 cells were transfected with DNA plasmid using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

Co-immunoprecipitation assay. The co-immunoprecipitation assays were performed as described previously (22). Briefly, NEC-8 cells were lysed with buffer X, centrifuged at 21,000 g for 10 min at 4°C and the supernatants (500 μg) were incubated overnight at 4°C with 2.0 μg of anti-mouse IgG or anti-E-cadherin antibody. Then, immune-complex was incubated for 2 h at 4°C with 20 μl of protein A/G agarose

Table I. The results of cDNA microarray analysis (NEC-8 vs. NEC-8/DDP).

| Gene name | Accession no. | Locus | Differential expression |
|--|---------------|----------------|-------------------------|
| Up-regulated genes in NEC-8/DDP cells | | | |
| Lymphocyte cytosolic protein 1 (L-plastin) | BF035921 | 13q14.3 | -4.1 |
| High density lipoprotein binding protein (vigilin) | NM_005336 | 2q37 | -3.7 |
| Spermidine/spermine N1-acetyltransferase | BF573292 | Xp22.1 | -3.6 |
| Peptidylprolyl isomerase A (cyclophilin A) | AV713597 | 7p13-p11.2 | -3.0 |
| Down-regulated genes in NEC-8/DDP cells | | | |
| Cathepsin F | BE502253 | 11q13 | 9.6 |
| Calbindin 1, (28 kD) | NM_004929 | 8q21.3-q22.1 | 4.8 |
| Hepatocellular carcinoma associated protein; breast cancer associated gene 1 EST | BF969633 | Xp11.4-p11.1 | 4.6 |
| TASP for testis-specific adriamycin sensitivity protein | BE219611 | 7q31.1-7q31.33 | 4.6 |
| <i>Homo sapiens</i> mRNA; cDNA DKFZp434P086 (from clone DKFZp434P086); partial c | AA813471 | | 4.4 |
| Immediate early response 3 | N32077 | 6p21.3 | 3.6 |
| ESTs | AW973290 | | 3.6 |
| Protein phosphatase 1, regulatory (inhibitor) subunit 1A | XM_006813 | 12q13.2 | 3.6 |
| Mal, T-cell differentiation protein | NM_022439 | 2q13 | 3.5 |
| Sjogren syndrome antigen A1 (52 kD, ribonucleoprotein autoantigen SS-A/Ro) | BE897247 | 11p15.5 | 3.4 |
| Interferon induced transmembrane protein 3 (1-8U) | BF699055 | 11p15.5 | 3.2 |
| RAB4, member RAS oncogene family | BC004309 | 1q42-q43 | 3.2 |
| Ectonucleoside triphosphate diphosphohydrolase 6 (putative function) | XM_009435 | 20q11.2 | 3.1 |
| Heat shock 70 kD protein 1B | AF134726 | 6p21.3 | 3.0 |
| Dihydropyrimidinase-like 4 | XM_011864 | 10q26 | 3.0 |

(Santa Cruz Biotechnology). The immunoprecipitated samples were washed three times with buffer X and the pre-immunoprecipitated samples (50 μ g) were subjected to Western blot analysis with the indicated antibodies.

Results

Nineteen genes were identified as differential-expressed genes between NEC-8 and NEC-8/DDP cells by cDNA microarray analysis. We established cisplatin-resistant NEC-8/DDP cells from testicular cancer NEC-8 cells by long-term culture under medium containing gradually increasing concentrations of cisplatin. Then, NEC-8/DDP cells were about 2.5-fold resistant to cisplatin compared with NEC-8 cells (Fig. 1A). To reveal the characteristics of gene expression in NEC-8 and NEC-8/DDP cells, we performed cDNA microarray analysis using NEC-8 and NEC-8/DDP cells. Then, microarray analysis revealed that 4 and 15 gene expressions were up-regulated and down-regulated in NEC-8/DDP cells compared with NEC-8 cells, respectively, when 3-fold change was employed as threshold value.

ENTPD6 expression is down-regulated in NEC-8/DDP cells compared with that in NEC-8 cells. Based on the

results of microarray analysis, we validated the expression of differentially-expressed genes between NEC-8 and NEC-8/DDP cells (Table I). First, we screened several candidate genes (*cathepsin F*, *calbindin 1*, *TASP for testis-specific adriamycin sensitivity protein*, *high density lipoprotein binding protein*, *spermidine/spermine N1-acetyltransferase*, *protein phosphatase 1* and *ENTPD6*) from the results of microarray analysis using semi-quantitative RT-PCR analysis. The results showed that the expression of any genes except *ENTPD6* was similar between NEC-8 and NEC-8/DDP cells (data not shown). However, only *ENTPD6* gene expression remarkably decreased in NEC-8/DDP cells as shown in Fig. 1B. Furthermore, this finding that *ENTPD6* gene expression was reduced in cisplatin-resistant cells was confirmed by quantitative real-time PCR analysis. As shown in Fig. 1C, *ENTPD6* gene expression was remarkably reduced in NEC-8/DDP as well as other cisplatin-resistant bladder cancer cells (T24/DDP10 and KK47/DDP20), which were established as described previously (14,15). Finally, whether this difference of gene expression contributed to protein expression was confirmed by Western blotting. Three cisplatin-resistant cancer cells including NEC-8/DDP, T24/DDP10 and KK47/DDP20 cells expressed less *ENTPD6* protein than their parental cells (Fig. 1D).

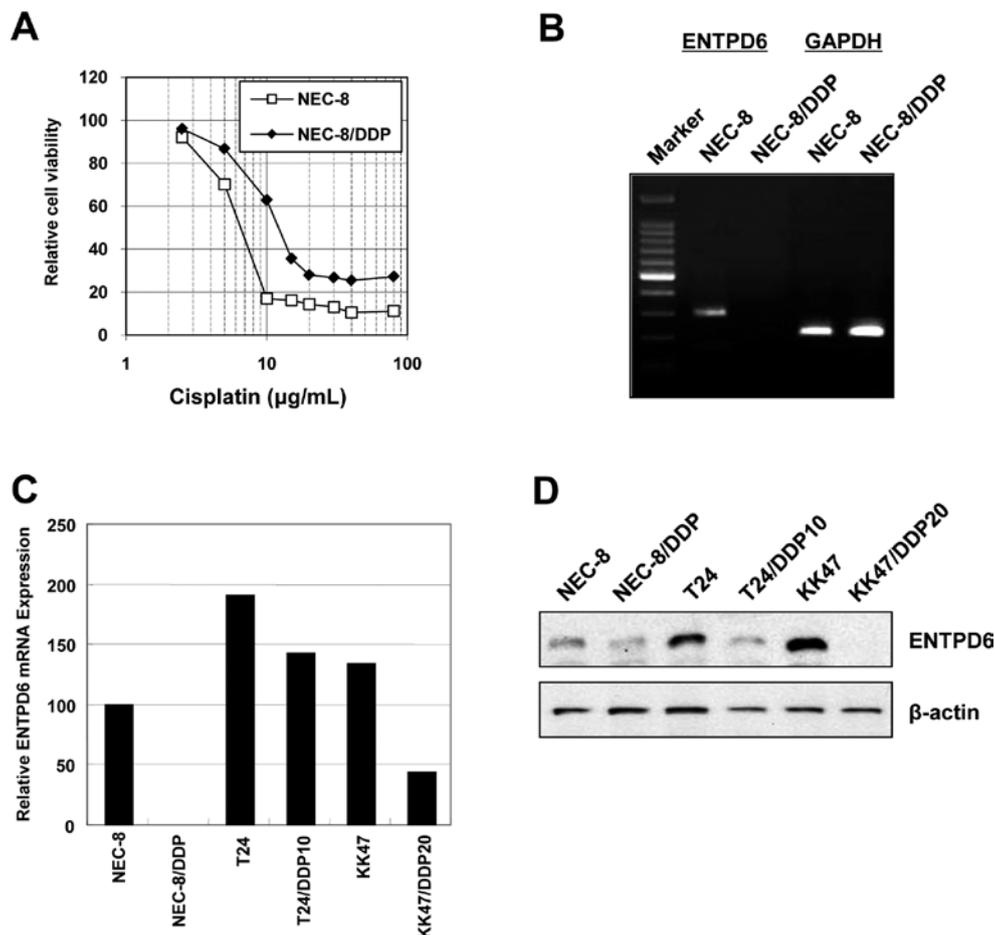


Figure 1. ENTPD6 is down-regulated in NEC-8/DDP cells compared with that in NEC-8 cells. (A) NEC-8 and NEC-8/DDP cells were seeded into 96-well plates. The following day, various concentrations of cisplatin were applied. After incubation for 48 h, cell survival was analyzed by a cytotoxicity assay. Cell survival in the absence of cisplatin corresponds to 1. All values are representative of at least three independent experiments. (B) Semi-quantitative RT-PCR was performed using cDNA from NEC-8 and NEC-8/DDP cells as template and the primers for *ENTPD6* and *GAPDH*. (C) Quantitative real-time PCR was performed using cDNA from NEC-8, T24, KK47 and their cisplatin-resistant cells and the primers and probes for *ENTPD6* and *GAPDH*. The *ENTPD6* transcript levels were corrected by the corresponding *GAPDH* transcript levels. All values are representative of at least three independent experiments. Each transcript level from NEC-8 cells was set as 100. (D) Whole-cell extracts of NEC-8, T24, KK47 and their cisplatin-resistant cells were analyzed for ENTPD6 and β -actin (loading control) by SDS-PAGE and Western blotting with specific antibodies.

ENTPD6 is differentially expressed in testis and testicular cancer. To explore the relevance of ENTPD6 expression in testis and its malignant tumor, we compared *ENTPD6* mRNA expression level between non-cancerous region and cancerous region using various testicular cancer tissues surgically obtained. First, we performed RT-PCR analysis. ENTPD6 mRNA expression in non-cancerous region and in cancerous region was detected in 9/9 (100%) and 26/31 (83.9%), respectively (data not shown). In addition, higher *ENTPD6* mRNA expression in non-cancerous region compared with that in cancerous region was confirmed by quantitative real-time PCR (Fig. 2A). Subsequently, we performed immunohistochemistry against human normal testis and testicular cancer. ENTPD6 localized mainly in cytosol and plasma membrane and was expressed to a weak or moderate extent in spermatoblast, seminiferous tubule, seminoma and its metastatic site, but abundantly expressed in Leydig cells in stroma (Fig. 2B).

ENTPD6 is differentially expressed between seminoma and other testicular tumors. To investigate ENTPD6 expression in testicular cancer, we analyzed *ENTPD6* mRNA expression in

testicular cancer. *ENTPD6* mRNA expressions in cancerous region of various testicular cancer tissues were measured. As shown in Fig. 3, *ENTPD6* expression was affected by tissue-type of testicular cancer (seminoma vs. other testicular tumor).

ENTPD6 is involved in cisplatin resistance through an interaction with *E-cadherin*. Finally, we investigated a mechanism of the relationship between ENTPD6 expression and cisplatin resistance. When ENTPD6 was overexpressed in NEC-8 cells by transfecting with ENTPD6 expression plasmid, NEC-8 cells were more sensitive to cisplatin (Fig. 4A). Secreted-form and membrane-bound form of ENTPD6 are known (12). *E-cadherin* is also known to localize in plasma membrane and to be involved in cisplatin resistance (23,24). Then, we investigated the possibility of interaction between *E-cadherin* and ENTPD6. When *E-cadherin* was immunoprecipitated using anti-*E-cadherin* antibody, ENTPD6 was detected in the resulting-immunoprecipitated complex (Fig. 4B). Furthermore, when ENTPD6 expression was suppressed using two kinds of ENTPD6 specific-shRNA, *E-cadherin* expression was reduced (Fig. 4C), suggesting that ENTPD6 is implicated in

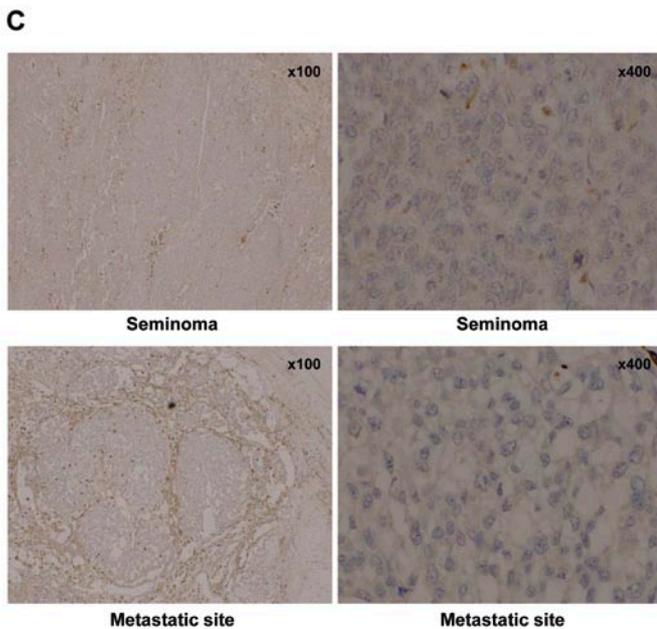
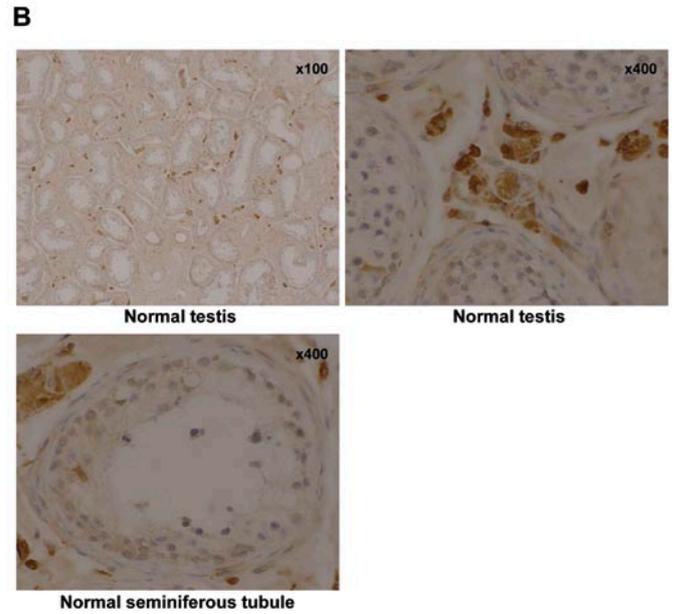
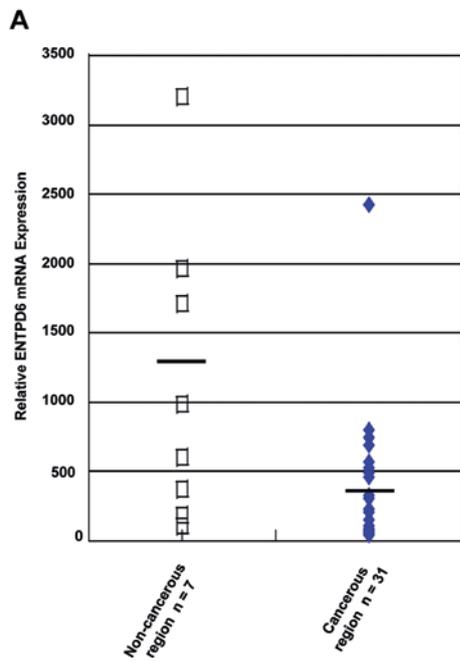


Figure 2. ENTPD6 is differentially expressed in testis and testicular cancer. (A) Quantitative real-time PCR using was performed cDNA from various non-cancerous region and cancerous region and the primers and probes for *ENTPD6* and *GAPDH*. The *ENTPD6* transcript levels were corrected by the corresponding *GAPDH* transcript levels. All values are representative of at least three independent experiments. The transcript level of NEC-8 cells was set as 100. (B and C) Immunohistochemistry against ENTPD6 in testis (B) and seminoma (C) is shown.

E-cadherin expression probably through E-cadherin protein stability.

Discussion

This study revealed that ENTPD6 is a candidate of key molecule determining cisplatin resistance in testicular cancer. Although ENTPD6 is so far reported to be expressed in various tissues including muscle, heart and in fetal brain (25), ENTPD6 expressions in testis and testicular cancer are unknown. In this study, ENTPD6 was detected in all testicular tissues of non-cancerous region and in most cancerous region, indicating that ENTPD6 plays a role in testis and testicular cancer. ENTPD6 is known to catalyze ATP and ADP to AMP. Since extracellular ATP is supposed to regulate many cellular

functions in various tissues, ENTPD6 in testis may play an indispensable role in its function.

Cisplatin resistance is thought to be complex and multi-causal phenomenon. Many resistance-related molecules including detoxifying enzymes, drug-efflux pumps, DNA repair enzymes and apoptosis-related genes have been reported (26,27). In this study, we identified that ENTPD6 may be a factor determining cisplatin sensitivity in testicular cancer. Seminoma is known to be highly-sensitive to chemotherapy including cisplatin-based one. This study revealed that ENTPD6 expression in seminoma was higher than that in other testicular tumor, suggesting that high sensitivity to cisplatin in seminoma might derive from relative-high expression of ENTPD6. Recently, epithelial-mesenchymal transition (EMT) is focused on in relationship with the resistance to

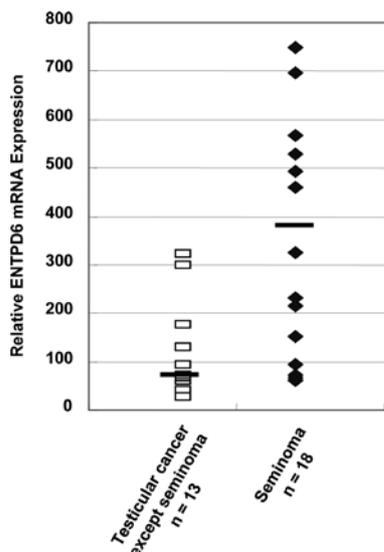


Figure 3. ENTPD6 is differentially expressed between non-seminoma and seminoma. Quantitative real-time PCR using cDNA from various non-seminoma and seminoma tissues and the primers and probes for *ENTPD6* and *GAPDH*. The *ENTPD6* transcript levels were corrected by the corresponding *GAPDH* transcript levels. All values are representative of at least three independent experiments. The transcript level of NEC-8 cells was set as 100.

anticancer therapeutics. E-cadherin is a key player of EMT, which expression is known to be regulated by Twist1 and other transcription factors including Snail and Slug. We and others previously found that Twist1 is a key player in cisplatin resistance (28-30), suggesting that Twist1 may exert its cisplatin-resistant function through a regulation of gene expressions of E-cadherin and others. E-cadherin recently is reported to be closely implicated in and inversely correlated with cisplatin resistance (23,24). In this study, ENTPD6 was found to interact with E-cadherin and to regulate E-cadherin expression. Although its mechanism remained unclear, it was suspected to regulate E-cadherin expression through protein stability because ENTPD6 was shown to interact with E-cadherin. Therefore, ENTPD6 may exert cisplatin resistance through a regulation of E-cadherin expression.

E-cadherin is known to be involved in carcinogenesis and tumor progression in various cancers (31). In mouse model of β -cell tumors of the pancreas, E-cadherin loss is a causal prerequisite for progression from adenoma to invasive carcinomas (32). In addition to many reports about E-cadherin expression in cancer by others, we have reported E-cadherin expression in prostate cancer (21) and renal cancer (33,34) and the methylation of E-cadherin promoter in bladder cancer (35). Then, the relationship between ENTPD6 and E-cadherin

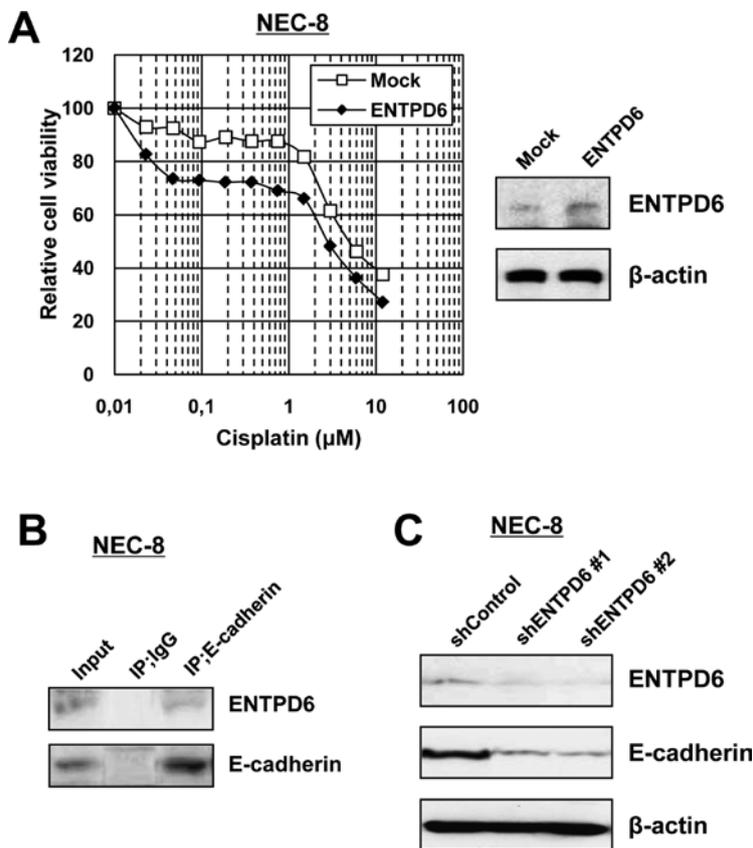


Figure 4. ENTPD6 is involved in cisplatin resistance through an interaction with E-cadherin. (A) NEC-8 cells were transfected with Mock or ENTPD6 expression plasmid. Various concentration of cisplatin was applied at 24 h after transfection, and then the cell viability was measured at 48 h after cisplatin addition. Values represent means from three independent experiments. NEC-8 cells were transiently transfected with Mock or ENTPD6 expression plasmid, and then the cells were harvested at 48 h after transfection. ENTPD6 and β -actin (loading control) expression was detected by Western blot analysis using anti-ENTPD6 and anti- β -actin antibodies. (B) Whole-cell extracts (500 μ g) were immunoprecipitated with 2.0 μ g of mouse IgG or anti-E-cadherin antibody. The pre-immunoprecipitants (input) and immunoprecipitants were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. (C) Whole-cell extracts of NEC-8 cells transfected with the indicated shRNA plasmid were analyzed for ENTPD6, E-cadherin and β -actin (loading control) by SDS-PAGE and Western blotting with specific antibodies.

might suggest its role of ENTPD6 in cancer carcinogenesis and progression.

In conclusion, we have revealed that ENTPD6 may be a tumor suppression gene and a determinant of cisplatin resistance in testicular cancer. Therefore, ENTPD6 is a promising diagnostic molecule of cisplatin resistance in testicular cancer and a molecular therapeutic target of testicular cancer.

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