Sensitization against Anticancer Drugs by Transfection with UBE2I Variant Gene into ras-NIH3H3 Mouse Fibroblasts

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Abstract. *We previously performed SEREX (serological identification of antigens by recombinant expression cloning) using the sera of patients with esophageal squamous cell carcinoma (SCC), and isolated a variant clone (AK093616) of ubiquitin-conjugating enzyme E2I (UBE2I). This clone was tentatively designated as UBE2I-v5 and analyzed for biological function by transient transfection of the cDNA into activated Ha-ras-transformed NIH3T3 (ras-NIH) mouse fibroblasts. Chemosensitivity to 92 cytotoxic drugs was compared between UBE2I-v5-transfected cells and the parental ras-NIH cells. The UBE2I-v5-transfected cells were more sensitive than the parental cells to anticancer drugs such as vincristine (VCR), mitoxantrone (MIT) and etoposide (VP-16). The regression analysis of the total chemosensitivity pattern of UBE2I-v5-transfected cells revealed that the function of UBE2I-v5 was positively related to RPA2 (replication protein A2), Rho-GDI (Rho guanine nucleotide dissociation inhibitor α), FUS (putative tumor suppressor) and TKT (transketolase) but negatively related to Per-1 (period-1), Ran (nuclear Ras-related protein), PTEN (phosphatase and tensin homolog), C/EBPα (CCAAT/enhancer binding protein α) and the tumor suppressor p53. Thus, it is possible that UBE2I-v5 plays a role in carcinogenesis by suppressing the function of C/EBPα and/or p53 via RPA2-like activity.*

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Human esophageal squamous cell carcinoma (SCC) is a highly malignant disease (1). Therefore, early diagnosis using tumor markers is indispensable for preventing tumor development. SEREX (serological identification of antigens by recombinant cDNA expression cloning) is an effective and convenient method for screening for tumor markers (2). It can be applied to the immunoscreening of cDNA libraries prepared from tumor specimens with autologous or allogeneic sera. Since the antigens are easily identified by sequencing the isolated cDNA clones, SEREX is suitable for large-scale screening of tumor antigens. SEREX has been applied to varying human tumor types and has identified more than 1,000 novel tumor antigens (3). We have previously performed large-scale SEREX screening and reported new tumor antigens of esophageal SCC (4-8). Among them, TROP2/TACSTD2, SLC2A1 and TRIM21 have gained much attention because their serum antibodies have frequently been developed in patients with esophageal SCC (4, 6, 7). In addition, most, if not all, of the esophageal SEREX antigens have been suggested to be related to carcinogenesis, apoptosis or cell growth (8). For example, the tumor suppressor p53, the oncoprotein phosphatidylinositol 3-kinase and stathmin have been isolated by our SEREX screening method. Thus, it is intriguing to analyze the function of the SEREX clones.

DSPA (drug-sensitivity pattern analysis) is a powerful method for comprehensive analysis of the functional relationship between two genes (9, 10). In principle, the chemosensitivity of gene A-transfected cells is examined for multiple drugs, and the total chemosensitivity pattern compared with that of gene B-transfected cells. When the regression analysis shows significant correlation between the two patterns, genes A and B could conceivably be functionally related.

In the present study, the function of a newly isolated SEREX antigen was investigated by transfection into mouse fibroblasts followed by DSPA.

Materials and Methods

Drugs. MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Etoposide (VP-16) was purchased from Nippon Kayaku (Tokyo, Japan), and 6-thioguanine (6-TG) was obtained from Wako Pure Chemicals (Kyoto, Japan). Tin protoporphyrin-IX (SnPP), parthenolide, Y-27632 (Rho-dependent protein kinase inhibitor), mitoxantrone (MIT) and quercetin were purchased from Alexis (Lausen, Switzerland). Vincristine (VCR), BAY-11-7082 (NF-κB inhibitor), C8 ceramide, difluoromethylornithine (DFMO), erbstatin, U73122 (phospholipase C inhibitor) and forskolin were obtained from EMD Biosciences (Damstadt, Germany). ACNU (1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3(2-chlorethyl)-3-nitrosourea) was provided by Sankyo Pharmaceutical (Tokyo, Japan).

Sera from patients with esophageal SCC. This work was approved by the Ethic Committee of Chiba University, Graduate School of Medicine. The sera were collected after obtaining the written, informed consent of the patients before treatment for esophageal SCC. Each serum sample was centrifuged at 3,000 xg for 5 min and the supernatant was stored at –80˚C until use.

Cells. A human esophageal SCC cell line, T.Tn (11, 12), was obtained from the Japan Cell Research Bank (Ibaraki, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum. Mouse fibroblasts, NIH3T3 and activated Ha-*ras*-transformed NIH3T3 (clone F25) (ras-NIH) (13), were cultured in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5% heat-inactivated bovine serum.

SEREX screening. A λZAP II phage cDNA library was prepared from the mRNA of the T.Tn cells and the immunological screening was performed as described previously (4-8). Monoclonalized phage cDNA clones were converted to pBluescript phagemids by *in vivo* excision using ExAssist helper phage (Stratagene, La Jolla, CA, USA). Plasmid DNA was obtained from the *E. coli* SOLR strain transformed by the phagemid.

Transfection. A full-length UBE2I-v5 (a clone of ubiquitin-conjugating enzyme) cDNA constructed in an expression plasmid, pME18SFL3 (14), was obtained from Toyobo Chemicals (Osaka, Japan). The plasmid was transiently transfected into the ras-NIH cells using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

The biological function of UBE2I-v5 in transformed cells was examined by an enforced expression of UBE2I-v5 cDNA in ras-NIH cells. Two days after transfection, ras-NIH and UBE2I-v5-transfected cells were treated with various concentrations of 92 cytotoxic drugs and further cultured for 3 days.

MTT method for assessing viability. The inhibitory effects of the drugs on the proliferation of cultured cells were examined by the MTT method of Mosmann (15) as described previously (16, 17). Five thousand cells were plated in each well and cultured for 3 days. Cell viability was quantified by measuring the absorbance at 570 nm after incubation with MTT for 4 h. The results are shown as percentages of the control results.

Results

Isolation of UBE2I-v5 cDNA by SEREX. A total of $1x10^6$ cDNA clones were screened using the sera from esophageal cancer patients, and a reactive clone was isolated. A search for homologous sequence in NCBI-accessible databases (http://www.ncbi.nlm.nih.gov/) revealed that the isolated clone was identical to an EST (expressed sequence tag) clone (Accession No:AK093616), which is a variant clone of ubiquitin-conjugating enzyme E2I (UBE2I). UBE2I is a human homolog of yeast ubc9 and is involved in a small ubiquitin-related modifier (SUMO)-conjugation (18-20). UBE2I interacts with many molecules such as FHIT (fragile histidine triad) (21), PTEN (phosphatase and tensin homolog) (22), C/EBPε (CCAAT/enhancer binding protein ε) (23), RANGAP1 (Ran GTPase activating protein 1) (24), IκBα (inhibitor α of NF-κB) (24) and the tumor suppressor p53 (24, 25). Because 4 variant clones are known for UBE2I, this clone (AK093616) was tentatively designated as UBE2I-v5 (UBE2I variant 5). UBE2I-v5 encodes the amino-terminal 76 amino acids of UBE2I variants 1-4. However, no information was available concerning the function of UBE2I-v5.

Comparison of chemosensitivity against cytotoxic drugs between ras-NIH and UBE2I-v5-transfected cells. The relative viability of parental ras-NIH and UBE2I-v5-transfected cells was evaluated by the MTT colorimetric assay. Some of the results are shown in Figure 1. The cell viability decreased as the drug concentration increased. UBE2I-v5-transfected cells were more resistant to SnPP, a competitive inhibitor of heme oxygenase (26), but more sensitive to anticancer drugs such as VCR, MIT and VP-16 than the parental ras-NIH cells.

To understand the difference in the chemosensitivity between the parental and transfected cells, a drug chemosensitivity index (DCI) was introduced (9, 10). Firstly, the ratios of the drug concentrations giving 40% inhibition in a test cDNA-introduced clone (IC_{40}test) *versus* those in the parental cells (IC_{40}parent) were calculated. Then, the logarithmic values of the ratios (DCI=log[IC_{40}test/IC_{40}parent]) were obtained. The DCI values lower than 0 represent sensitization by transfection. Some examples of low and high DCI values are shown in Table I. As compared to the parental cells, the UBE2I-v5-transfected cells were more sensitive to anticancer drugs such as VP-16, MIT, VCR and 6-TG but more resistant to SnPP, ceramide, DFMO, erbstatin, Y-27632, parthenolide, ACNU, quercetin, U73122 and BAY-11-7082. No obvious difference in chemosensitivity was detected between these cells for the other 78 drugs (data not shown).

Functional analysis of UBE2I-v5 by DSPA. The DSPA method for assessment of protein function was utilized. In

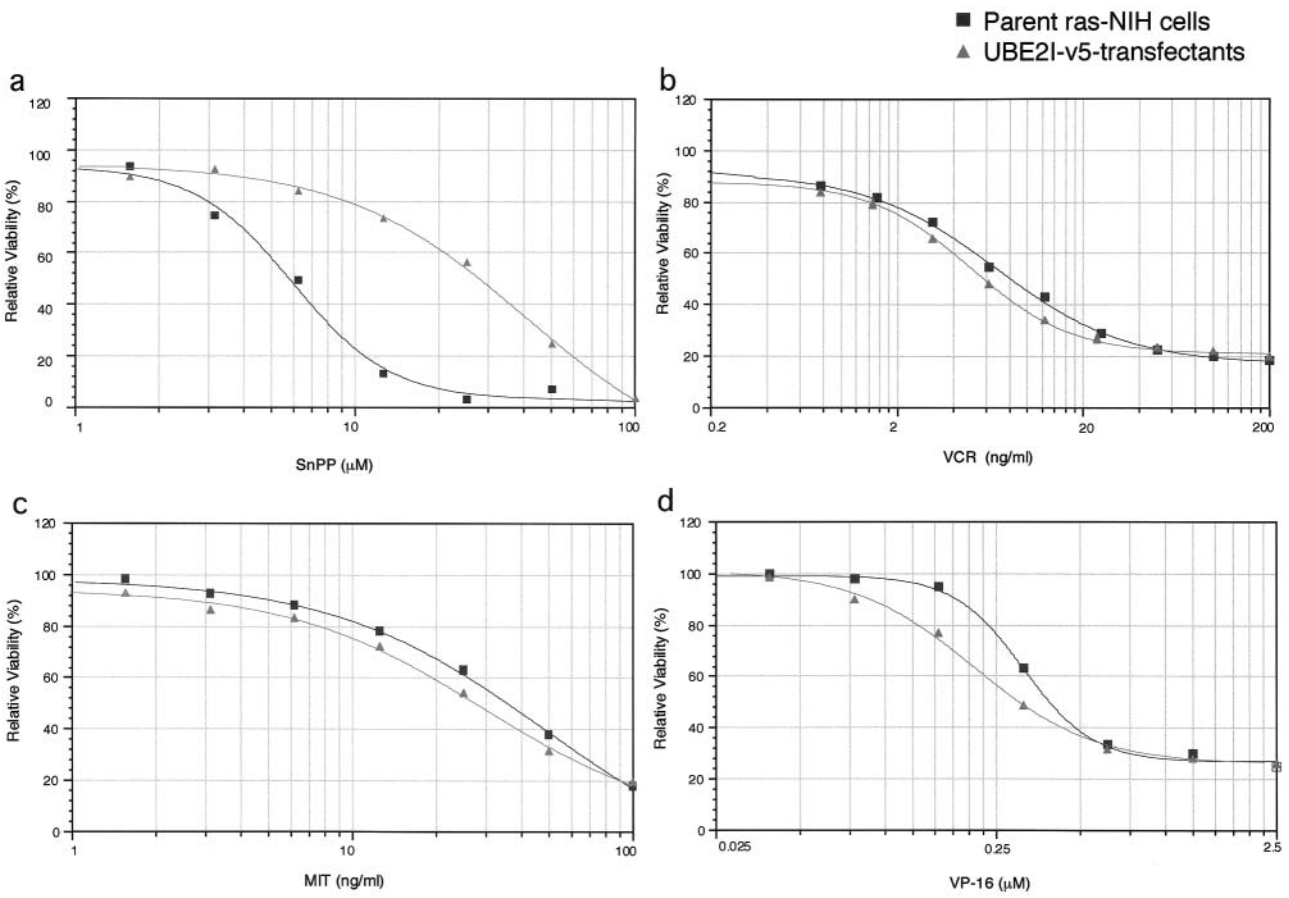

Figure 1. *The effect of UBE2I-v5 on the chemosensitivity toward cytotoxic drugs. Parental ras-NIH (squares) and UBE2I-v5-transfected cells (triangles) were cultured in the presence of (a) SnPP, (b) VCR, (c) MIT and (d) VP-16 for 3 days. The viabilities were measured by the MTT assay. The abscissa and ordinate represent the concentration of the test drugs and the relative viability versus that in the absence of test drugs, respectively.*

this way, the DCI values of UBE2I-v5-transfected cells were compared with those of other transfected cells. Some of the results of regression analysis are shown in Figure 2 and summarized in Table II. It was suggested that UBE2I-v5 was functionally related to RPA2 (replication protein A2), TKT (transketolase), RhoGDIα (Rho guanine nucleotide dissociation inhibitor α), FUS2 (putative tumor suppressor) and so on (Table IIa). Because RPA2 was related to UBE2I-v5 most closely, UBE2I-v5 may have RPA2-like activity.

An inverse relationship as shown in Figures 2e and f may imply that Per-1 (period-1) and RAN (nuclear Ras-related protein) have opposite roles in cell survival. Thus, the function of UBE2I-v5 may be inversely related to PTEN, C/EBPα, dominant negative Akt, p53 and so on (Table IIb). Because these genes posssess tumor-suppressive roles in carcinogenesis, UBE2I-v5 may have a tumor-promoting activity.

Discussion

In our previous large-scale screening of esophageal SCC (4-8), it was found that SEREX antigens may induce their serum antibodies possibly by the overexpression of wild-types, the expression of alternative splicing variants and/or altered protein processing. The SEREX antigens are not only possible tumor markers but also potential participants in tumor development. In fact, most of the esophageal SEREX antigens we identified were related to cancer (8).

The MTT analysis is highly reproducible (9, 15) and, therefore, the results may reflect subtle molecular changes in cells. As compared to the parental cells, the UBE2I-v5-transfected cells were more resistant to quercetin (Table I) which is an inhibitor of PI3-kinase (27). On the other hand, DSPA suggested a functional relationship between UBE2I-v5 and PTEN and Akt (Table II). Thus, UBE2I-v5 may participate in

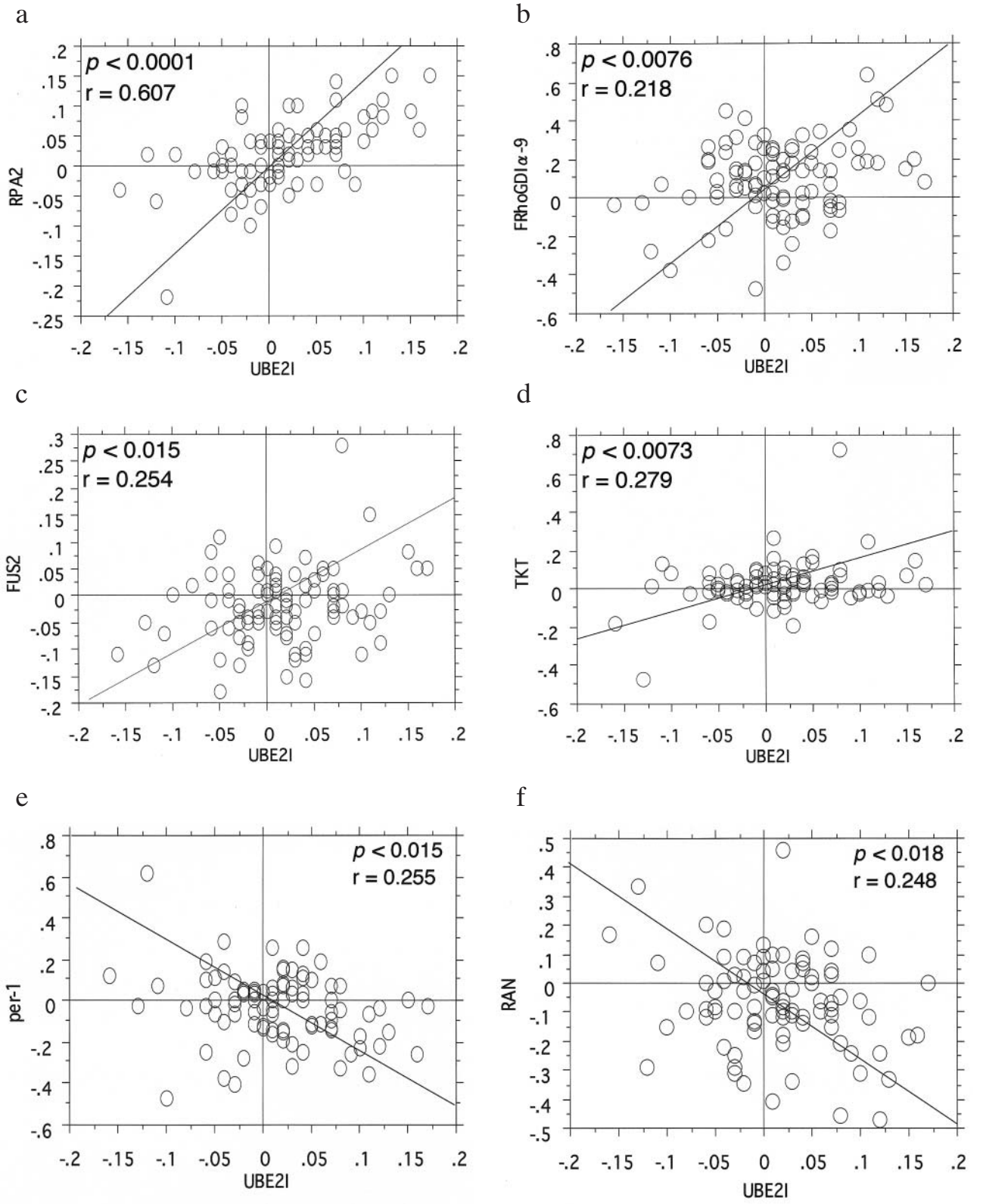

Figure 2. *Results of regression analysis between UBE2I-v5-transfectants and other transfected cells. Shown are distribution maps of DCI values of tranfected cells. The abscissa and ordinate represent the DCI values of UBE2I-v5-transfectants and (a) RPA2-, (b) RhoGDIα-, (c) FUS-, (d) TKT-, (e)Per-1- and (f) RAN-transfected cells, respectively.*

Table I. *Results of MTT assay.*

a. Drugs with low DCI values		
Drug	DCI value	Function/Action
VP-16	-0.16	Topoisomerase II inhibitor
MIT	-0.13	DNA strand break
VCR	-0.12	Tubulin inhibitor
6-TG	-0.11	Purine metablism inhibitor
Forskolin	-0.10	Adenylate cyclase activator
b. Drugs with high DCI values		
Drug	DCI value	Function/Action
SnPP	0.64	Heme oxygenase inhibitor
C8 ceramide	0.17	Apoptosis inducer
DFMO	0.16	Ornithine decarboxylase inhibitor
Erbstatin	0.15	EGFR kinase inhibitor
Y27632	0.13	ROCK inhibitor
Parthenolide	0.12	NF-κB inhibitor
Quercetin	0.11	PI3-K inhibitor
U73122	0.11	Phospholipase C inhibitor
BAY-11-7082	0.10	NF-κB inhibitor

Table II. *Results of DSPA analysis.*

a. Positive correlation		
Gene	r-value	*p*-value
RPA2	0.607	<0.001
Col-XVIII	0.308	0.003
TKT	0.279	0.007
RhoGDIα	0.278	0.008
FUS2	0.254	0.015
HNF4	0.249	0.017
OPRT	0.232	0.027
PARK7	0.231	0.027
PSEN1	0.216	0.040
b. Negative correlation		
Gene	r-value	*p*-value
PTEN	0.273	0.084
DAN	0.266	0.020
E2F	0.266	0.071
C/EBPα	0.263	0.088
Per-1	0.255	0.015
TK	0.252	0.095
RAN	0.248	0.018
PDGFR	0.238	0.182
HSP40	0.234	0.025
Akt-DN	0.231	0.028
TSC	0.224	0.033
p53	0.219	0.037

phosphoinositide metabolism. The UBE2I-v5-transfected cells were also resistant to Y27632 (Table I) which inhibits Rho-associated protein kinase (28). This may be related to the DSPA result that UBE2I-v5 was functionally related to Rho-GDIα (Table II).

It should be noted that transfection of UBE2I-v5 caused resistance to parthenolide and BAY-11-7082 (Figure 1, Table I), both of which are NF-κB inhibitors (29, 30). UBE2I has been reported to mediate sumoylation of IκBα, which is an endogenous NF-κB inhibitor (24). Thus, UBE2I-v5 may also affect the activity of NF-κB, possibly by affecting UBE2I variants 1-4. In addition to NF-κB, DSPA showed that the function of UBE2I-v5 was related to C/EBPα, RAN and p53 (Figure 2, Table II), which have been reported to be sumoylated by the other UBE2I variants (22-25). Compared to the other variants (158 amino acids), UBE2I-v5 is much smaller in the size (76 amino acids). Therefore, it is implausible that UBE2I-v5 possesses sumoylation activity. Rather, UBE2I-v5 may interfere with the activity of the other UBE2I variants because some of the truncated gene products have a dominant negative role on the full-length counterparts (31). Thus, naturally occurring UBE2I-v5 may have an important role as a negative regulator in full-length UBE2I-induced sumoylation pathways. UBE2I-v5 may be a valuable specific tool for the investigation of sumoylation pathways.

The results of DSPA suggested that the function of UBE2I-v5 was most closely related to RPA2 (Figure 2a, Table II). RPA2 is a component of replication protein A (RPA), which is required for multiple processes in eukaryotic DNA metabolism including DNA replication, DNA repair and recombination (32). RPA interacts with p53 and suppresses its DNA-binding activity (33). After DNA damage, RPA is dissociated from p53 by single-stranded DNA and/or hyperphosphorylation (33, 34). Thus, functional similarity between UBE2I-v5 and RPA suggested by DSPA may account for the sensitization of UBE2I-v5-transfected cells to the DNA-damaging anticancer drugs such as VP-16 and MIT (Figure 1, Table I). Modulation of ubiquitination and the following proteasomal degradation pathway have already been proposed as new anticancer drugs (35, 36). The present study suggests that sumoylation may also be a target for cancer chemosensitivity.

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