Inhibition of Ubiquitin-conjugating Enzyme E2 May Activate the Degradation of Hypoxia-inducible Factors and, thus, Overcome Cellular Resistance to Radiation in Colorectal Cancer

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Abstract. *Background:* NSC697923, a ubiquitin-conjugating enzyme E2 (UBE2) inhibitor, was suggested as an agent to degrade hypoxia-inducible factor 1 alpha subunit (HIF1 α), a key factor in radiation resistance. We attempted to clarify whether NSC697923 could overcome radiation resistance. *Materials and Methods:* Radiation resistance and expression of HIFs were evaluated in radiation-sensitive HCT116 and -resistant SW480 cells treated with or without NSC697923 and radiation under normoxia and hypoxia in vitro and in vivo. We examined NSC697923-regulated genes using RNA sequencing. *Results:* HIF expression significantly increased under hypoxia with an increase of cellular radiation resistance in vitro and in vivo. The therapeutic activity of NSC697923 was higher in radiation-resistant SW480 than radiation-sensitive HCT116 in vivo. Next-generation RNA sequencing revealed that NSC697923 regulated the expression of cell migration-inducing protein, hyaluronan binding (CEMIP) and apelin (APLN) genes, that are related to HIF pathways. *Conclusion:* NSC697923 might effectively regulate HIF families, and be a promising partner with radiation to overcome resistance.

Progress in perioperative management and adjuvant therapy has led to improved survival for patients with colorectal cancer (CRC) (1-3). However, advanced CRC presents serious obstacles to radical resection: Advanced CRC, especially in the rectum and anal canal, enhances the risk of local recurrence and colostomy following abdominal peritoneal resection (4). It has been reported that adjuvant radiation therapy is very effective and is often performed to eliminate CRC cells near the anal canal (5, 6). Overcoming radiation resistance is vital for avoiding abdominal peritoneal resection and local recurrence.

Significant areas of rapid growth in tumors are located at a distance from supporting blood vessels, which results in the formation of a microenvironment low in oxygen and nutrients (7-9). Hypoxia-inducible factor 1-alpha subunit (HIF1 α) and hypoxia-inducible factor 2-alpha subunit (HIF2 α), also known as EPAS1, are essential factors in maintaining cellular oxygen homeostasis and adaptation to hypoxia (10-12). Generally, tumor cells that respond to these hypoxic conditions acquire resistance to radiation therapy and most types of chemotherapy in various cancer types (13-16).

High expression of HIF1 α and -2 α has been associated not only with radiation resistance but also poor prognosis in CRC (17-19). Lee *et al.* reported that HIF1 inhibitor alone strongly suppressed proliferation of the radiation-sensitive CRC cell line HCT116 in *in vivo* analysis (20), but the combination effects of HIF1 α targeting and radiation on CRC cells, especially radiation-resistant cell lines, have not yet been analyzed as far as we are aware.

Recently it was reported that targeting the ubiquitin-conjugating enzyme E2 (UBE2) family prevents HIF1 α and -2 α degradation by proteasome systems (21, 22). Some

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Figure 1. Radiation sensitivity in colorectal cancer (CRC) cell lines under normoxia. A: Cell viability in different CRC cell lines. These cells were irradiated at 2, 6, and 10 Gy. Radiation sensitivity in HCT116 was higher than that in SW480 cells ($p < 0.0001$). From these data, SW480 and HCT116 cells were identified as being radiation-resistant and radiation-sensitive CRC cell lines, respectively. B: Western blotting of hypoxia-inducible factor 1 alpha subunit (HIF1α) and -2α baseline expression in SW480 and HCT116 cells under normoxia. The baseline expression of HIF1α and -2α was higher in radiation-resistant SW480 cells than in radiation-sensitive HCT116 cells. Heat-shock cognate protein 70 (HSC70) was used as a loading control.

researchers reported several inhibitors of UBE2 family genes as antitumor agents (23-25), but the potential of these UBE2 inhibitors as radiation modulators has not yet been established. We, thus, focused on the UBE2 inhibitor NSC697923, which was recently reported as a promising candidate compound that can induce strong inhibition of proliferation and survival in diffuse large B-cell lymphoma and neuroblastoma (23-25).

The purpose of this study was to elucidate whether NSC697923 can actively overcome radiation resistance in CRC cells by regulating HIF1α and -2α *in vitro* and *in vivo* using radiation-sensitive HCT116 and radiation-resistance SW480 cells. We also examined other probable action determinants of NSC697923, using a next-generation sequence (NGS) analysis system.

Materials and Methods

Cell lines and reagents. The human CRC cell lines HCT116, SW480, HCC56, CCK-81, WiDr, LoVo, and DLD-1 were purchased

from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan), the RIKEN Cell Resource Center of Biomedical Research (Tsukuba, Japan), and American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI-1640, Ham's, Eagle's minimum essential medium or Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin antibiotics, and then incubated at 37°C and 5% CO₂. For hypoxia induction, the cells were exposed to hypoxia (1% O₂) using a hypoxic chamber (Billups-Rotenberg, SanDiego, CA, USA) with gas mixtures (95% N₂ and 5% CO₂). Hypoxic plates were coated with a paper sealer, which maintains the hypoxic O₂ level inside the well. UBE2 inhibitor NSC697923 and cisplatin were purchased from SIGMA-ALDRICH Japan (Tokyo, Japan) and Wako laboratory chemicals (Osaka, Japan), respectively.

Cytotoxic analysis. Cell viability was analyzed using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded at 4×10^3 /100 μl per well in 96-well plates and allowed to attach for 24 h with or without hypoxia. After 24 h incubation, cells were exposed to radiation with/without drug for 72 h. Then 10 μl of CCK-8 solution was added to each well and

Figure 2. Hypoxia-inducible factor 1 alpha subunit (HIF1 α) accumulation by hypoxia-induced radiation resistance in SW480 and HCT116 cells. A: Cell viability in SW480 and HCT116 cells treated with 6 Gy radiation under normoxic and hypoxic environments. Hypoxia induced radiation resistance in both cell lines. B: Time course of the expression of HIF1 α and -2 α under hypoxia. Western blotting was used to evaluate expression in SW480 and HCT116 cells. HIF1 α and -2 α expressions were at their highest level 6 hours from initial exposure to hypoxia in both cell lines. The expression of the proteins was normalized to the level of that of heat-shock cognate protein 70 (HSC70). Intensity was measured using the Image J software.

incubated at 37°C for 2 h. Absorbance was detected at 450 nm using a plate reader (BioRad, Hercules, CA, USA), and then the half maximal inhibitory concentration (IC₅₀) was calculated with MS Excel for each cell line.

Nude mouse experimental system (mouse xenograft model). Eight-week-old male BALB/c *nu/nu* nude mice received subcutaneous injections of 1×10^7 CRC cells. Tumor volume was calculated using the formula: $\text{Volume} = S^2 \times L / 2$, where S is the short length of the tumor in mm and L is the greatest length of the tumor in mm. The tumor volumes were determined by caliper measurements every 4 days. After the tumor volume had reached 100 mm³, the mice were divided randomly into five groups: Control, radiation alone, NSC697923 alone, NSC697923 plus radiation, and cisplatin plus radiation. Each group had six mice. Cisplatin and NSC697923 were administered intraperitoneally on days 0, 4, and 8. The doses of cisplatin and NSC697923 were set as one-third of the lethal dose resulting in 50% mortality (LD₅₀); 2 mg/kg cisplatin and 7.5 mg/kg NSC697923. Radiation of 6 Gy was performed at day 0 (initial day of treatment) in a single fraction. Mouse tissue samples were

harvested on 0, 4, 8 and 12 of the experiment. The evaluation of tumor volume and mouse body weight was continued until day 28.

Radiation. For *in vitro* study, HCT116 and SW480 in 96-well plates were exposed to hypoxia (1% O₂) for 24 h, then treated with radiation, and evaluated by CCK-8 assay after 72 h incubation in normoxia. An X-ray machine (FAXITRON, RX-650, Faxitron X-Ray LLC, Lincolnshire, IL, USA) with 100 kV, Al 0.3 mm filter was used for radiation treatment. We performed local radiation against tumor with 6 mm lead shielding using a subcutaneous xenograft mouse model. Radiation was performed at a dose of 6 Gy in single fraction operated (TITAN-225S; Shimadzu Mectem, Inc., Tokyo, Japan) with 200 kV, 14.6 mA with Al 0.5 mm and Cu 0.5 mm filtration at a distance of 47.3 cm from the target.

Protein extraction and western blotting. The proteins were extracted using lysis buffer [10 mM Tris-HCl (pH7.5), 1 mM EDTA, 10% Glycerol, 0.5% NP40, 400 mM NaCl, 4 μ g/ml aprotinin, phenylmethylsulfonyl fluoride, proteasome inhibitor MG-132 and 1 mM dithiothreitol. Total protein (10 μ g) was electrophoresed

Figure 3. Ubiquitin-conjugating enzyme E2 (UBE2) inhibitor NSC697923 can repress hypoxia-inducible factor 1 alpha subunit (HIF1α) and -2α expression in radiation-resistant SW480 cells under hypoxia. A: HIF1α and -2α expression in SW480 and HCT116 cells treated with half maximal inhibitory concentration (IC₅₀) concentration of NSC697923 (NSC) under 6 h hypoxia. Western blotting was used to evaluate the expression in both cells. NSC697923 significantly repressed expression of both HIF1α and -2α induced by hypoxia in SW480 cells, but not in HCT116 cells. The expression of these proteins was normalized to the level of heat-shock cognate protein 70 (HSC70). B: Radiation combination with NSC697923 suppressed cell viability of SW480 and HCT116 cells under hypoxia more than radiation alone (HCT116: 5.61 μM and SW480: 1.87 μM). n.s. Not significant; **p*<0.01; ***p*<0.001.

on a 10% polyacrylamide gel, and then electroblotted at 300 mA for 90 min on a nitrocellulose membrane (Invitrogen). Western blotting was used to confirm the expression of HIF1α and -2α, and heat-shock cognate protein 70 (HSC70). These proteins were detected using rabbit monoclonal antibody to HIF1α and to HIF2α (1:1,000 *in vitro* and 1:500 *in vivo*) (Cell Signaling Technology Inc., Danvers, MA, USA) and mouse monoclonal antibody to HSC70 (1:3000 *in vitro* and *in vivo*) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). HSC70 expression was used as a loading control. The signals were detected using the ECL Western Blotting Detection System and an Image Quant LAS 4000 machine (GE Healthcare Life Sciences Inc., Chicago, IL, USA).

RNA-sequencing. Total RNA was extracted by using NucleoSpin RNA kit (Clontech) following the manufacture's instructions. One microgram of total RNA was used to generate sequencing libraries of the barcoded fragment using the TruSeq Stranded mRNA Sample

Prep Kit (Illumina, Inc., San Diego, CA, USA) following the manufacturer's instructions. Libraries were subjected to paired-end sequencing of 45-bp reads on the NextSeq500 System (Illumina, Inc.) using NextSeq500 High Output v2 Kit (Illumina). Briefly, the reads were aligned to the University of California, Santa Cruz, reference human genome 19 (hg19) using Spliced Transcripts Alignment to a Reference (STAR) software v2.3.1 (DNA STAR, Inc. Madison, WI, USA). Differentially expressed genes were detected using DESeq v.1.24.0 (Bioconductor open-development software project).

Statistical analysis. For continuous variables, the data are expressed as the mean±standard deviation. Cell viability and differences between treatment groups and *in vitro* assay data were analyzed using MS Excel. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA).

Figure 4. The combination effect of NSC697923 with radiation induced tumor-growth inhibition in mouse xenograft models. A: Tumor volume in mouse xenograft models of SW480 and HCT116 cells treated with radiation (IR) with/without NSC697923 (NSC). The combination was effective against tumor xenografts of both HCT116 and SW480, especially against radiation-resistant SW480 xenografts. Tumor volume was normalized to that of non-treated controls. Radiation was performed on day 0 and intra-peritoneal injection of NSC697923 on 0, 4 and 8. B: Time course of hypoxia-inducible factor 1 alpha subunit (HIF1 α) and -2 α expression and volume of the tumor xenografts. Western blotting was used to evaluate HIF1 α and -2 α expression. HIF1 α and -2 α suppression with the combination of NSC697923 and radiation were not prominent even at day 12. The expression of these proteins was normalized to the level of heat-shock cognate protein 70 (HSC70).

Figure 5. NSC697923 overcomes radiation resistance in SW480 cells more than radiation-modulator cisplatin does. A: Tumor volume in mouse xenograft models of SW480 and HCT116 cells treated with radiation (IR) with cisplatin (CDDP) or NSC697923 (NSC). Tumor volume was normalized to non-treated controls. B: Percentage of body weight loss in xenografted mice treated with IR alone, and IR with CDDP or NSC697923. Body weight was normalized to that of the non-treated control.

Results

In vitro radiation sensitivity of CRC cell lines in a normoxic environment. We treated several CRC cells with radiation in doses of 0, 2, 6, and 10 Gy to examine their sensitivity to radiation (Figure 1A). Among a total of seven cell lines investigated, we selected HCT116 and SW480 cells as the most radiation-sensitive and -resistant lines, respectively, to use in the subsequent experiments. Cell viability in radiation-sensitive HCT116 cells was significantly suppressed compared to radiation-resistant SW480 cells at all treatment doses ($p < 0.0001$) (Figure 1A). We then checked the expression of HIF1 α and -2 α in the 2 cell lines. Western-blot analysis showed that baseline-expression levels of both HIF1 α and -2 α were higher in SW480 than those in HCT116 cells, which was likely related to cellular level of resistance to radiation (Figure 1B).

Accumulation of HIFs under hypoxia and radiation resistance. To clarify whether hypoxia causes an increase in cell resistance to radiation as a result of induction of HIF1 α or HIF-2 α , HCT116 and SW480 cells were exposed to hypoxia (1% O₂) for 24 h, and then treated with radiation (6 Gy). Cellular sensitivity to radiation was evaluated by CCK-8 assay after 72 h incubation under normoxia. Hypoxia caused a statistically significant increase of cellular resistance to radiation in both cell lines as compared with normoxia (Figure 2A). Cell viability increased from 37.2% to 52.7% in HCT116 cells ($p < 0.001$) and from 77.3% to 88.5% in SW480 cells ($p < 0.05$) under hypoxia (Figure 2A). We also performed time-course analysis of HIF1 α and -2 α expression under hypoxia. It demonstrated that expression of both proteins was significantly induced and reached a peak 6 h after the initial exposure to hypoxia in both cell lines. However, expression subsequently declined distinctly rapidly

Figure 6. Analytical method of RNA sequencing to identify promising targets of NSC697923 under normoxic and hypoxic conditions. We chose 68 (Table I) and 17 (Table II) genes as targets down-regulated by NSC697923 under normoxic and hypoxic conditions, respectively. From these targets, we found two genes, apelin (APLN) and hyaluronan binding (CEMIP), that were classified as downstream pathway genes of HIF1 α and -2 α , respectively, under both normoxic and hypoxic conditions.

in radiation-sensitive HCT116 cells and gradually in radiation-resistant SW480 cells (Figure 2B).

UBE2 inhibitor can repress HIF1 α and -2 α expression in radiation-resistant SW480 cells even under hypoxia. Increased expression of HIF1 α or -2 α was probably a critical cause of the cellular resistance to radiation. Hence we examined NSC697923 as a possible modulator to overcome radiation resistance in CRC cells. HCT116 and SW480 cells were pre-incubated for 24 h in 96-well plates, exposed to NSC697923, and then treated with 6 Gy radiation after 24 h of drug treatment. We found that NSC697923 significantly repressed expression of both HIF1 α and -2 α induced by hypoxia in SW480 cells, although such remarkable suppression was not observed in HCT116 cells (Figure 3A). Associated with the repressive effect on HIF1 α and -2 α , NSC697923 induced significant cytotoxicity, especially against SW480 cells, even when used by itself, and its combination with radiation yielded the most remarkable cell-growth inhibition of both HCT116 and SW480 cells even under hypoxic conditions (Figure 3B).

Effect of NSC697923 combination with radiation in a mouse xenograft model. Nude mouse experiments were performed to determine the activity of NSC697923 *in vivo*. Similar to the *in vitro* analyses, we confirmed that the combination of NSC697923 with radiation was the most active treatment against both tumor xenograft types, HCT116 and SW480, among all treatments investigated (Figure 4A). The combination inhibited tumor growth more than single treatment with IR or NSC697923 alone, although the therapeutic advantage was limited against HCT116 tumors.

Expression of HIF1 α and -2 α in tumors increased with increasing tumor growth in the control groups (Figure 4B). In the HCT116 xenograft system, HIF expression was repressed by all treatments investigated, likely due to tumor growth inhibition (Figure 4B). Nevertheless, these correlations were not clearly observed in the SW480 xenograft system. A decrease of HIF1 α and -2 α expression in the combination group of NSC697923 and radiation was not so prominent and expression was similar to those in the other treatment groups.

Nevertheless, radiation combined with NSC697923 was most active against both radiation-sensitive and -resistant

Table I. *Genes significantly suppressed after NSC697923 treatment under normoxia. We chose 68 genes altered under normoxic conditions as possible targets of NSC697923 from the results of the next-generation RNA sequencing.*

Related pathway	Symbol	Gene name	HCT116		SW480	
			Log2 fold change	p-Value	Log2 fold change	p-Value
HIF-1α	IGFBP7	Insulin-like growth factor binding protein 7	0.23	1.00E+00	-0.77	8.64E-05
	CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)	1.23	8.11E-01	-0.76	3.03E-06
	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	0.33	4.23E-01	-0.48	2.16E-03
	FABP3	Fatty acid binding protein 3, muscle and heart	1.23	8.11E-01	-0.46	5.97E-03
	APLN	Apelin*	0.13	9.40E-01	-0.42	2.01E-02
	VLDLR	Very low density lipoprotein receptor	1.23	8.11E-01	-0.40	1.04E-02
	SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	1.49	2.02E-05	-0.34	1.54E-02
	ASCL2	Achaete-scute family bHLH transcription factor 2	1.23	8.11E-01	-0.33	3.77E-02
	NEDD9	Neural precursor cell expressed, developmentally down-regulated 9	1.97	4.22E-01	-0.33	3.13E-02
	OXR1	Oxidation resistance 1	-0.23	4.31E-01	-0.33	2.00E-02
HIF-1α/-2α	BACE2	Beta-site APP-cleaving enzyme 2	0.10	7.57E-01	-0.33	2.48E-02
	CXCR4	Chemokine (C-X-C motif) receptor 4	3.47	2.68E-03	-0.40	1.03E-02
HIF-2α	CEMIP	Cell migration-inducing protein, hyaluronan binding*	-0.42	4.06E-01	-0.72	4.41E-06
	ACSS2	Acyl-CoA synthetase short-chain family member 2	-0.01	9.74E-01	-0.59	5.53E-05
p53	CYBRD1	Cytochrome b reductase 1	-0.12	6.99E-01	-0.29	4.44E-02
	IFITM1	Interferon induced transmembrane protein 1	-0.18	1.00E+00	-0.90	3.83E-06
	CDK15	Cyclin-dependent kinase 15	0.23	1.00E+00	-0.66	4.85E-04
	IFI27	Interferon, alpha-inducible protein 27	-0.22	8.87E-01	-0.47	8.41E-03
	ABCB4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	2.56	5.87E-01	-0.46	3.76E-02
	C2orf54	Chromosome 2 open reading frame 54	1.42	2.69E-01	-0.39	1.29E-02
	STRA6	Stimulated by retinoic acid 6	0.82	9.49E-01	-0.34	1.92E-02
	KCNJ5	Potassium channel, inwardly rectifying subfamily J, member 5	1.23	5.90E-01	-0.32	3.05E-02
	CXCL14	Chemokine (C-X-C motif) ligand 14	0.23	9.85E-01	-1.18	6.32E-08
	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	-0.77	8.79E-01	-0.70	2.53E-05
NF-kB	RGCC	Regulator of cell cycle	-0.06	9.37E-01	-0.39	1.01E-02
	ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa	2.23	4.56E-01	-0.37	2.10E-02
	TNFRSF19	Tumor necrosis factor receptor superfamily, member 19	-0.91	4.44E-01	-0.31	4.43E-02
	MPZL2	Myelin protein zero-like 2	0.14	6.87E-01	-0.29	4.40E-02
	JNK	CST1	Cystatin SN	0.23	9.85E-01	-0.97
JNK	DKK3	Dickkopf WNT signaling pathway inhibitor 3	1.23	3.63E-01	-0.58	7.87E-04
	SAA1	Serum amyloid A1	0.23	1.00E+00	-0.47	1.37E-03
	KRT5	Keratin 5, type II	2.18	1.04E-01	-0.33	1.85E-02
	TGM2	Transglutaminase 2	0.76	9.99E-03	-0.32	2.29E-02
	WNT6	Wingless-type MMTV integration site family, member 6	-0.13	9.27E-01	-0.33	2.97E-02
	Cholesterol biosynthesis	APOE	1.59	9.25E-02	-0.53	7.96E-04
Cholesterol biosynthesis	CCDC3	Coiled-coil domain containing 3	-0.31	3.14E-01	-0.35	1.62E-02
	KANK4	KN motif and ankyrin repeat domains 4	0.23	1.00E+00	-1.11	1.98E-07
	MYH7B	Myosin, heavy chain 7B, cardiac muscle, beta	0.13	9.79E-01	-0.93	1.13E-10
	UROCI	Urocanate hydratase 1	1.23	8.26E-01	-0.84	8.27E-07
	RASL11B	RAS-like, family 11, member B	0.89	6.74E-01	-0.83	7.84E-06
	RLBP1	Retinaldehyde binding protein 1	0.23	1.00E+00	-0.79	1.59E-04
	VIL1	Cillin 1	0.82	9.49E-01	-0.64	2.46E-03
	CD8B	CD8b molecule	0.23	1.00E+00	-0.58	1.55E-02
	CBS	Cystathionine-beta-synthase	-0.35	1.00E+00	-0.57	7.81E-04
	KCNA6	Potassium channel, voltage gated shaker related subfamily A, member 6	0.23	1.00E+00	-0.57	5.29E-03
	GYG2	Glycogenin 2	0.23	1.00E+00	-0.54	3.75E-03
	QPRT	Quinolate phosphoribosyltransferase	-0.18	1.00E+00	-0.51	2.11E-03
	CDH13	Cadherin 13	0.23	1.00E+00	-0.48	9.33E-03
	SDK1	Sidekick cell adhesion molecule 1	2.23	5.21E-01	-0.48	1.92E-03
	MYO1D	Myosin ID	-0.24	4.00E-01	-0.47	7.03E-04
	PSG1	Pregnancy specific beta-1-glycoprotein 1	0.23	9.85E-01	-0.44	4.14E-03
	ACSS1	Acyl-CoA synthetase short-chain family member 1	0.23	9.85E-01	-0.43	5.71E-03
Other	SLC7A7	Solute carrier family 7 (amino acid transporter light chain, y+L system), member 7	1.82	6.19E-01	-0.40	1.47E-02

Table I. *Continued*

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Related pathway	Symbol	Gene name	HCT116		SW480	
			Log2 fold change	p-Value	Log2 fold change	p-Value
	<i>APCDD1</i>	Adenomatosis polyposis coli down-regulated 1	2.82	3.27E-01	-0.39	5.14E-03
	<i>TMEM163</i>	Transmembrane protein 163	1.23	9.65E-01	-0.39	3.46E-02
	<i>SERPINA1</i>	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	-0.03	9.92E-01	-0.39	1.41E-02
	<i>ISM1</i>	Isthmin 1, angiogenesis inhibitor	0.12	9.29E-01	-0.38	1.90E-02
	<i>HUNK</i>	Hormonally up-regulated Neu-associated kinase	0.23	1.00E+00	-0.37	1.11E-02
	<i>ADAMTS15</i>	ADAM metalloproteinase with thrombospondin type 1 motif,15	-1.27	1.30E-01	-0.35	4.47E-02
	<i>KIF26B</i>	Kinesin family member 26B	0.30	7.25E-01	-0.35	4.46E-02
	<i>KRT13</i>	Keratin 13, type I	0.06	1.00E+00	-0.33	3.58E-02
	<i>TMEM139</i>	Transmembrane protein 139	0.74	3.51E-01	-0.32	3.53E-02
	<i>CLSTN2</i>	Calsynenin 2	0.23	9.85E-01	-0.31	4.72E-02
	<i>HEG1</i>	Heart development protein with EGF-like domains 1	0.31	3.43E-01	-0.29	4.65E-02
	<i>TMEM45B</i>	Transmembrane protein 45B	0.23	9.85E-01	-0.29	4.72E-02
	<i>SPTB</i>	Spectrin, beta, erythrocytic	-0.87	3.06E-02	-0.45	3.11E-03
	<i>ARL4C</i>	ADP-ribosylation factor-like 4C	-0.72	4.35E-02	-0.20	1.78E-01
	<i>KRT23</i>	Keratin 23, type I	-0.88	2.94E-02	-0.11	4.35E-01

Significant *p*-values (<0.05) are shown in bold and reflect genes significantly up-/down-regulated. *Genes down-regulated under both normoxia and hypoxia.

tumor xenografts, and had a therapeutic advantage in combination to radiation even compared with a well-known radiation-modulator, cisplatin (Figure 5A). For SW480 tumors, the combination treatment of radiation with NSC697923 inhibited tumor growth more than the combination of radiation and cisplatin, with tolerable toxicity (Figure 5B).

These results led us to hypothesize that the repression of HIF1 α and -2 α would not be always the main action mechanism of NSC697923, and that the nude mouse xenograft system would not always be able to correctly evaluate the activity of drugs targeting HIFs. NSC697923 might repress HIF1 α and -2 α and shrink lesions with high expression of these proteins. This might explain why the expression of HIF1 α and -2 α in residual tumors was apparently not related to the antitumor activity of NSC697923.

NSC697923 modulates several radiation-resistant pathways including, HIF1 α and -2 α , in SW480 cells. NSC697923 may have more pertinent molecular targets than HIF1 α and -2 α . We attempted to search for other probable action determinants of NSC697923 using NGS. RNA sequencing of radiation-sensitive HCT116 and radiation-resistant SW480 cells was performed after incubation under normoxia or hypoxia, with and without NSC697923 treatment. We then chose genes expressed at higher levels in radiation-resistant SW480 than in radiation-sensitive HCT116 cells under normoxic conditions. We also chose genes that were more

up-regulated under hypoxic conditions, and further down-regulated after NSC697923 treatment under both normoxic and hypoxic conditions in both cell lines, matched for any anticancer activity induced by the drug (Figure 6). We chose 68 genes (Table I) in normoxic, and 17 genes (Table II) in hypoxic conditions as possible targets of NSC697923. Among these genes, we noted apelin (*APLN*) and cell migration-inducing protein, hyaluronan binding protein (*CEMIP*), which have recently attracted attention as factors in HIF1 and -2 pathways.

Discussion

In this study, we found that NSC697923 can overcome radiation resistance *via* suppression of HIF1 α and -2 α expression in *in vitro* and *in vivo* analysis. For *in vivo* analysis, the combination of NSC697923 with radiation was more effective than that with an established radiation modulator, cisplatin. Next-generation RNA sequencing showed that NSC697923 treatment can suppress HIF family pathway genes, including *APLN* and *CEMIP*, under both normoxic and hypoxic conditions.

Radiation resistance is a serious problem for patients with CRC. Resistance is induced by various pathways, including hypoxia, receptor tyrosine kinase/protein kinase B (RTK/AKT), DNA damage repair, adhesion pathway, inflammation and developmental pathway (26, 27). In this study, we focused on the HIF pathway to overcome radiation

Table II. Genes significantly suppressed after NSC697923 treatment under hypoxia. We chose 17 genes altered under hypoxic conditions as possible targets of NSC697923 from the results of the next-generation RNA sequencing.

Related pathway	Symbol	Gene name	HCT116		SW480	
			Log2 fold change	p-Value	Log2 fold change	p-Value
HIF-1 α	<i>FGF11</i>	Fibroblast growth factor 11	-0.22	6.54E-01	-0.51	1.20E-02
	<i>APLN</i>	Apelin*	-0.24	8.33E-01	-0.48	1.90E-03
	<i>ETS2</i>	V-ets avian erythroblastosis virus E26 oncogene homolog 2	0.05	8.67E-01	-0.38	1.00E-02
	<i>ASNS</i>	Asparagine synthetase (glutamine-hydrolyzing)	-0.23	4.00E-01	-0.31	3.68E-02
	<i>MIR210HG</i>	MIR210 host gene	-0.77	1.36E-02	-0.42	3.04E-02
	<i>ZNF395</i>	Zinc finger protein 395	-0.62	2.31E-02	-0.36	1.58E-02
	<i>WDR54</i>	WD repeat domain 54	-0.85	4.70E-03	-0.22	2.18E-01
	<i>BNIP3</i>	BCL2/adenovirus E1B 19kDa interacting protein 3	-0.54	4.71E-02	-0.69	9.71E-01
HIF-2 α	<i>CEMIP</i>	Cell migration-inducing protein, hyaluronan binding*	-0.90	5.98E-02	-0.65	1.22E-05
p53	<i>FGD5</i>	FYVE, RhoGEF and PH domain containing 5	-1.42	7.47E-01	-0.36	1.82E-02
JNK	<i>TRPV4</i>	Transient receptor potential cation channel, subfamily V, member 4	-0.87	4.87E-01	-0.47	1.77E-02
Others	<i>KCNJ10</i>	Potassium channel, inwardly rectifying subfamily J, member 10	-0.92	5.70E-01	-0.49	9.52E-03
	<i>CHST15</i>	Carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	0.20	4.67E-01	-0.40	6.59E-03
	<i>GPR146</i>	G protein-coupled receptor 146	-1.29	1.33E-02	-0.25	6.27E-01
	<i>KIAA0895L</i>	KIAA0895-like	-0.72	1.91E-02	-0.08	7.69E-01
	<i>BCKDHA</i>	Branched chain keto acid dehydrogenase E1, alpha polypeptide	-0.67	2.98E-02	-0.18	2.77E-01
	<i>H1FO</i>	H1 histone family, member 0	-0.55	3.89E-02	-0.13	3.65E-01

Significant *p*-values (<0.05) are shown in bold and reflect genes significantly up-/down-regulated. *Genes down-regulated under both normoxia and hypoxia.

resistance because HIF1 α activity is well known to be associated with radiation resistance, angiogenesis, cell survival, and proliferation in cancer cells (28-30). Some representative HIF downstream genes have already been reported as angiogenesis mediators, including nitric oxide synthase (*NOS*), and vascular endothelial growth factor (*VEGF*); cell-survival regulators include insulin-like growth factor (IGF)-related factors and cell-proliferation regulators include *c-MYC*, *NOS*, and *IGF2*. Such genes are induced and associated with radiation resistance under hypoxic conditions. Numerous studies have analyzed putative HIF1 α and -2 α suppression mechanisms focusing on transcriptional level, translation, stabilization, dimerization, DNA binding, and transcriptional activity of down-stream genes (31, 32).

We also focused on the UBE2 family, which was already reported to suppress the Von Hippel-Lindau tumor suppressor, E3 ubiquitin protein (VHL) (28). VHL degrades HIF1 α and -2 α via proteasome systems, hence UBE2 family inhibitors can induce HIF suppression via VHL accumulation. Our data showed in both *in vitro* and *in vivo* analyses that the UBE2 inhibitor NSC697923 can suppress HIF1 α and -2 α expression and overcome radiation resistance. Interestingly, it was already reported that NSC697923 can induce anticancer effects by targeting some cancer-related genes, including nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- κ B) (24, 25), c-Jun N-terminal kinase (JNK) and *p53* (23), which are linked to radiation resistance in various types of cancer

(33, 34). Based on our results, we believe that the combination therapy of NSC697923 and radiation might be a promising strategy to overcome radiation resistance by suppressing the HIF1 α and -2 α family pathways.

We focused on NSC697923 as a radiation modulator under hypoxic conditions, but single-drug treatment can induce antitumor effects in both normoxia and hypoxia. Another possible pathway might be related to the action of NSC697923. Several recent studies have shown that NSC697923 induced cancer cell death by targeting the JNK pathway, DNA damage signaling, NF- κ B signaling, the mitogen-activated protein kinase (MAPK) pathway and *p53*-mediated apoptosis (23-25): *p53* is an important tumor suppressor, regulating cellular apoptosis, survival and HIF stabilization (35, 36). In our study, baseline expression of HIFs in SW480 cells was higher than that of HCT116 cells under normoxic conditions. It is possible that survival of SW480 cells, which harbor *p53* mutation, depends on the inactivation of *p53* and HIFs, compared with *p53* wild-type HCT116 cells. From these previous reports, it has been suggested that NSC697923 monotherapy might have antitumor effects via induction of *p53*-induced apoptosis.

From our *in vivo* results, it is possible that NSC697923 might have molecular targets other than HIF1 α and -2 α : Our RNA sequencing data showed that HIF1 α -related *APLN* and HIF-2 α -related *CEMIP* genes were down-regulated after NSC697923 treatment under both normoxic and hypoxic

conditions. Previous findings showed that *CEMIP* is a downstream effector of HIF2 α -mediated CRC aggressiveness, and directly facilitated colon tumor growth; high expression correlated with poor outcome in advanced stages of CRC (37, 38). In addition, *APLN* protected cells from apoptosis and was a potent activator of tumor angiogenesis (39, 40). Therefore, tumor-suppressive mechanisms of NSC697923 treatment under normoxic and hypoxic conditions might depend on *APLN* and *CEMIP*, making them promising downstream targets for HIF family in CRC.

Neo-adjuvant radiation and chemoradiotherapy (CRT) are very important therapeutic tools in preventing local recurrence and permanent colostomy after abdominal peritoneal resection (41-43). In the case of radiation-sensitive CRC cells, a pathological complete response is estimated as being achieved in 15-27% of patients treated with CRT (44), patients with CRC refractory to these therapies often have poor outcomes (45). CRT causes side-effects including pain, urological problems, gastrointestinal bleeding, proctitis and ileus (46, 47). From our *in vivo* data, the toxicity of the combination of NSC697923 and radiation was tolerable, therefore NSC697923 might be useful in patients with colorectal cancer because its combination with radiation can significantly induce cell death even in radiation-resistant CRC cells. During treatment, modern radiation technologies, including tomotherapy, intensity-modulated radiotherapy, volumetric intensity-modulated arc therapy and image-guided radiotherapy can protect surrounding normal tissues, and precisely focus on the target radiation field (48-50). These advantages might enable use of this combination therapy with less toxicity for patients with CRC.

In conclusion, this study showed that the UBE2 inhibitor NSC697923 mitigates radiation resistance through suppression of HIF1 α and -2 α . NSC697923 might be particularly effective against hypoxia-induced radiation resistance by targeting HIF downstream pathway genes, including *APLN* and *CEMIP*. In considering new molecular cancer therapies, NSC697923 may be a promising radiation modulator against refractory CRC.

Ethics Approval and Consent to Participate

Mouse experiments were performed in compliance with the guidelines of the Institute for Laboratory Animal Research at Gunma University, Maebashi, Japan.

Competing Interests

The Authors declare that they have no competing interests in regard to this study.

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