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Up-regulation of UVRAG by HDAC1 Inhibition Attenuates 5FU-induced Cell Death in HCT116 Colorectal Cancer Cells

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Abstract. *The ultraviolent irradiation resistance-associated gene (UVRAG), a component of the Beclin 1/autophagy-related 6 complex, regulates the autophagy initiation step and functions in the DNA-damage response. UVRAG is frequently mutated in various cancer types, and mutations of UVRAG increase sensitivity to chemotherapy by impairing DNA-damage repair. In this study, we addressed the epigenetic regulation of UVRAG in colorectal cancer cells. UVRAG expression was increased in cells treated with histone deacetylase (HDAC) inhibitors, such as valproic acid and suberoylanilide hydroxamic acid. Down-regulation of HDAC1 enhanced UVRAG expression in colorectal cancer cells. In addition, both chemical and genetic inhibition of HDAC1 reduced the activation of caspase-3 and cytotoxicity in 5-fluorouracil (5FU)-treated cancer cells. In contrast, UVRAG overexpression inhibited caspase activation and cell death in 5FU-treated cells. Taken together, our findings suggest that up-regulation of UVRAG by HDAC1 inhibition potentiates DNA-damage–mediated cell death in colorectal cancer cells.*

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Autophagy has important roles in cellular homeostasis through the degradation of useless or damaged proteins and organelles *via* lysosomes (1, 2). The primordial function of autophagy is a response to stress, such as starvation, oxidative stress, and ion stress (1, 2). Given that multiple signaling pathways are involved in the regulation of autophagy progress, various autophagy-related (ATG) proteins and other autophagy-regulatory proteins have been identified (2). The early years of autophagy research focused on the dynamic membrane rearrangements and post-translational modifications of ATG proteins, whereas recent progresses has elucidated its regulation by gene expression (3-6). Several transcription factors, such as farnesoid X receptor (FXR), cAMP response element-binding protein, and transcription factor EB (TFEB) coordinately control major autophagy regulators (3, 4). In addition, autophagy-related and lysosomal genes are up-regulated through direct binding of TFEB to co-activator-associated arginine methyltransferase 1 (CARM1) under starvation conditions (5). Recently our group also reported that methyltransferase G9 inhibition leads to increased expression of autophagy-related genes and directly regulates BECLIN 1 (BECN1)/ATG6 expression (6).

Among various autophagy regulatory proteins, the ultraviolent irradiation resistance-associated gene (*UVRAG*) is a putative mammalian ortholog of the yeast Vps38 component recruiting BECN1/PI3K complex protein. UVRAG promotes autophagy *via* activating the BECN1/PI3K complex, but suppresses apoptosis and tumorigenicity of cancer cells by inhibiting cell proliferation and activation of BCL2 associated X, apoptosis regulator (7, 8). *UVRAG* frameshift leads to its expression as a truncated form in colorectal cancer (9). Moreover, UVRAG prevents cells from accumulating

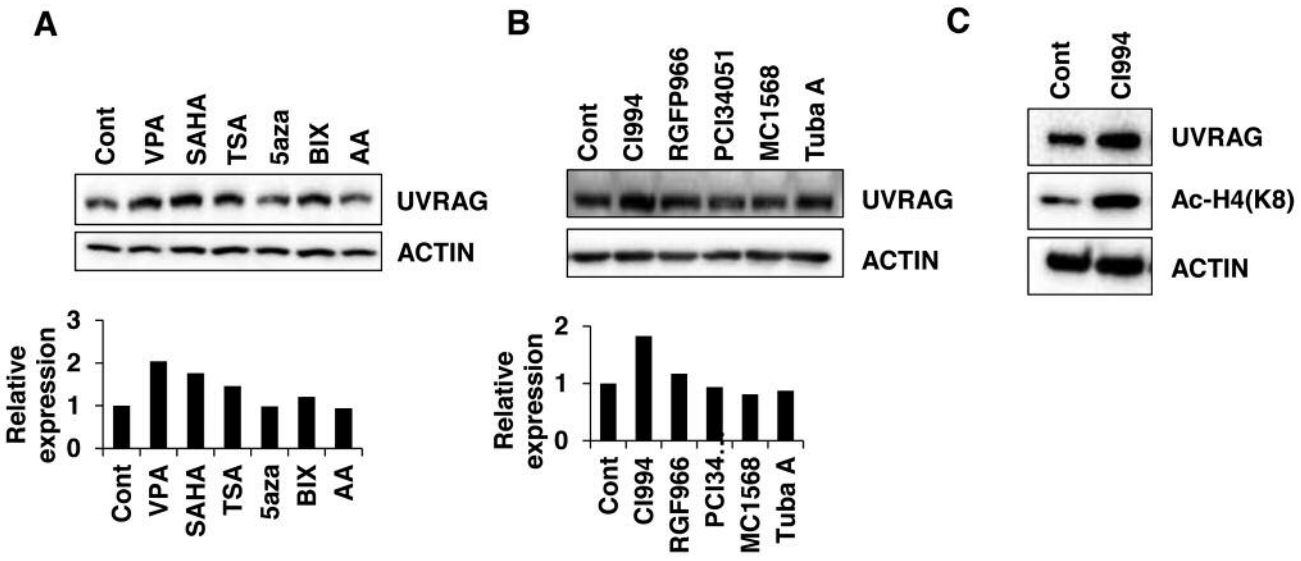

Figure 1. *Ultraviolent irradiation resistance-associated gene (UVRAG) expression is induced by histone deacetylase (HDAC) inhibitor in HCT116 cells. A: HCT116 cells were treated with dimethylsulfoxide (Cont) or epigenetic regulators valproic acid (VPA;1 mM for 72 h), suberoylanilide hydroxamic acid (SAHA; 5 μM for 24 h), trichostatin A (TSA; 300 nM for 24 h), 5-aza-2'-deoxycytidine (5aza; 5 μM for 24 h), BIX-01294 (BIX; 10 μM 24 h) or anacardic acid (AA; 5 μM for 24 h). The cells were harvested and analyzed by western blotting with anti-UVRAG. B: HCT116 cells were incubated with specific HDAC inhibitors, CI994 (2.5 μM), RGFP966 (10 μM), PCI34051 (5 μM), MC1568 (10 μM) or tubastatin A (Tuba A; 10 μM) for 24 h. UVRAG expression was assessed by western blotting and normalized with actin expression. C: HCT116 cells were treated with CI994 (2.5 μM) for 24 h treated and expression of UVRAG and acetyl histone (Ac-H4, K8) were analyzed by western blotting.*

abnormal chromosomes, thereby, developing oncogenic mutation (10). Because autophagy maintains cellular homeostasis under various stress conditions, dysregulation of autophagy is involved in many diseases such as cancer, diabetes and neurodegeneration (11). However, the precise regulatory mechanism of autophagy is not fully understood. In this study, we addressed epigenetic regulation for autophagy regulators and found that UVRAG is modulated by histone deacetylase (HDAC) in colorectal cancer cells.

Materials and Methods

Reagents. Valproic acid (VPA), trichostatin A, BIX-01294, 5-aza-2'-deoxycytidine, anacardic acid, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Suberoylanilide hydroxamic acid (SAHA) was provided by Crystal Genomics Co. (Seoul, S. Korea). Inhibitors CI994, RGFP966, PCI34051, MC1568 and tubastatin A were purchased from Selleckchem (Houston, TX, USA). 5-Fluorouracil (5FU) was purchased from JW Pharma (Seoul, S. Korea).

Cell culture and transfection. HCT116 colorectal cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas VA, USA). The cells were cultured at 37˚C in a 5% CO_2 incubator and maintained in RPMI-1640 containing 10% fetal bovid serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Validated siRNAs targeting *HDAC1* (#1, 5'-CACCCGGA GGAAAGTCTGTTA-3'; #2, 5'-GACGAGTCCTATGAGGCCATT-3') or scrambled control siRNA (Sc; 5'-CCUACGCCACCAA UUUCGU-3') were synthesized from Genolution (Seoul, S. Korea). Both plasmids and siRNAs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

MTT cell viability assay. HCT116 cells (~10,000) cultured in 96-well plates were either treated with CI994 (2.5 μM for 24 h) or transfected with *HDAC1* siRNA for 48 h. Then the cells were treated with or without 20 mg/ml 5-fluorouracil for additional 24 h. For MTT assay, 10 μl MTT solution (5 mg/ml) was added to the cells which were then incubated at 37˚C for 4 h. The reaction was stopped with solubilizing solution (10% SDA and 0.01 M HCl) to each well. The absorbance change was measured at 570 nm by a microplate reader (VICTOR X3; PerkinElmer, Waltham, MA, USA). The cell viability was calculated as follows: chemical treated cells/untreated cells ×100.

Western blotting. HCT116 cells treated with VPA (1 mM for 72 h), SAHA (5 μM for 24 h), trichostatin A (300 nM for 24 h), 5-aza-2'-deoxycytidine (5 μM for 24 h), BIX-01294 (10 μM 24 h), anacardic acid (5 μM for 24 h), CI994 (2.5 μM for 24 h), RGFP966 (10 μM for 24 h), PCI34051 (5 μM for 24 h), MC1568 (10 μM for 24 h) or tubastatin A (10 μM for 24 h) were harvested using cell lysis buffer. All cell lysates were prepared with 2× Laemmli sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 25% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue] (BioRad, Hercules, CA, USA). Proteins (approximately 50 μg) were

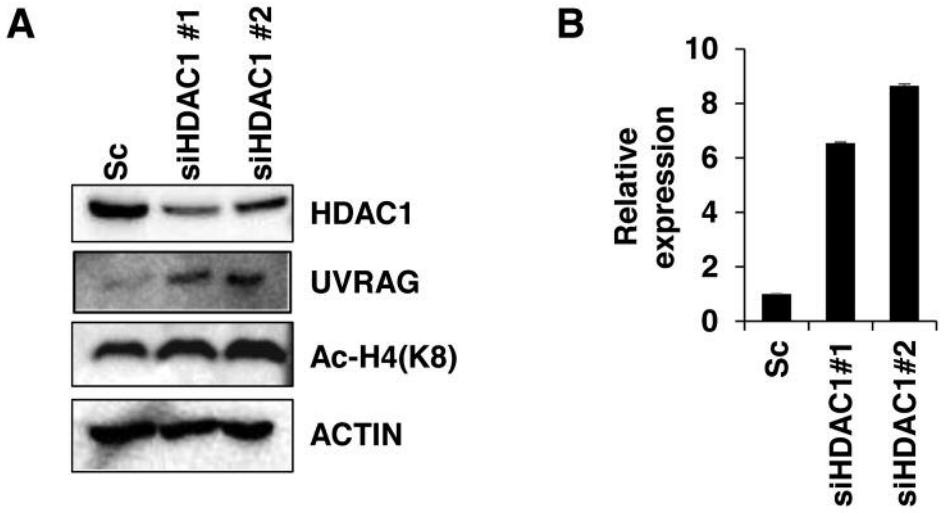

Figure 2. *Histone deacetylase 1 (HDAC1) suppression induces ultraviolent irradiation resistance-associated gene (UVRAG) expression in HCT116 cells. A: HCT116 cells were transiently transfected with either siRNA against HDAC1 (siHDAC1 #1 and #2) or scrambled siRNA (Sc) as a negative control. After 3 days, the cells were analyzed by western blotting and the level of HDAC1 and UVRAG protein was detected with specific antibodies. B: The protein expression was quantified by densitometric image analysis of the western blots.*

quantified by using Bradford solution (BioRad). Then the samples were resolved by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane. After blocking, the membranes were incubated with specific primary antibodies; anti-UVRAG (ab174550, Abcam, Cambridge, UK), anti-acetyl histone (sc-8660; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HDAC1 (sc7872; Santa Cruz Biotechnology), anti-poly (ADP-ribose) polymerase (#9542; Cell Signaling, Beverly, MA, USA), anti-cleaved caspase 3 (#9661; Cell Signaling), anti-green fluorescent protein (GFP) (sc8334; Santa Cruz Biotechnology) and anti-actin (#MAB1501; Milipore, Temecula, CA, USA). For protein detection, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and signals were detected with EzWestLumi Plus (ATTO, Tokyo, Japan).

Statistical analysis. Data were obtained from least three independent experiments, and presented as means±S.E.M. Statistical evaluation of the results was performed with one-way ANOVA. Data represent±standard error of the mean (S.E.M.) from more than three independent experiments, n=3.

Results

UVRAG expression is increased by HDAC inhibitors in colorectal cancer cells. To identify novel epigenetic target proteins, we addressed expression change of various autophagy regulatory proteins in the context of epigenetic changes in colorectal cancer cells. HCT116 cells were treated with different chromatin remodeling inhibitors, including HDAC inhibitors. Then we analyzed the level of ATG proteins such as ATG4, ATG6, ATG7, ATG10, WD repeat protein interacting with phosphoinositide 2 (WIPI2), and UVRAG. Among the investigated proteins, we found that UVRAG expression was highly increased in HCT116 cells-treated with VPA and SAHA (Figure 1A). However, other proteins were not notably altered under those conditions (data not shown), suggesting that expression of UVRAG is influenced by HDAC inhibitors. HDAC proteins are grouped into four classes (class I, IIa, IIb, III, and IV) based on their sequence similarity and function (12).

As *UVRAG* was up-regulated by HDAC inhibitors VPA and SAHA, we further addressed which HDAC proteins regulate UVRAG expression. HCT116 cells were treated with subtype-selective inhibitors: class I inhibitors: CI994, RGF966 and PCI34051; class IIa inhibitor MC1568; and class IIb inhibitor tubastatin A. Interestingly, among the tested HDAC inhibitors, UVRAG expression was substantially increased in cells treated with CI994 (Figure 1B). HDAC1, HDAC2, HDAC3, and HDAC8 are class I HDAC proteins, but CI994 has higher selective inhibition of HDAC1 and HDAC3 than other HDAC proteins. However, UVRAG expression was not changed by another class I inhibitor, RGFP966, which has highly selective inhibition for HDAC3 (Figure 1B). These results imply that HDAC1 is involved in UVRAG expression in HCT116 cells (Figure 1B). We also confirmed increased histone acetylation in cells treated with HDAC inhibitor CI994 (Figure 1C).

To further examine whether UVRAG expression is controlled by HDAC1, HDAC expression was suppressed by specific siRNA and UVRAG expression was investigated. Consistent with the chemical inhibitor experiment, reduction

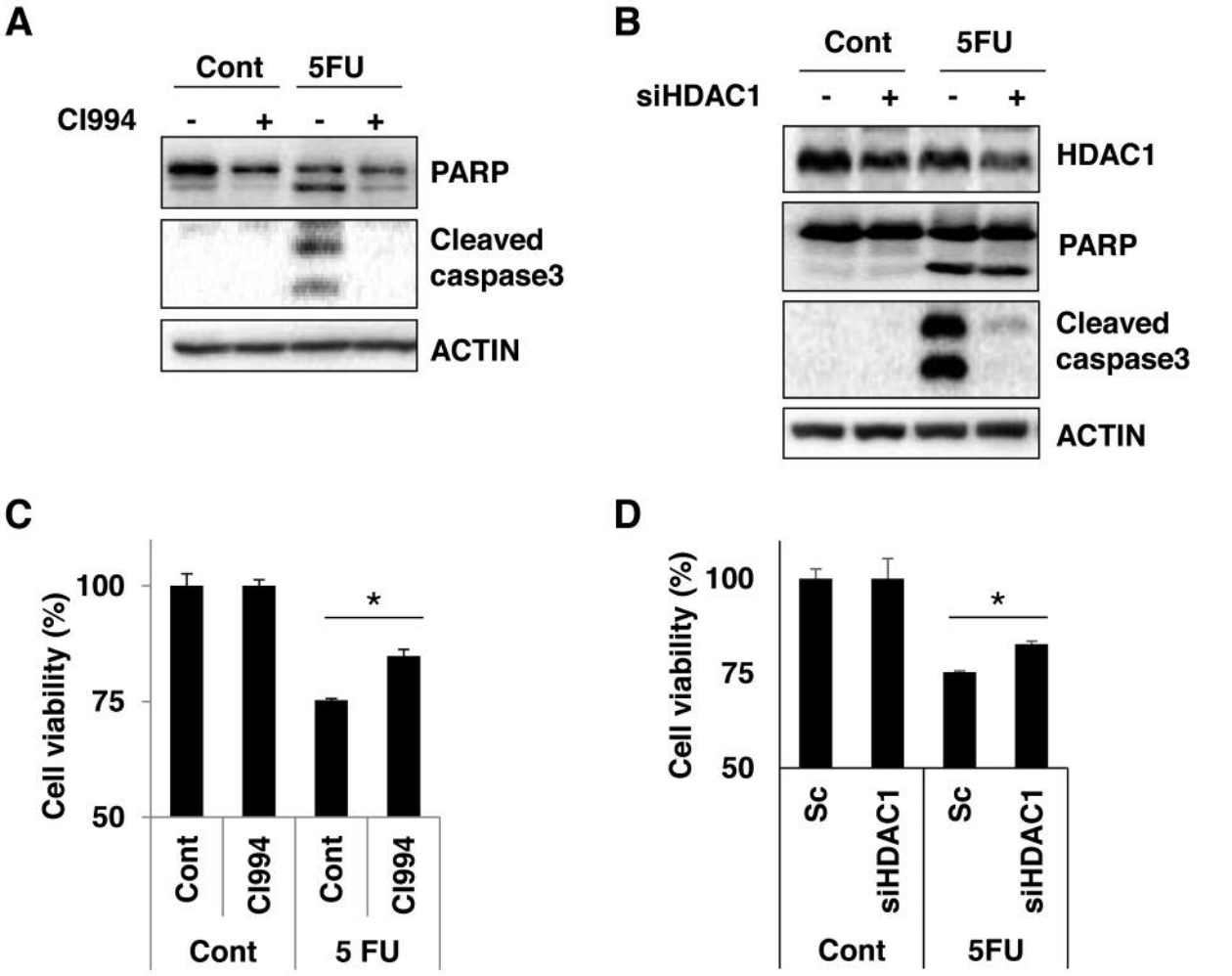

Figure 3. *Inhibition of histone deacetylase 1 (HDAC1) attenuates cell death in 5-fluorouracil (5FU)-treated cells. A: HCT116 cells pre-treated with HDAC inhibitor CI994 were additionally incubated in the presence or absence of 5FU (20 mg/ml) for 24 h. The cells were subjected to western blotting with antibodies to poly (ADP-ribose) polymerase (PARP) and cleaved caspase-3. B: HCT116 cells were transfected with HDAC1 siRNA. After 48 h transfection, the cells were further incubated with 5FU (20 mg/ml) for 24 h. The cells were analyzed with western blotting with antibodies to HDAC1, PARP and cleaved caspase-3. C: HCT116 cells pre-treated with CI994 were additionally incubated in the presence or absence of 5FU (20 mg/ml) for 24 h. Then, cell viability was measured by the MTT assay. D: HCT116 cells were transfected with HDAC1 siRNA. After 48 h transfection, the cells were incubated with 5FU (20 mg/ml) for 24 h. Then cell viability was measured by the MTT assay. Data are presented as the mean±SEM from three independent experiments (n>3). *Significantly different at p<0.05).*

of HDAC1 by RNAi resulted in increased UVRAG expression as well as histone acetylation in HCT116 cells (Figure 2). Taken together, these results indicated that HDAC1 negatively controls UVRAG expression in colorectal cancer cells.

Inhibition of HDAC1 attenuates 5FU-induced cell death in HCT116 cells. Since UVRAG is a multifunctional protein that suppresses cell death, we further investigated a role of induced UVRAG expression by inhibiting HDAC1 in 5FU-treated cells. 5FU, a pyrimidine analog, functions *via* the irreversible inhibition of thymidylate synthase and has been used in the treatment of various types of cancers, including colorectal, breast, gastrointestinal and cervical cancer. In accordance with this notion, we observed an increase of caspase-3 activation and cleavage of PARP1 in 5FU-treatd HCT116 cells (Figure 3A). However, both the treatment with CI994 and knockdown of *HDAC1* significantly suppressed the increased activation of caspase-3 in 5FU-treated cells (Figure 3A and B). In addition, cell death induced by 5FU was slightly, but significantly suppressed in both CI99-treated cells and HDAC1 knockdown cells (Figure 3C and D). These results suggest that *HDAC1* knockdown reduces 5FU-mediated cell death in HCT116 cells.

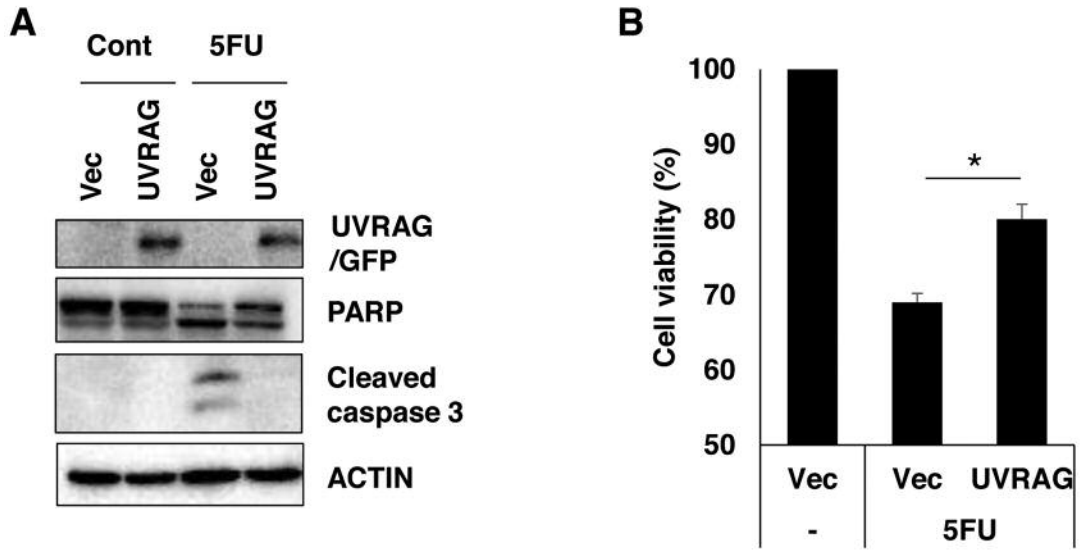

Figure 4. *Overexpression of ultraviolent irradiation resistance-associated gene (UVRAG) suppresses 5-fluorouracil (5FU)-mediated cytotoxicity in HCT116 cells. A: HCT116 cells were transfected with a green fluorescent protein (GFP) control plasmid (Vec) or GFP-fused UVRAG plasmid (UVRAG). After 24 h, the cells were treated with 20 mg/ml 5FU for 24 h and expression of poly (ADP-ribose) polymerase (PARP) and cleaved caspase-3 was determined by western blotting. B: HCT116 cells were transfected with GFP control plasmid (Vec) or GFP-fused UVRAG plasmid (UVRAG) and treated with 10, 20, or 50 mg/ml 5FU for 24 h, and cell viability of these cells was then determined using a MTT assay. Data are presented as the mean±SEM from three independent experiments (n>3). *Significantly different at p<0.05.*

UVRAG suppresses 5FU-induced cell death in HCT116 cells. We addressed the effect of UVRAG overexpression on 5FU-induced cell death. HCT116 cells transiently transfected with *UVRAG* plasmid were treated with 5FU, and cell death was determined. As shown in Figure 4, ectopic expression of UVRAG suppressed cell death, as well as caspase activation in 5FU-treated cells. Thus, these results suggest that HDAC1-mediated up-regulation of *UVRAG* expression inhibits 5FU-induced cell death in HCT116 cells.

Discussion

Epigenetics affects various cellular and physiological traits, and a number of studies have shown that epigenetic changes also regulate autophagy (3, 4, 13, 14). Recently, our group also reported that ATG6 is transcriptionally repressed by euchromatic histone-lysine *N*-methyltransferase 2 in cancer cells (6). Nonetheless, epigenetic regulation of the expression of various autophagy regulatory proteins has not been elucidated clearly. In this study, we revealed that UVRAG expression is also epigenetically regulated by HDAC1. It was previously reported that UVRAG expression is controlled at both transcriptional and post-transcriptional levels (15-18). Some microRNAs, such as *Mir125a* and *Mir351*, directly target and reduce *UVRAG* expression, which lead to the inhibition of autophagy (15). In addition, *Mir183* targets *UVRAG* resulting in negative regulation of *UVRAG* expression in colorectal cancer (16). Furthermore, protein kinase B (PKB/AKT1)1 inhibits autophagy by reducing UVRAG expression in breast cancer, whereas hepatitis C virus increases autophagy and UVRAG expression by its replication (17, 18). Here, we further revealed that *UVRAG* is epigenetically regulated. Inhibition of HDAC by chemical inhibitors or genetic knockdown resulted in up-regulation of *UVRAG* in colorectal cancer cells (Figures 1 and 2).

UVRAG has been implicated in the formation and maturation of autophagosomes and suppression tumorigenicity (19, 20). In addition, UVRAG activates the DNA double-strand-break repair system through the increase of DNA protein kinase (21). Consequently, dysregulation of UVRAG increases genetic instability and sensitivity in irradiated cells (21). Deficiency of DNA mismatch repair occurs in over 15% of sporadic colorectal cancer and 90% of hereditary nonpolyposis colorectal cancer cases, resulting in microsatellite instability (MSI) (22), and truncated mutations of *UVRAG* with MSI were reported in colorectal cancer (23). Interestingly, loss of function by truncated mutations of *UVRAG* increases chemosensitivity to common anticancer agents, such as 5FU, oxaliplatin, and irinotecan in colorectal cancer (9). In accordance with the reports, we also found that forced expression of *UVRAG* expression suppressed 5FU-mediated cell death in colorectal cancer cells (Figure 4).

Among the class I HDACs, HDAC1, HDAC2, HDAC3 are found in the nucleus, while HDAC8 is localized in both nucleus and cytoplasm (24), and class I HDACs show diverse effects in tumors. In particular, HDAC1 is enhanced in breast, gastric, hepatocellular, lung, pancreatic, and prostate cancer (25, 26). Moreover, increased HDAC1 is associated with invasion, differentiation and poor prognosis in various cancer types (25, 26). In contrast, inhibition of HDAC1 accelerates leukemogenesis in the early stages, and depletion of HDAC1 leads to increase survival in established tumor cells (26). In this study, we also found that inhibition of HDAC1 suppressed 5FU-mediated cell death in colorectal cancer cells (Figure 3). However, *UVRAG* is frequently found to be mutated in common types of cancer (9, 27). For example, He *et al*. recently showed that *UVRAG* is expressed as a truncated mutation (frame shift mutation) which abrogates the normal functions of UVRAG such as autophagy in colorectal cancer (9). The frame shift mutation of *UVRAG* found in colorectal cancer renders SW480 cancer cells more sensitive to 5FU-mediated chemotherapy(9). Given that individual tumors display extensive intra tumoral heterogeneity, and HDACs epigenetically regulate multiple targets, the effects of inhibition of HDACs on cancer are very complicated and controversial in different cancer cells (28). Nonetheless, HDAC inhibitors represent a promising class of new anticancer agents (29). The disruption of multiple pathways by HDAC inhibitors could contribute to the cytotoxicity found in many of the clinical trials (29). Thus, further investigations are necessary to understand the molecular mechanism of HDAC on UVRAG expression.

In conclusion, the results from our study suggest that UVRAG expression is epigenetically regulated by HDAC1, and increased UVRAG expression suppresses 5FU-mediated cell death in colorectal cancer cells.

Acknowledgements

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