TP53 mutation influences the efficacy of treatment of colorectal cancer cell lines with a combination of sirtuin inhibitors and chemotherapeutic agents

HAO YANG[1,2], YA CHEN[2], YUAN JIANG[2], DONGLIANG WANG[2], JUN YAN[1] and ZHAOLI ZHOU[2]

[1]Department of Oncology, Jiading District Central Hospital Affiliated to Shanghai University of Medicine and Health Sciences, Shanghai 201800; [2]Shanghai Key Laboratory of Molecular Imaging, Shanghai University of Medicine and Health Sciences, Shanghai 201318, P.R. China

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Abstract. Chemoresistance of colorectal cancer (CRC) leads to tumor recurrence and metastasis and new strategies are urgently needed to improve the outcomes of conventional chemotherapy. Sirtuin (SIRT) inhibitors prevent tumor cell growth by increasing the levels of acetylated histones and non-histones, as well as disrupting survival-related pathways. The aim of the present study was to determine the effect of SIRT inhibitors on CRC chemotherapy. The CompuSyn software program was used to evaluate the synergistic or antagonistic effects of various drugs, and the status of the protein deacetylation regulatory genes in microarray datasets were analyzed using bioinformatics. In HCT116 cells expressing wild-type (wt) *TP53*, SIRT inhibitors were found to act antagonistically with multiple chemotherapeutic agents (cisplatin, 5-fluorouracil, oxaliplatin, gefitinib, LY294002 and metformin), and decreased the anti-tumor effects of these agents. By contrast, SIRT inhibitors sensitized *TP53*-mutant (mut) SW620 cells to various chemotherapeutic drugs. Bioinformatics analysis indicated that SIRT1 and protein deacetylation related genes were highly expressed in $TP53^{wt}$ CRC cells when compared to $TP53^{mut}$ cells. Therefore, it was hypothesized that the likely mechanism underlying the antagonistic effect of SIRT inhibitors on $TP53^{wt}$ CRC cells was a reduction in the level of stable p53 protein. The present results indicated that divergent *TP53* status may translate to a different chemosensitivity profile, and suggested that a combination therapy of SIRT inhibitors and first-line chemotherapeutic drugs may be beneficial for the treatment of patients with $TP53^{mut}$ CRC.

Correspondence to: Professor Zhaoli Zhou, Shanghai Key Laboratory of Molecular Imaging, Shanghai University of Medicine and Health Sciences, 279 Zhouzhu Road, Shanghai 201318, P.R. China
E-mail: zhouzl@sumhs.edu.cn

Dr Jun Yan, Department of Oncology, Jiading District Central Hospital Affiliated to Shanghai University of Medicine and Health Sciences, 1 Chengbei Road, Shanghai 201800, P.R. China
E-mail: yanjun1040@126.com

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Introduction

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide and is usually not diagnosed until it is at the advanced or metastatic stage (1). Surgical resection is the primary treatment option for CRC, followed by chemotherapy for patients who cannot undergo surgery (2). First-line drugs, such as irinotecan, oxaliplatin, fluorouracil, capecitabine and calcium folinate, and more recently developed targeted drugs, such as bevacizumab, cetuximab and gefitinib, as well as combinations of these drugs have been used against CRC (3). However, patients frequently develop chemoresistance, which is the major cause of treatment failure (4). Some new drug combinations (5,6) and genetic interventions (7,8) have achieved tumor cell chemo-sensitization. For example, the combination therapy of oxaliplatin and coxsackievirus A11 increases the oncolytic activity in oxaliplatin-resistant CRC cells (5). Guanine nucleotide-binding protein subunit β-5 knockdown enhances cetuximab cytotoxicity in KRAS-mutant CRC cells (8). Nevertheless, drug resistance is still a major challenge that needs to be managed in order to improve therapeutic efficacy.

Sirtuins (SIRTs) are NAD^+-dependent protein deacetylases that are localized to specific cellular compartments, including the nucleus (SIRT1, SIRT6 and SIRT7), cytoplasm (SIRT2 and SIRT5) and mitochondria (SIRT3 and SIRT4) (9). SIRTs act as tumor activators or suppressors through regulating metabolism, genomic stability or cancer stem cell proliferation (10,11). SIRT expression levels in CRC cells are correlated with chemosensitivity (12). Prolonged exposure to drugs can promote SIRT1-induced mitochondrial oxidative phosphorylation, resulting in chemoresistance and tumor survival (13), while deletion of SIRT2 confers resistance to MEK inhibitors in KRAS mutant (mut) CRC cells (14). Resveratrol-mediated inhibition of CRC cells is accompanied by DNA damage and SIRT6 upregulation (15). SIRT inhibitors, including

EX527 (an inhibitor of SIRT1), AGK2 (an inhibitor of SIRT2) and sirtinol (an inhibitor of SIRT1 and SIRT2) have shown anti-neoplastic effects in CRC cells (16-18). However, it is unclear whether different SIRT inhibitors act synergistically or antagonistically when combined with other chemotherapeutic drugs against CRC.

In the present study the effect of multiple SIRT inhibitors and other drugs on *TP53* in wild-type (wt) and mut CRC cell lines was analyzed. Bioinformatics analysis was additionally used to indicate the status of SIRT1 and protein deacetylation regulatory genes in $TP53^{wt}$ CRC cells compared to the $TP53^{mut}$ cells. The likely mechanism underlying the antagonistic effect of SIRT inhibitors with other agents was explored in $TP53^{wt}$ CRC cells. These data suggested that the sensitivity of CRC cells to multiple drug combinations is governed by the p53 mutation status.

Materials and methods

Cell lines and culture conditions. CRC cell lines HCT116 (ATCC® CCL-247™, $KRAS^{mut}$ and $TP53^{wt}$) and SW620 (ATCC® CCL-227TM, $KRAS^{mut}$ and $TP53^{mut}$ R273H) were obtained from American Type Culture Collection and tested for mycoplasma contamination and STRs were confirmed. The characteristics of SW620 cells have been previously defined in relevant studies (19,20) and on the ATCC website (https://www.atcc.org/products/all/CCL-227.aspx#characteristics). Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37˚C under 5% CO_2.

Chemotherapeutic agents. All chemotherapeutic agents were purchased from Selleck Chemicals LLC and the following stock solutions were prepared in dimethyl sulfoxide (DMSO) or PBS: 1 M nicotinamide (NAM), 50 mM EX527, 10 mM AGK2, 2 mg/ml cisplatin, 25 mg/ml 5-fluorouracil (5-FU), 10 mM irinotecan, 10 mg/ml oxaliplatin, 10 mg/l paclitaxel, 10 μM gefitinib, 5 mg/ml LY294002, 2 M dichloroacetate (DCA) and 1.5 M metformin. All drugs were freshly added to the medium for the experiments.

Cytotoxicity assay. HCT116 and SW620 cells were seeded at a density of $1x10^4$ cells/well in 96-well plates and allowed to adhere for 24 h. The cells were then treated with drugs in triplicate at the indicated concentrations for 72 h. After the medium was discarded, fresh medium containing 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each well. The absorbance values at 450 nm were measured and cell viability was calculated as the ratio of the absorbance values between the drug-treated and equal dose vehicle (up to 0.5% DMSO in PBS)-treated cells. IC_{50} values of the different drugs were determined using inhibition dose-response curves with variable slopes, as previously described (21).

Drug screening. The synergistic or antagonistic effects of various drugs were analyzed according to the Chou-Talalay method (22) using the CompuSyn software program (Version 1.0.1; ComboSyn, Inc.). The combination index (CI) was calculated as $D_1/D_{x1} + D_2/D_{x2}$, wherein D_1 or D_2 are the inhibitory concentrations of the individual drugs and D_{x1} or D_{x2} the inhibitory concentration of the drugs when used in combination. A CI<1 and >1 indicate synergistic and antagonistic effects, respectively (22). The $-\log_{10}$ of the CI value was used to define chemo-sensitization (positive value) or antagonism (negative value). At least three independent experiments were performed.

Cell cycle assay. CRC cell lines were treated with vehicle (0.1% DMSO in PBS), cisplatin (2 and 0.2 μg/ml), NAM (3 and 5 mM) or a combination of these drugs for 72 h at 37˚C. The treated cells were fixed in 70% ethanol for at least 12 h at -20˚C, followed by incubation with 500 μl propidium iodide/RNase Staining Buffer Solution (BD Pharmingen; BD Biosciences) for 15 min at room temperature. The stained cells were assessed by flow cytometry using a FACSMelody Flow Cytometer (BD Biosciences) and the proportion of cells in the different cell cycle stages were analyzed using the Modfit LT software (version 3.1; Verity Software House).

Western blotting. HCT116 and SW620 cells were treated with 5 mM NAM or vehicle (PBS) and lysed on ice with RIPA buffer (Nanjing KeyGen Biotech Co., Ltd.) containing protease inhibitors. The lysates were centrifuged at 15,000 x g, 4˚C for 20 min to remove the cell debris and the concentration of total protein was determined using the BCA Protein Quantification kit [Yeasen Biotechnology (Shanghai) Co., Ltd.]. 20 μg (5-20 μl volume) protein in each lane were separated by SDS-PAGE (7.5% separating gel) and transferred to PVDF membranes (GE Healthcare). The membranes were sequentially incubated with the primary antibodies overnight at 4˚C and secondary antibodies for 1 h at room temperature and the bands were detected using ECL HRP substrate (Sigma-Aldrich; Merck KGaA) in the bioanalytical imaging system c300 (Azure Biosystems, Inc.). The following primary antibodies were used: anti-p53 (cat. no. 10442-1-AP; ProteinTech Group, Inc.), anti-histone H3K9 acetylation (cat. no. A7255; ABclonal, Inc.), anti-histone H3 (cat. no. 17168-1-AP; ProteinTech Group, Inc.), anti-phospho-p53 (cat. no. 9284; Cell Signaling Technology, Inc.), anti-p21 (cat. no. 10355-1-AP; ProteinTech Group, Inc.), anti-SIRT1 (cat. no. 60303-1-Ig; ProteinTech Group, Inc.) and anti-GAPDH (cat. no. G9545; Sigma-Aldrich; Merck KGaA). The anti-GAPDH antibody was used as the reference antibody. HRP-labeled goat anti-rabbit (cat. no. KGAA35; Nanjing KeyGen Biotech Co., Ltd.) and goat anti-mouse (cat. no. KGAA37; Nanjing KeyGen Biotech Co., Ltd.) secondary antibodies were used.

Microarray datasets and gene set enrichment analysis (GSEA). Gene expression profiles of $TP53^{wt}$ and $TP53^{mut}$ CRC and other tumor cell lines (GSE41258, GSE57343) were downloaded from the Gene Expression Omnibus (GEO) database (accessed on April 22nd, 2019) (23,24). The GSE41258 dataset included the expression data of the $TP53^{wt}$ lines HTB39 (GSM1012660), LNCaP (prostate cancer cell line, GSM1012661) and LOVO (GSM1012662). The datasets also contained the $TP53^{mut}$ lines DLD1 (S241F, GSM1012656), HCT15 (S241F P153A, GSM1012657), HT29 (R273H, GSM1012659), SW1116 (A159D, GSM1012665), SW620 (R273H, GSM1012666) and WiDr (R273H, GSM1012667). The GSE57343 dataset

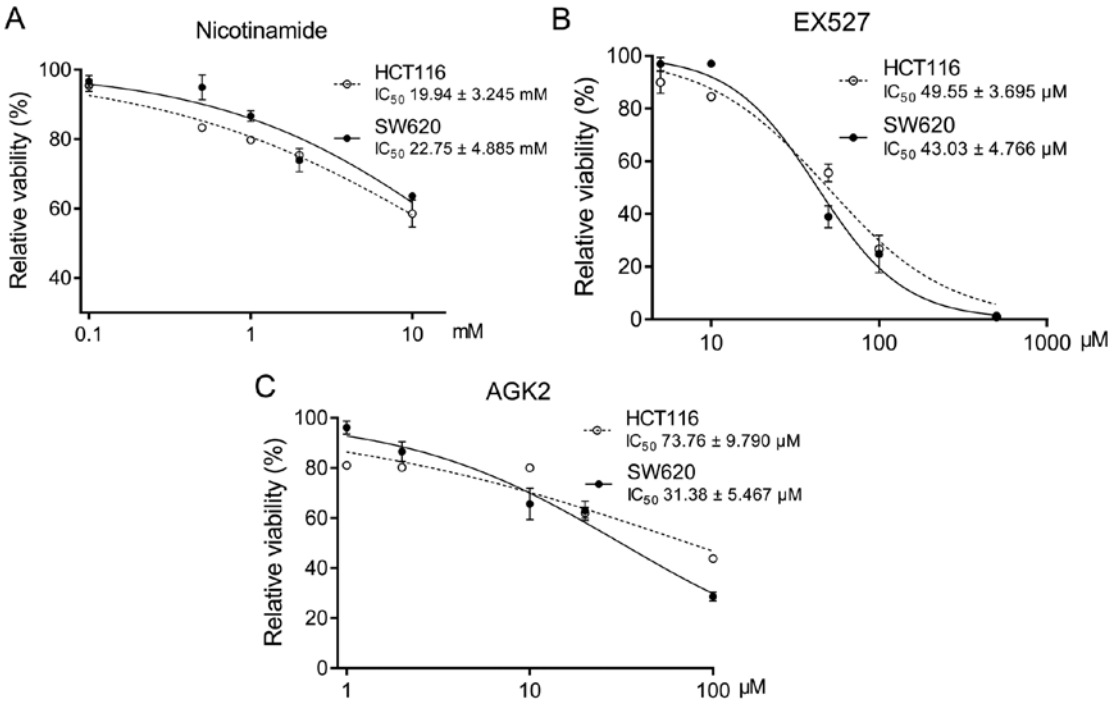

Figure 1. Cytotoxicity of SIRT inhibitors on *TP53*^{wt} and *TP53*^{mut} CRC cells. The viability of HCT116 (*TP53*^{wt}) and SW620 (*TP53*^{mut}) cells treated with (A) 0.1, 0.5, 1, 2 and 10 mM nicotinamide; (B) 5, 10, 50, 100 and 500 μM EX527; or (C) 1, 2, 10, 20 and 100 μM AGK2 for 72 h. The IC_{50} values for each agent in both cell lines are presented as the mean ± SD of three independent experiments. CRC, colorectal cancer; SIRT, sirtuin; wt, wild-type; mut, mutant.

included SW620 (GSM1380254-GSM1380259) and HCT116 (GSM1380296-GSM1380301) cell lines. GSEA (version 4.0.3; Broad Institute, Inc.) was performed using the above datasets to explore potential Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and protein acetylation or deacetylation-related Gene Ontology (GO) gene sets using the Molecular Signatures Database (MsigDB, version 7.0; Broad Institute, Inc.). Heat maps of differentially expressed genes (DEGs) were drawn using GraphPad Prism 7 (GraphPad Software, Inc.), as demonstrated in a previous study (25).

Statistical analysis. GraphPad Prism 7 was used for statistical analysis of cell cycle data. One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used to analyze the differences between each two groups in the cell cycle assays. Data are expressed as the mean ± SD of three independent experiments and $P<0.05$ was considered statistically significant.

Results

SIRT inhibitors show synergistic effects with chemotherapeutic agents in TP53 mut CRC cells. The HCT116 (*KRAS*^{mut} and *TP53*^{wt}) and SW620 (*KRAS*^{mut} and *TP53*^{mut}) cells were treated with NAM (a broad spectrum SIRT inhibitor), EX527 (a SIRT1 inhibitor) or AGK2 (a SIRT2 inhibitor) and the respective IC_{50} values were calculated (Fig. 1). Similarly, the IC_{50} values of cisplatin, 5-FU, irinotecan, oxaliplatin, paclitaxel, EGFR inhibitor gefitinib, PI3K inhibitor LY294002, pyruvate dehydrogenase kinase inhibitor DCA and the gluconeogenesis inhibitor metformin were determined (Fig. 2). These findings suggested that, SIRT inhibitors and chemotherapeutic agents had tumor inhibitory effects on CRCs.

Table I. Estimated concentration of each agent that led to 30% inhibition (70% survival) of the two CRC cell lines.

	Concentration in each cell type	
Drug	HCT116	SW620
Nicotinamide	3 mM	5 mM
EX527	20 μM	35 μM
AGK2	15 μM	10 μM
Cisplatin	2 μg/ml	0.2 μg/ml
5-Fluorouracil	0.2 μg/ml	0.9 μg/ml
Irinotecon	4.6 μM	15 μM
Oxaliplatin	0.45 μg/ml	0.05 μg/ml
Paclitaxel	4 μg/l	5.7 μg/l
Gefitinib	0.02 μM	0.01 μM
LY294002	3.6 μg/ml	10 μg/ml
Dichloroacetate	50 mM	26 mM
Metformin	17 mM	3.4 mM

CRC, colorectal cancer.

Inhibition curves were used to predict the 30% inhibitory concentration of each agent in both the HCT116 and SW620 cells (Table I), followed by the calculation of the CI and the $-\log_{10}$ CI. In the *TP53*^{wt} HCT116 cells, cisplatin, 5-FU, oxaliplatin, gefitinib, LY294002 and metformin were antagonistic to the SIRT inhibitors, whereas irinotecan and paclitaxel acted synergistically (Fig. 3A-C). In the *TP53*^{mut} SW620 cells, the majority of the chemotherapeutic agents showed a weak

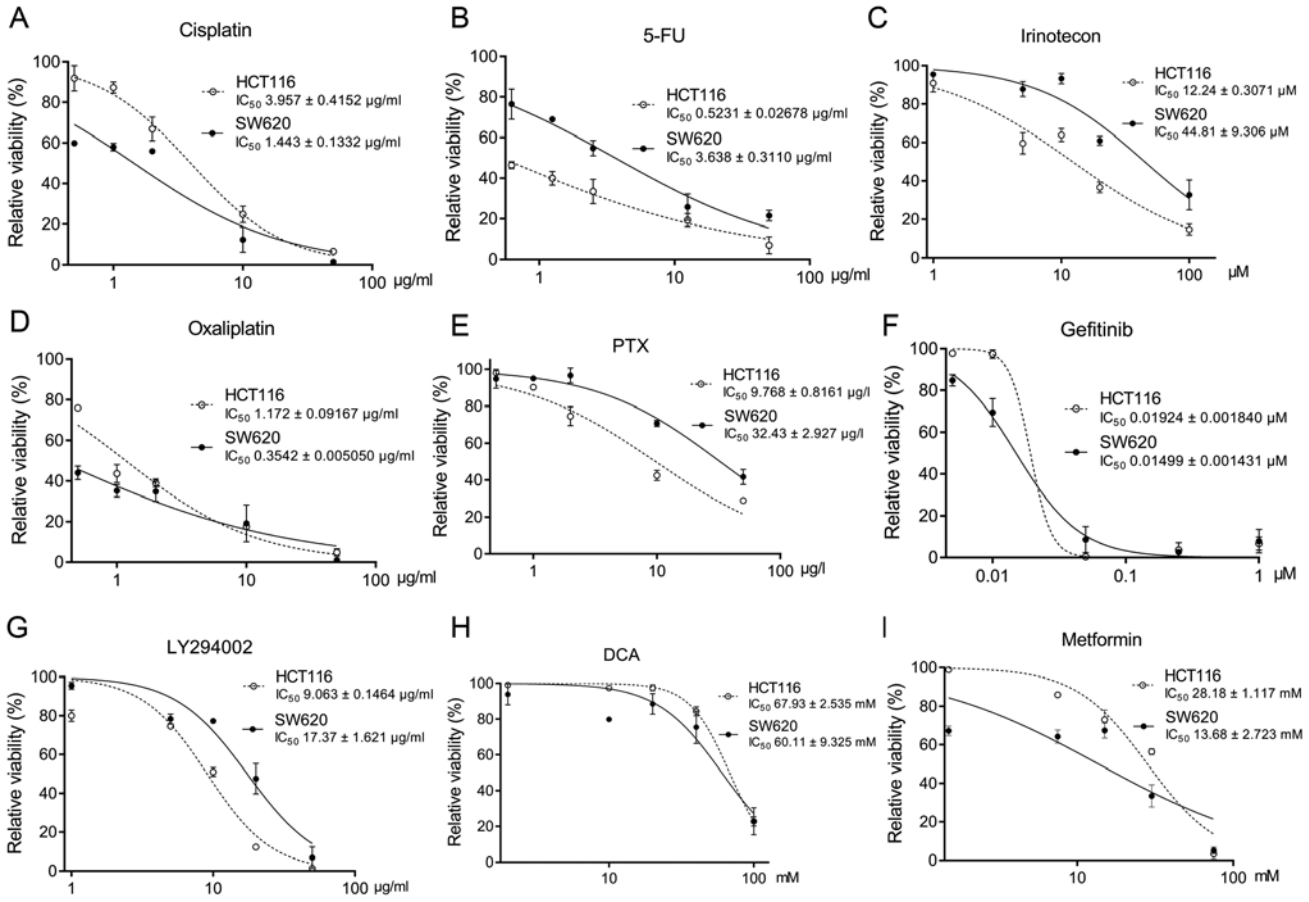

Figure 2. Cytotoxicity of chemotherapeutic agents on *TP53*^{wt} or *TP53*^{mut} CRC cells. Viability of HCT116 and SW620 cells treated with (A) 0.5, 1, 2, 10 and 50 μg/ml cisplatin; (B) 0.625, 1.25, 2.5, 12.5 and 50 μg/ml 5-FU; (C) 1, 5, 10, 20 and 100 μM irinotecan; (D) 0.5, 1, 2, 10 and 50 μg/ml oxaliplatin; (E) 0.5, 1, 2, 10 and 50 μg/l PTX; (F) 0.005, 0.01, 0.05, 0.25 and 1 μM gefitinib; (G) 1, 5, 10, 20 and 50 μg/ml LY294002; (H) 2, 10, 20, 40 and 100 mM DCA; and (I) 1.5, 7.5, 15, 30 and 75 mM metformin for 72 h. The IC_{50} values are presented as the mean ± SD of three independent experiments. 5-FU, 5-fluorouracil; CRC, colorectal cancer; SIRT, sirtuin; wt, wild-type; mut, mutant.

synergism with the SIRT inhibitors. Cell cycle analysis also confirmed that when used in combination, cisplatin and NAM were not more effective for preventing the G1-S transition of HCT116 cells compared with cisplatin alone (Fig. 3D). However, the G1-S transition was significantly reduced with a combination of cisplatin and NAM when compared to the use of each drug individually in the SW620 cells (Fig. 3E). These results suggested that SIRT inhibitors may antagonize chemotherapeutic drugs in *TP53*^{wt} CRC cells, while inhibition of SIRTs may make *TP53*^{mut} cells more sensitive to other chemotherapeutic agents in this assay.

P53 status and its level in CRC cells determines the combined effects of SIRT inhibitor and chemotherapeutic agents. *TP53* is frequently mutated into a proto-oncogene in tumor cells (26), which encodes a highly acetylated protein that is unstable and degrades easily (27). Therefore, the present study aimed to determine whether similar p53 protein levels and acetylation status existed in HCT116 and SW620 cells. In the present study the level of the deacetylase SIRT1 was significantly higher in HCT116 cells when compared to SW620 cells, with high levels of wt p53. In SW620 cells, the level of the deacetylase SIRT1 was lower than that in HCT116 cells, which may lead to increased levels of acylated and easily-degraded p53 mut (Fig. 3F). Treatment with NAM reduced the levels of the wt p53 protein, activated p-p53Ser15 and its downstream target p21 in HCT116 cells. This effect was not observed in the SW620 cells with a mutated p53 protein (Fig. 3G).

Enrichment of genes associated with the GO-term protein deacetylation in CRC cells with wt p53 expression. The transcriptome data of *TP53*^{wt} and *TP53*^{mut} cancer cell lines was extracted from the RNA-Seq GSE41258 dataset and submitted to GSEA for enrichment analysis. KEGG pathway analysis did not reveal any significant differences in the enrichment of genes related to the term 'p53 signaling pathway' between the cell lines (Fig. 4A). However, *TP53*^{mut} cells were enriched in genes related to the GO-term 'protein acetylation' (Fig. 4B), which were also differentially expressed compared to that in the *TP53*^{wt} cells (Fig. 4C). Consistent with this, genes associated with the GO term 'protein deacetylation' were enriched in the *TP53*^{wt} cells (Fig. 4D and E). GSEA of the HCT116 and SW620 transcriptomes (from the GSE7343 dataset) similarly showed a downregulation of genes associated with the KEGG term 'p53 signaling pathway' and the GO-term 'protein deacetylation' in SW620 cells (Fig. 4F-H). Taken together, these results suggested that the protein deacetylation machinery may be more activated in the *TP53*^{wt} compared to *TP53*^{mut} CRC cells. With the blockage of the SIRTs inhibitors, the stable wt p53 was significantly reduced, which may

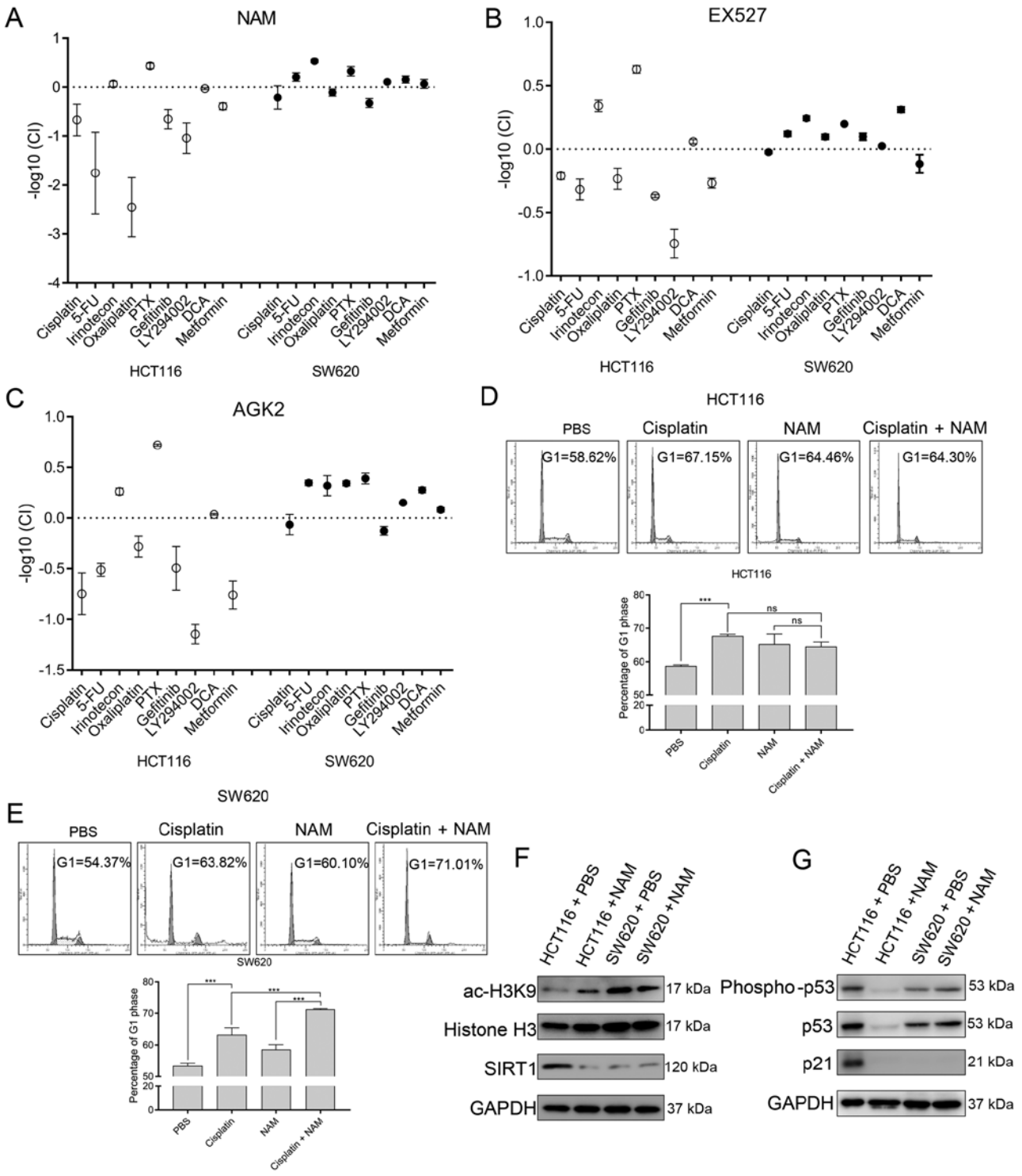

Figure 3. SIRT inhibitors antagonize chemotherapeutic agents in *TP53*^{wt} CRC cells by reducing wild-type 53 protein levels. The log CI values between (A) NAM, (B) EX527 or (C) AGK2 and various chemotherapeutic agents. Cell cycle profile of (D) HCT116 and (E) SW620 cells treated with cisplatin and/or NAM for 72 h. Data are presented as the mean± SD of three independent experiments. (F and G) Immunoblots showing levels of p53, phospho-p53, p21, SIRT1 and histone H3K9 acetylation in HCT116 and SW620 cells treated with 5mM NAM or vehicle for 72 h. ***P<0.001; ns, no significance. CRC, colorectal cancer; CI, combination index; NAM, nicotinamide; SIRT, sirtuin; wt, wild-type; mut, mutant.

antagonize the action of SIRT inhibitors in combination with a chemotherapeutic drug.

Discussion

Studies show that ~60% of CRC cases harbor *TP53* mutations, which correlate with greater malignancy (28). The tumor suppressor *TP53* is a 'master regulator' of cellular processes including the cell cycle, apoptosis and DNA damage repair (29). Gain-of-function mutations in *TP53*, such as V143A, R248Q, R273H and R280K, confer a malignant phenotype on tumor cells by promoting proliferation, invasion, metastasis and chemo-resistance (30). In CRC cells, mut p53 protein binds to STAT3 and activates the pro-tumorigenic Jak2/STAT3

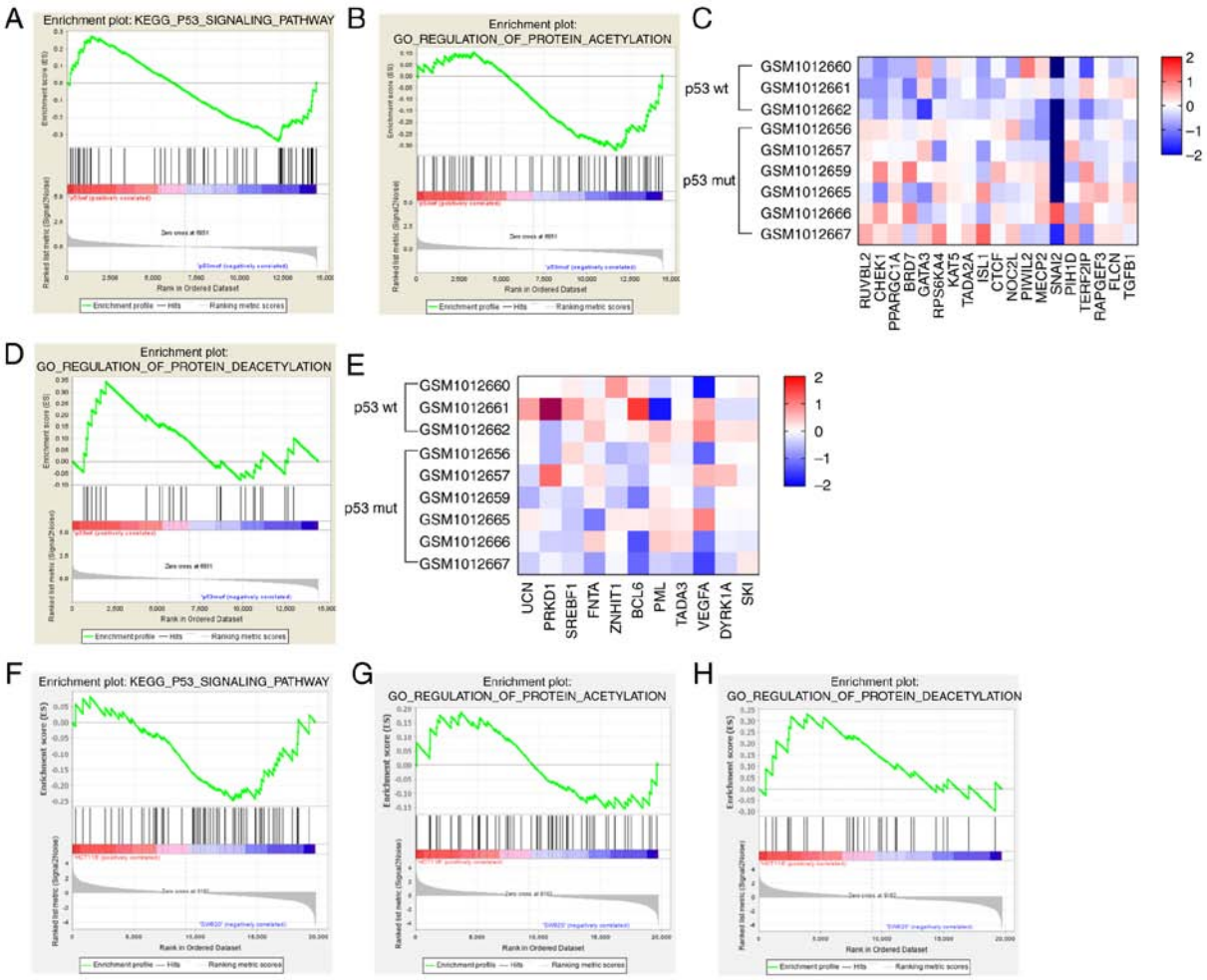

Figure 4. Genes associated with the GO-term protein deacetylation were enriched in *TP53*^{wt} CRC cells. (A) GSEA results showing enrichment of genes related to the KEGG term p53 signaling pathway in the GSE41258 dataset. (B) GSEA results showing enrichment of genes related to the GO-term protein acetylation in the GSE41258 dataset. (C) Heat map showing differentially expressed genes related to protein acetylation in the GSE41258 dataset. (D) GSEA results showing enrichment of genes related to the GO-term protein deacetylation in the GSE41258 dataset. (E) Heat map showing differentially expressed genes related to protein deacetylation in the GSE41258 dataset. (F) GSEA results showing enrichment of KEGG terms related to p53 signaling pathway in the GSE7343 dataset. (G) GSEA results showing enrichment of GO-term related to protein acetylation in the GSE7343 dataset. (H) GSEA results showing enrichment of GO-term related to protein deacetylation in the GSE7343 dataset. GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.

signaling pathway, which increases tumor invasiveness, leading to a worse prognosis (31). In addition, mut p53 also drives CRC progression and chemo-resistance by increasing cancer stem cell renewal and reprogramming tumor-associated macrophages (32,33). Distinct therapeutic strategies are needed against *TP53*^{wt} and *TP53*^{mut} CRCs, as they are likely to differ in their chemo-sensitivities. For example, the therapeutic potential of ascorbic acid is higher when used in combination with first-line drugs such as 5-FU or oxaliplatin in *TP53*^{mut} CRC cells (34). Furthermore, the Wee1 inhibitor MK1775 induces apoptosis in the *TP53*^{mut} HT29 and SW480 cells and sensitizes them to irinotecan (35). In contrast, the histone deacetylase inhibitors, valproic acid and capecitabine, are antagonistic in p53-deficient CRC cells, but act synergistically in cells expressing normal or mut p53 (36). In the present study, SIRT inhibitors sensitized *TP53*^{mut} CRC cells to chemotherapeutic agents and *TP53*^{wt} CRC cells to only irinotecan or paclitaxel, while antagonizing the other drugs. These findings suggested that SIRT inhibitors are promising for *TP53*^{mut} refractory or drug-resistant CRC but not suitable for *TP53*^{wt} CRC.

The role of SIRTs in tumorigenesis, tumor progression and metastasis is controversial. SIRT1 acts as tumor suppressor in *TP53*^{mut} hepatocellular carcinoma and its high levels predict a favorable prognosis (37). In esophageal squamous cell carcinoma; however, miR-34a-mediated inhibition of SIRT1 and induction of p53 exerted an anti-tumor effect (38). Some SIRT inhibitors retard tumor growth by attenuating the deacetylase activity of SIRTs and downregulating tumorigenic signaling pathways. For example, the antipsychotic drug chlorpromazine induces apoptosis in CRC cells by downregulating SIRT1 (39) and the SIRT inhibitor benzimidazole also inhibits growth of CRC cells (40). SIRT inhibitors used in the present cytotoxicity and cell cycle experiments appeared to show an inhibitory effect on two CRC cell lines. However, cell cycle analysis only determines the proportion of viable dividing cell populations, which presents limitations in determining the proportion of apoptotic cells (41). In addition, SIRT levels were not the decisive reason for the different chemosensitivity in HCT116 and SW620 cells in the present study. This can be concluded because after NAM treatment SIRT1 was induced to be the same level in the two

cell lines, which suggested that the baseline levels of SIRT1 were the same for the combination usage of the SIRT inhibitors and those chemotherapeutic agents. From these data, it was concluded that the baseline difference of SIRT1 in these two tested cell lines could not be a driving or effective factor that leads to the efficacy-divergency of the combination treatment.

p53 protein binds to HDAC6 and HSP90 to form a complex, which protects it from ubiquitin protease-mediated degradation (42). HDAC6 inhibitors interfere with the formation of this complex and degrade p53 to an unstable state (43). In the present study, NAM suppressed SIRT activity and reduced the stability of both wt and mut p53. However, the overall levels of acetylated p53 were low in the *TP53*^{wt} HCT116 cells when compared with *TP53*^{mut} SW620 cells, which corresponded with high levels of the stable p53 protein. Therefore, a reduced pool of stable p53 was underlying the antagonism between NAM and multiple chemotherapeutic agents.

In conclusion, the experimental data and bioinformatics analysis in the present study suggested that *TP53* status may be responsible for the divergence in CRC cell chemosensitivity profiles. The findings also suggested that a combination of SIRT inhibitors and first-line drugs may be beneficial for patients with *TP53*^{mut} CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HY performed experiments and wrote the manuscript. JY and ZZ designed the study and revised the manuscript. HY, JY and ZZ contributed to the cell cycle assay and GSEA data analysis. HY, DW and YC performed the cytotoxicity assay and drug sensitivity analysis. YC and YJ performed statistical and bioinformatics analysis. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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