

## Original Article

# APE1/Ref-1 regulates 5-FU resistance in colon cancer cells through its redox and endonuclease activity

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Received January 13, 2018; Accepted October 8, 2018; Epub April 15, 2019; Published April 30, 2019

**Abstract:** 5-Fluorouracil (5-FU) is one of the most widely used drugs for treatment of patients with advanced colon cancers. However, recurrence of colorectal cancer is frequent due to the development of drug resistance. The understanding of drug resistance is still limited now. Here in this study, we showed that the dual-functional protein APE1/Ref-1 (APE1 in short) was upregulated in 5-FU resistance HCT116 colon cancer cell lines. Inhibiting APE1 protein by siRNA sensitized cells to 5-FU again. By deactivating the DNA repair and redox activity respectively, we found inhibiting either DNA repair activity or the redox activity enhanced 5-FU's effect in both cell model and xenograft animal model. Moreover, inhibiting the redox activity showed a more robust effect on reversing the drug resistance. Inhibiting endonuclease activity of APE1 increased sensitivity to 5-FU in a p53 dependent manner. A major implication of our studies is that inhibition of APE1 may have considerable therapeutic potential to overcome drug resistance in colorectal cancer cells.

**Keywords:** APE1, colon cancer, 5-FU, drug resistance, P53

## Introduction

5-Fluorouracil (5-FU), a thymidylate synthase inhibitor is one of the most common used chemotherapy drug for colorectal cancer treatment [1]. 5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen, after uptaken by cells, 5-FU is converted into several metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) which are incorporated into DNA or RNA, leading to DNA strand breakage and a decrease in protein synthesis [2].

Drug resistance is the major cause of cancer relapse and death. Current knowledge about mechanism of drug resistance in cancers includes upregulation of transporters, overexpression of survival genes, inhibition of tumor suppressors, DNA repairs, autophagy and etc. [3]. Even much effort has been taken, drug resistance is still an obstacle for cancer treatment.

Human apurinic/aprimidinic endonuclease 1/redox effector factor 1 (APE1/Ref-1) is a multifunctional protein [4]. The C terminal domain of APE1/Ref-1 replies for its DNA base excision repair (BER) activity. High expression of APE1 have been shown in different types of cancers, including melanoma, pancreatic cancer and ovarian cancer [4, 5]. Over-expression of APE1 and enhanced AP endonuclease activity were shown linked to monofunctional alkylators and radiation resistance [6, 7]. APE1 is also an important transcriptional regulator of gene expression [8]. APE1 regulates the redox status of several transcription factors such as the early growth response protein-1 (Egr-1), nuclear factor kappa B (NF- $\kappa$ B), hypoxia inducible factor 1 (HIF-1), cAMP response element binding protein (CREB), activator protein-1 (AP-1), and paired boxcontaining proteins (Pax) [9]. Many of them have been proved important regulators of drug resistance in breast cancer, colon cancer and other cancers [10-13].

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The p53 tumor suppressor is a transcription factor that can be activated by various stresses including chemotherapeutic agents [14]. p53 and its target genes have been shown directly involved in DNA repair after oxidative and alkylation damage [15]. Upon DAN damage, p53 becomes activated by a number of post-translational regulating factors, including kinases, ubiquitin ligases, histone deacetylase and acetylating proteins [16]. After stabilization by those regulators, p53 elicits several biological responses including DNA damage repair, cell-cycle arrest, senescence and apoptosis [17]. However, more than 50% of human cancers lost p53 function because of mutations, loss of the short arm of chromosome 17 or other mechanisms [17]. Loss of p53 function can result in enhanced cell proliferation, genomic instability and resistance to cell death, which raises an obstacle for cancer chemotherapy.

### Material and methods

#### *Cell lines and reagents*

HCT116 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The HCT116 5-FU resistance cell line was generated by incubating HCT116 cells with increasing concentrations (from 20 ng/ml to 1 ug/ml) of 5-FU (Sigma-Aldrich, St. Louis, MO, USA) for 2 months [18].

5-FU and was purchased from Sigma (St. Louis, MO). The antibodies for western blot were purchased as indicated: anti-APE1, abcam (Cambridge, England), anti-p53, Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and anti-GAPDH, Sigma (St. Louis, MO).

#### *Q-PCR and western blot assay*

Total cell mRNA was prepared with (RNeasy Mini Kit, Qiagen, Hilden, Germany) Expression of APE1 was determined using SYBR® Green PCR Mastermixes (Qiagen, Hilden, Germany) The primers for APE1 and GAPDH are APE1: F: 5'-ACAGAGCCAGAGCCAAGAA-3', R: 5'-CCTGCTGCCTTTGTGTCATTT-3'. GAPDH: F: 5'-AATCCCATCACCATCTTCCA-3', R: 5'-TGGACTCCACGACTACTCA-3'. Whole cell lysates for western blot assay were prepared in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM

EDTA and a protease inhibitor cocktail (Sigma, St. Louis, MO)). Equivalent amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA). Proteins were detected using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

#### *Cell viability assay*

Cells were seeded in 96-well plates and treated with 5-FU or E3330 for indicated time. Cells were then stained with Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma, St. Louis, MO) for 2 hours. The OD at 570 nm was read on a ELx808™ Absorbance Microplate Reader (BioTek, Winooski, VT) after the purple precipitates were dissolved by MTT detergent reagent (Sigma, St. Louis, MO). Normalized cell viability was calculated as a ratio of OD values of treated samples to those of controls.

#### *DNA fragmentation assay*

DNA fragmentation was detected using a DNA fragmentation ELISA assay kit (Roche) according to manufacturer's protocol. Briefly, cells were seeded in 96-well plates and subjected 5-FU for 24 hours. The cells were stained with MTT to determine cell numbers or lysed for ELISA assays to determine DNA fragmentation. The relative DNA fragmentation was determined by dividing ELISA values by MTT values of each sample.

#### *Plasmid and construct*

Oligonucleotides used for siRNA of APE1 were as follows: sense 5'-CCTGCCACTCAAGATCTGC-3'; antisense, 5'-GCAGATCTTGAGTGTGGCAGG-3'. the scrambled oligonucleotide sequences: sense, 5'-AGTCTAACTCGCCACCCCGTA-3'; antisense, 5'-TACGGGGTGGCGAGTTAGACT-3'. APE1 knock-in constructs were generated following the instruction described previous [19]. All the mutants were confirmed by DNA sequencing (genescript China). shp53 pLKO.1 puro was a gift from Bob Weinberg (Addgene plasmid # 19119), shRNA oligo sequence is 5'-CCGACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCACTGGAGTCTTTTT-3'.

#### *Luciferase reporter assay*

HCT116R Cells were co-transfected with plasmid with luciferase driven by NF-κB, AP-1, or

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HIF1 (pLuc-MCS with the NF $\kappa$ B, AP-1, or HIF1 responsive promoter; PathDetect cis-Reporting Systems, Stratagene, La Jolla, CA) and a Renilla luciferase control reporter vector pRL-TK (Promega Corp., Madison, WI). After transfected, cells were treated with E3330 and the luciferase activity was assayed as described previously [20]. Cells expressing inducible siAPE1 construct were treated with doxycycline for 2 days to eliminate the endogenous APE1. The luciferase activity was determined in the same experiment.

### *In vivo xenograft model*

Animal experiments were approved by the Institutional Animal Ethical Committee. Cells expressing different constructs were injected into the flank of 4-6 weeks old male athymic nude mice on both sides. Dox was added in the drink water to induce the knockdown of APE1. 5-FU (40 mg/kg/day) administered by i.p. injection every other day for 16 days. Tumor volumes were measured as described previously [21]. The estimated tumor volumes (V) were calculated by the formula  $V = W^2 \times L \times 0.5$ , where W represents the largest tumor diameter in centimeters and L represents the next largest tumor diameter. The relative tumor volumes (RTV) were calculated by  $RTV = V_x/V_0$  where  $V_x$  is the volume in cubic millimeters at a given time [21].

### *Statistical analysis*

Statistical analyses were performed using two-way ANOVA or Student's t-test.

## Results

### *HCT116 cells obtained drug resistance after 5-FU treatment for 2 months*

5-FU has been using for treating colon cancers for more than 40 years [2]. Drug resistance is the major cause of failure of treatment [22]. HCT116 is a type of poorly differentiated colon cell line [23]. It is well responded to 5-FU treatment. Drug resistance occurred when HCT116 cells were treated with increasing levels of 5-FU for two months. As shown in **Figure 1A**, the IC<sub>50</sub> of 5-FU resistant cell line was 6 folds higher than its parental counterpart. The major mechanism for 5-FU is to incorporate into DNA and induce the DNA damage in the tumor cells [2]. As shown in **Figure 1B**, The DNA fragmentation assay showed that

5-FU resistant cells exhibited reduced DNA fragmentation compared to parental HCT116 cells.

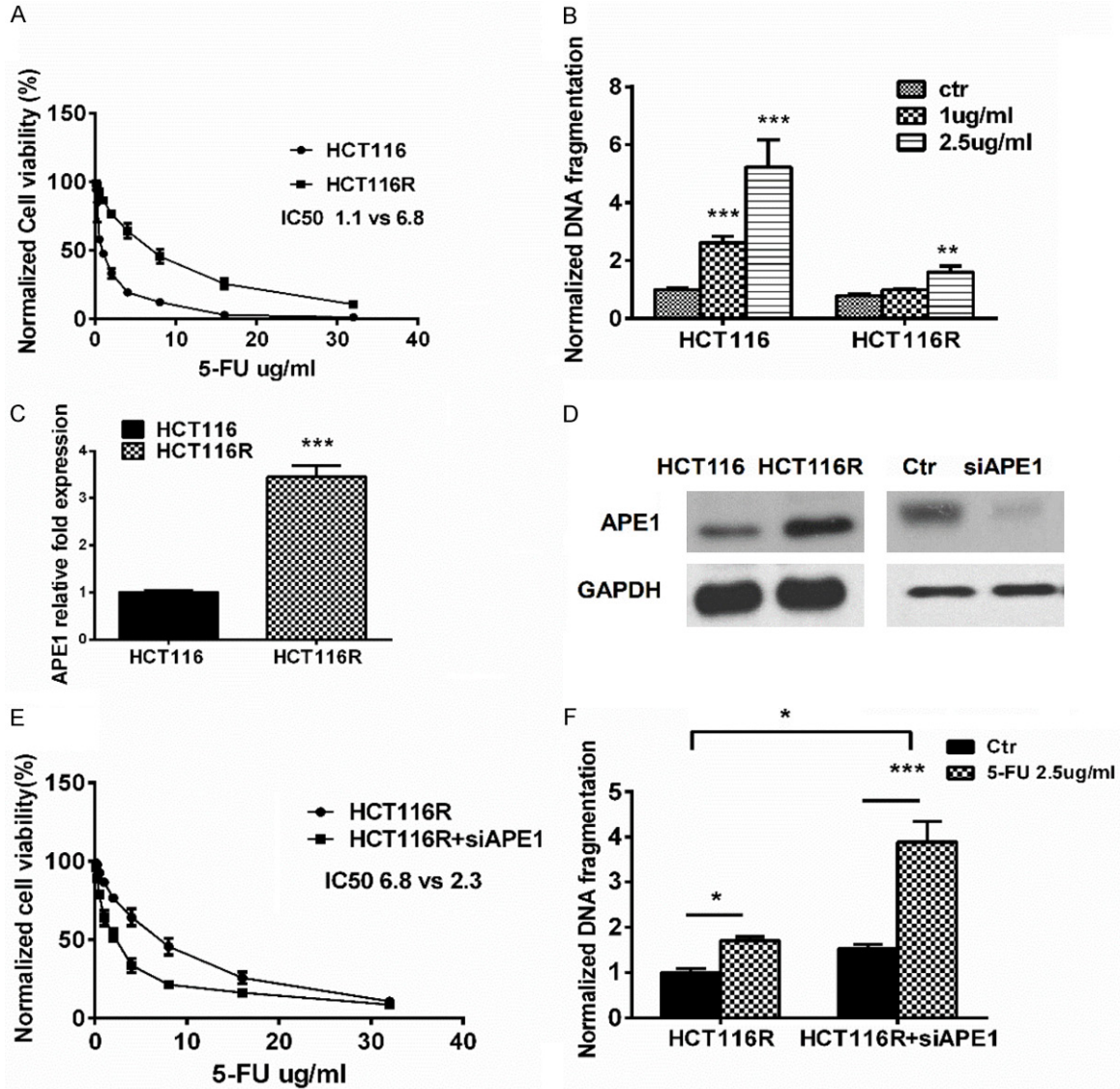
### *APE1 was upregulated in 5-FU resistance HCT116 cells*

Drug resistance is a complex process and many mechanisms are involved. APE1 is a multiple functional protein, whose endonuclease and redox functions are both shown playing important roles in tumor development and drug resistance [24, 25]. As expected, we found both mRNA and protein were increased in the 5-FU resistant HCT11R cells (**Figure 1C, 1D** left), indicating the involvement of APE1 in 5-FU resistance. Then, we used siRNA to knockdown the expression of APE1 in HCT116 R cells. We found that knockdown APE1 significantly sensitized HCT116R cells to 5-FU (**Figure 1E**). The APE1 knockdown cells also showed more DNA fragmentation compared to HCT116R control cells (**Figure 1F**).

### *Both endonuclease activity and redox activity contributed to drug resistance of 5-FU*

APE1 is the only protein performing both endonuclease and redox activities [26]. Since the two functions are independent on each other [26], it will be very interesting to investigate which function is more important for 5-FU resistance in our model. We introduced ectopic flagged forms of wild-type (WT) and mutant APE1 proteins on an inducible APE1 siRNA background to HCT116R cells [19] (**Figure 2A**). The APE1-H309N mutant lacks the DNA repair function [27, 28] and the APE1-C65S mutant has no redox activity [28]. As shown previously, knockdown APE1 sensitized cell to 5-FU (**Figure 1E**). Introducing the wild-type APE1 led the cells regain the resistant phenotype (**Figure 2B**). Interestingly, cells expressing APE1-H309N recovered parts of its 5-FU-resistant phenotype, but APE1-C65S had little effect on recovering drug resistance (**Figure 2B**). This result indicated redox activity of APE1 played a dominant role in 5-FU resistance in HCT116R cells. To confirm this result, we treated HCT116R cells with E3330, which is a potent APE1 redox activity inhibitor that does not impede its DNA repair function [26]. The activity of E3330 was reflected by the decrease of promoter activity of NF- $\kappa$ B, AP-1 and HIF1 $\alpha$  (**Figure 3B-D**). When combined with 10  $\mu$ M E3330, 2.5  $\mu$ g/ml 5-FU

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**Figure 1.** APE1 was upregulated in 5-FU resistant HCT116R cells and knockdown APE1 sensitized cells to 5-FU. A. HCT116 cells and 5-FU resistant HCT116R cells were seed in 96 well plate at 4000 cells/well and then subjected to a series concentration of 5-FU for 72 hours. Cell viability was determined with MTT assay. Normalized cell viability was shown. B. HCT116 and HCT116R cells were seed in 96 well plates at 8000 cells/well and treated with 2 ug/ml or 2.5 ug/ml 5-FU for 24 hours. DNA fragmentation was determined using a Cellular DNA Fragmentation ELISA kit. C. The relative mRNA expression of APE1 in HCT116 and HCT116R cells was determined using a real-time PCR assay. D. APE1 protein expression in HCT116 and HCT116R cells (left) and APE1 expression after siRNA knockdown (right) were determined using western blot assay. E. HCT116R and HCT116R-siAPE1 cells were seed in 96 well plate at 4000 cells/well and then subjected to a series concentration of 5-FU for 72 hours. Cell viability was determined with MTT assay. Normalized cell viability was shown. F. HCT116R and HCT116R-siAPE1 cells were treated with 2.5 ug/ml 5-FU. DNA fragmentation was determined. The data are presented as the mean  $\pm$  SD of three replications. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

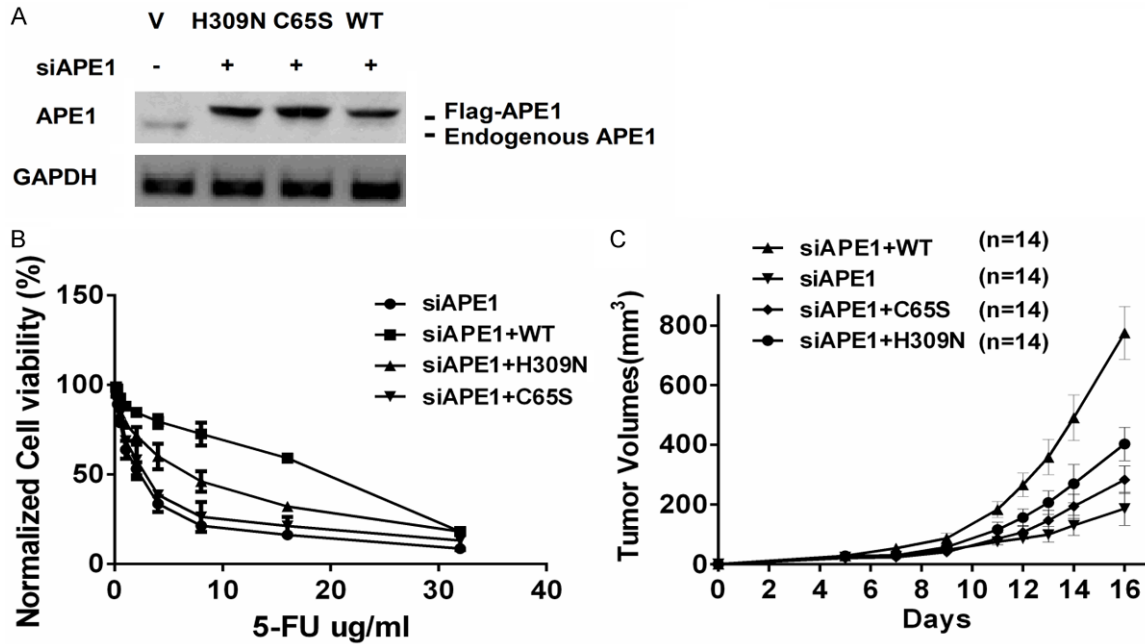
significantly inhibited HCT116R cell proliferation (Figure 3A).

*APE1's endonuclease activity in 5-FU resistance was depended on p53*

p53 has been shown participated in many DNA repair pathways [14]. It is also an important

regulator of chemotherapy drug induced cell death [15]. HCT116 cell line is a poorly differentiated colon cell line which expresses wild type of P53 protein. We hypothesized that p53 was an important mediator for 5-FU and APE1-H309N induced cell death in HCT116R cells. To determine the function of p53 in this model, we knocked down p53 in HCT116R cells. The

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**Figure 2.** Cell lacking APE1 endonuclease or redox activity sensitized cells to 5-FU treatment. A. Wild type and mutant APE1 was introduced into HCT116R cells and then endogenous APE1 was inhibited by siRNA. APE1 protein was determined by western blot assay. B. Cell were seed in 96 well plate at 4000 cells/well and then subjected to a series concentration of 5-FU for 72 hours. Cell viability was determined with MTT assay. Normalized cell viability was shown. C. 2 million cells expressing different constructs were injected into the flank of 4-6 week old male athymic nude mice on both sides. Dox was added in the drink water to induce the knockdown of APE1. 5-FU (40 mg/kg/day) administered by i.p. injection every other day for 16 days. Tumor volumes were measured at the beginning of the treatment and every other day after that till the mice were euthanized. Xenograft tumor growth curves of HCT116R cells is shown. n = 14.

efficiency of p53 knockdown was shown in **Figure 4A**. When p53 was knocked down, the inhibitor effect of 5-FU (5 ug/ml) on APE-N309H cell growth was attenuated (**Figure 4B**). And 5-FU induced apoptosis was decreased by p53 knockdown too (**Figure 4C**).

### *Impair endonuclease/redox activity of APE1 modulated 5-Fu resistance in vivo*

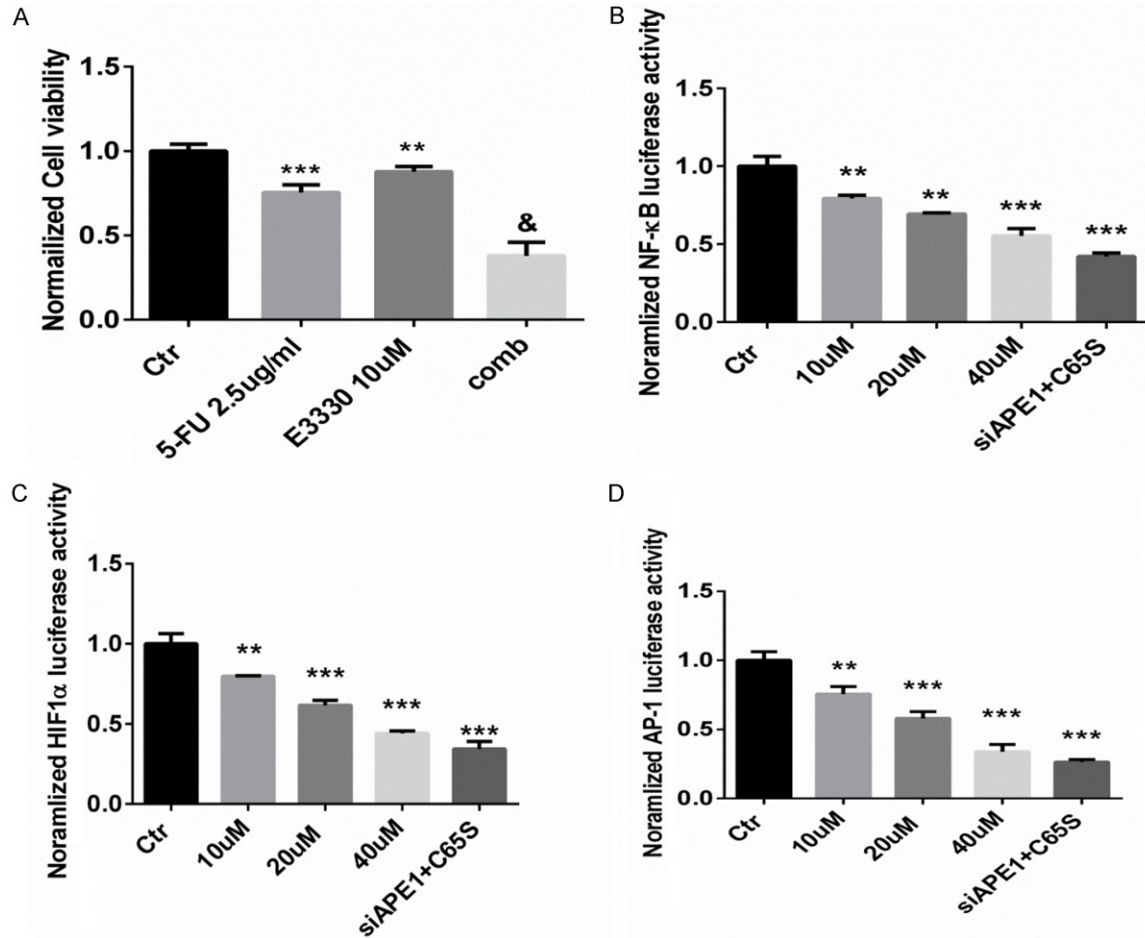
To test the function of APE1 on 5-FU resistance *in vivo*, we established a colon tumor xenograft model by subcutaneously inoculating athymic nude mice with two million HCT116R cells that express APE1-WT or mutants. After cell injection, doxycycline was added to the drinking water to induce the knockdown of endogenous APE1. After 7 days, mice were treated with 5-FU (40 mg/kg/day) administered by i.p. injection every other day for 16 days. Tumor growth was monitored during the course of 5-FU treatment. Consistent with *in vitro* result. 5-FU significantly inhibited HCT116R-siAPE1 tumor growth. Reconstitute the redox activity by expressing

H309N recovered a small portion of resistant phenotype, but APE1-C65S, which lacks redox activity, has little effect to help tumors to regain resistance to 5-FU (**Figure 2C**).

### Discussion

In this study, we have shown that APE1 was upregulated in 5-FU resistant HCT116 colon cancer cells. The amplification of APE1 mRNA and protein indicated that the DNA base excision repair function as well as redox regulation activity were enhanced during 5-FU resistance. Knockdown APE1 in HCT116R cells made the cells sensitive to 5-FU again. By inhibiting the endonuclease or the redox activity respectively, we found that redox activity was more dominant in regulating 5-FU resistance in HCT116R cells. Moreover, inhibiting endonuclease activity of APE1 was not sufficient to sensitize cells to 5-FU when p53 is absent.

5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen.



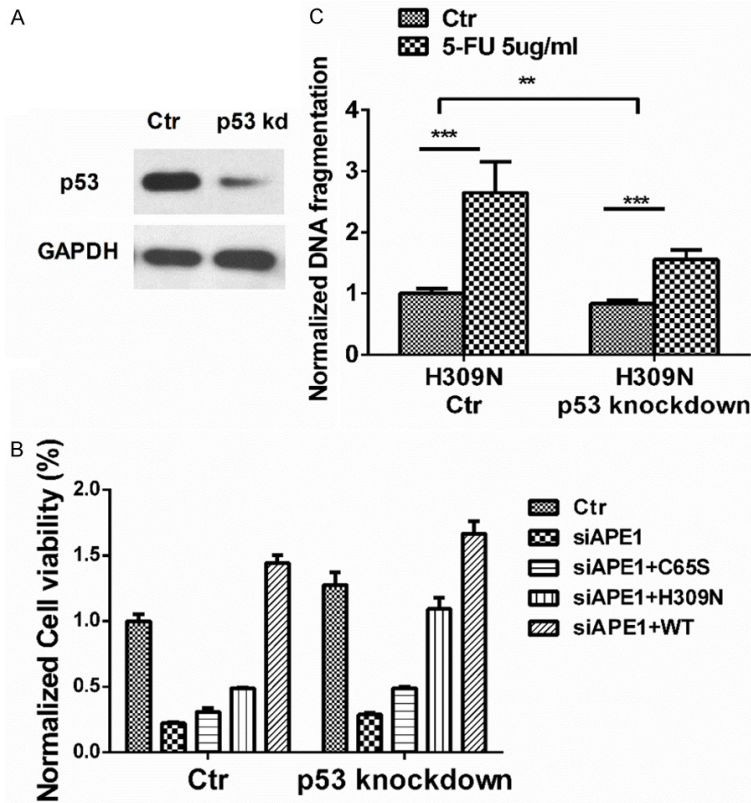
**Figure 3.** E3330 enhances 5-FU's effect and inhibits the activity of NF-κB, HIF1, and AP-1. A. HCT116R cells were treated with E3330, 5-FU or combination of both. Cell viability was determined by MTT assay. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs ctr. & $P < 0.01$  vs 5-FU or E3330 group. B-D. HCT116R cells were transfected with NFκB, HIF1, or AP-1-Luc construct and cotransfected with a Renilla vector, pRL-TK. 16 hours after transfection, cells were treated with E3330 for 24 hours, and Firefly and Renilla luciferase activities were assayed using Renilla luciferase activity for normalization. Luciferase activity of siAPE1/C65S cells were determined in the same experiment.

5-FU exerts its anticancer effects through inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA [29]. 5-FU has been used for colon cancer for more than 40 years. However, in advanced colorectal cancers, the overall response rate is only 10-15% because of the development of drug resistance through various mechanisms [30]. We showed in this study, 5-FU induced DNA fragmentation was reduced in HCT116R cells (**Figure 1B**), indicating the cells developed mechanisms to defend cytotoxicity of chemotherapy drugs.

APE1 exhibits both endonuclease and redox activities. The two functions are completely independent in their actions [26]. It has been

well described that APE1 was one of the major enzyme in the EBR pathway, which is the most used pathway to cope with the single base lesion [26]. People found that a mutation form of APE1, lacking detectable nuclease activity, sensitized cancer cells to DNA-damaging agents carmustine and dideoxycytidine but had no effect on mitomycin C and cisplatin [31]. In our study, inhibiting nuclease activity had a moderate effect on 5-FU induced cell death in HCT116R cells. Those results indicated that function the APE1's nuclease activity was very likely to rely on the genetic background of cancer cells.

The redox function of APE1 is found only in mammals [26], APE1's reduced the disulfide



**Figure 4.** Endonuclease activity of APE1 was depended on p53 during 5-FU resistance. A. P53 was knocked down by shRNA, protein expression was determined by western blot assay. B. HCT116R cells and HCT116R p53 knockdown cells with different modification of APE1 were seed to 96 well plate at 4000 cells/well, and then treated with 5 ug/ml 5-FU for 72 hours. Cell viability was determined by MTT assay. Normalized cell viability was shown. C. HCT116R-H309N cells and HCT116R-H309N-p53kd cells were treated with 5 ug/ml 5-FU for 24 hours. DNA fragmentation was determined. The data are presented as the mean  $\pm$  SD of three replications. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

bonds of numerous transcription factors and enhanced their DNA binding [32]. Lots of transcription factors under the regulation of APE1 are involved in cancer promotion and progression, notably AP-1 (Fos/Jun), NF- $\kappa$ B, PAX, HIF-1 $\alpha$ , HLF, p53 and others [28, 33-35]. The dual function of APE1 makes it a good therapy target for anti-cancer drug development. In this study, we inhibited the endonuclease and redox activity respectively in HCT116R cells, we found inhibiting redox activity was more potent in reversing drug resistance both *in vitro* and *in vivo*.

Many small molecules targeting the redox activity of APE1 have been developed. E3330 ([5-(2,3dimethoxy-6-methyl-1,4-benzoquinolyl)]-2-nonyl-2-propenoic acid) is a quinone

compound that functions as a redox inhibitor of APE1 by increasing disulfide bond formation with Cys 65 and Cys 93 residues of APE1 [36]. In many studies, E3330 has shown great potential to be a good drug candidate by inhibiting tumor cell functions [4, 37, 38]. In this study, we showed that E3330 significantly increased the cytotoxicity of 5-FU, which was constant with previous report of combination of E3330 and 5-FU on treating colon cancer stem cells [39]. So, taken together, redox activity of APE1 might serve as a good therapeutic target in treating 5-FU resistant cancers.

The development of drug resistance involves the alteration of multiple genes and pathways. The DNA repair function of APE1 has been suggested playing a vital role in cell viability and proliferation in colon cancer cells [40]. In our study, we noticed that loss of endonuclease activity had a moderate effect on the drug resistance of HCT116R cells, but this phenotype was attenuated by p53 knockdown. p53 and its target genes have been shown participating in DNA repair pathways as well as in DNA-damage induced cell death [14, 17].

In HCT116R cells the wild type p53 might stimulate cell cycle arrest and apoptosis upon the DAN damage caused by 5-FU and APE1 knockdown. Moreover, other mechanisms like NEIL1/2/PNK-dependent base excision repair can bypass the APE1 [41]. This might explain why inhibiting endonuclease activity of APE1 only had a moderate effect on the drug resistance. Due to the complexity of drug resistance, more efforts are still needed to unveil the function of APE1 during drug resistance in different cell context.

**Disclosure of conflict of interest**

None.

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