BPB Reports

Report

Sensitization of Gastric Cancer Cells to Irinotecan by p53 Activation

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Irinotecan (camptothecin-11 [CPT-11]) is a topoisomerase I inhibitor that has been used in the treatment of a wide spectrum of cancers including gastric cancer. Recent reports suggest that the expression of CES2, a serine hydrolase that converts irinotecan to its active compound SN-38, is regulated by the tumor-suppressor p53. In this study, we investigated whether irinotecan acted synergistically with a p53 activator nutlin-3a in human gastric cancer cells. Nutlin-3a treatment enhanced the expression of CES2 in gastric cancer cell lines with wild-type p53. However, this effect was not observed in cells with non-functional p53. Irinotecan showed synergistic antitumor effects in combination with nutlin-3a in gastric cancer cells with wild-type p53, whereas the survival of cells with non-functional p53 was not significantly affected by the presence of nutlin-3a. These results provide evidence that p53 activation can enhance the antitumor effect of irinotecan or other anticancer prodrugs activated by CES2 in gastric cancer cells through upregulation of CES2 expression.

Key words CES2, gastric cancer, irinotecan, p53, nutlin-3a

INTRODUCTION

Gastric cancer is relatively prevalent malignancy and ranks the fifth most commonly diagnosed malignancy and the third in cancer-related death worldwide.[1)] Gastric cancer is generally asymptomatic in early stages and has progressed to an advanced unresectable stage by the time of presentation.[2)] The prognosis of patients with gastric cancer remains extremely poor.[3)] Even patients with resectable tumor usually have a high rate of local recurrence and distant relapse.[4)] The standard palliative treatment for patients with advanced gastric cancer is chemotherapy, which both controls tumor-related symptoms and improves overall survival. Clinical trials have shown the survival benefit of irinotecan (camptothecin-11 [CPT-11]) in advanced gastric cancer as second-line chemotherapy.[5)]

Irinotecan is an anticancer drug that is used for the treatment of a wide spectrum of cancers including gastrointestinal cancer. Irinotecan is a prodrug and converted to its active compound 7-ethyl-10-hydroxy-camptothecin (SN-38) by the carboxylesterase CES2.[6)] However, the expression of CES2 is frequently downregulated in many types of cancers including gastric cancer,[6)] which may affect the therapeutic efficacy of irinotecan. Recent studies have indicated that CES2 can be transcriptionally activated by p53, a tumor suppressor that controls the transcription of plethora of genes in response to cellular stresses such as DNA damage, oxidative stress, and hypoxia.[7)] Therefore, it is conceivable that activation of p53 could sensitize gastric cancer cells to irinotecan by upregulating CES2 expression.

In this study, using various cell lines of human gastric cancer (Table 1), we provide evidence that irinotecan exerts a synergistic antitumor effect in combination with a p53 activator in human gastric cancer cells. We used nutlin-3a as a p53 activator, which inhibits binding of the E3 ubiquitin ligase MDM2 to p53 and thereby directly activates p53 signaling without genotoxic side effects.[13)] Nutlin-3a treatment enhanced CES2 expression in gastric cancer cells and sensitized these cells to irinotecan in a p53-dependent manner. Our results highlight the importance of *TP53* gene status and the combination use of p53-activating drugs for the efficacy of irinotecan and other antitumor prodrugs that are activated by CES2 in human gastric cancer.

MATERIALS AND METHODS

Cell Lines and Reagents The human gastric cancer cell lines MKN1, MKN7, MKN74 and NUGC4 were obtained from the RIKEN BRC Cell Bank (Tsukuba-shi, Ibaraki, Japan). TMK1 cells were provided by Hiroshima University (Hiroshima-shi, Hiroshima, Japan). NUGC3 cells were provided by the Health Science Research Resources Bank (Sennan-shi, Osaka, Japan). AGS cells were from the American Type Culture Collection (ATCC). The cells were cultured at 37°C and 5% CO_2 in RPMI 1640 medium (Wako) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Nutlin-3a was purchased from AdooQ BioScience. All

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[1] The first two authors equally contributed to this study.

Table 1. Human Gastric Cancer Cell Lines Used in This Study

Name	Histological type[a]	Origin	*TP53* Status	*TP53* Mutation found at				Reference
				cDNA description	Exon	Codon	Amino acid change	
AGS	as	Stomach	wt	-	-	-	-	8
NUGC4	sig	metastasis (LN)	wt	-	-	-	-	8
MKN74	tub2	metastasis (Liver)	wt	-	-	-	-	9
NUGC3	por	metastasis (Brachialis muscle)	mt	c.659A>G	6	220	Tyr to Cys	8
MKN1	por	metastasis (LN)	mt	c.428T>C	5	143	Val to Ala	10
MKN7	tub1	metastasis (LN)	mt	c.832C>T	8	278	Pro to Ser	11
	tub1	metastasis (LN)	mt	c.751A>C	7	251	Ile to Leu	12
TMK1	por	metastasis (Liver)	mt	c.517G>A	5	173	Val to Met	10

[a]According to the Japanese Classification of Gastric Carcinoma. por, poorly differentiated adenocarcinoma; as, adenosquamous carcinoma; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; sig, signet ring cell carcinoma; LN, lymph node; wt, wild type; mt, mutant.

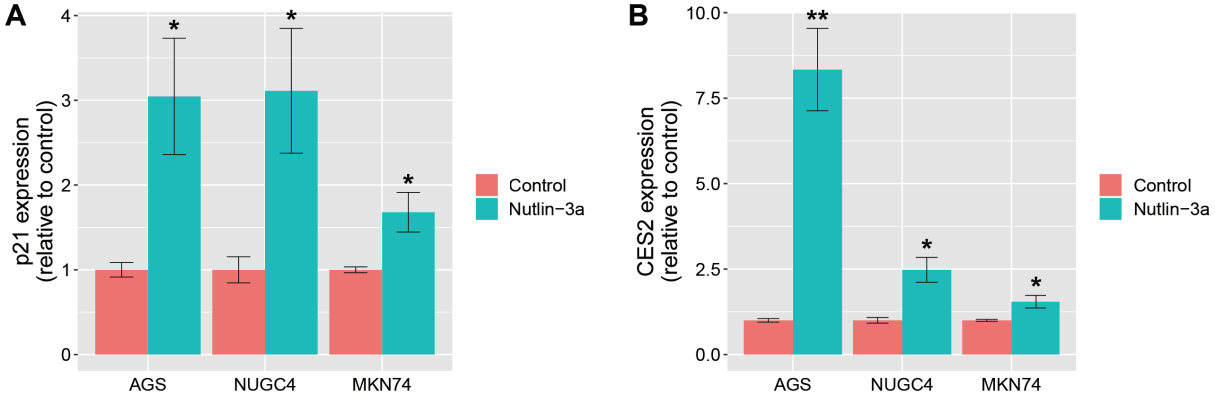

Fig. 1. Upregulation of CES2 Expression by Nutlin-3a in Gastric Cancer Cells with Wild-type p53.

Human gastric cancer cell lines with wild-type p53 (AGS, NUGC4, and MKN74) were treated with 5 μM nutlin-3a for 24 h. The expression of p21 (A) and CES2 (B) was quantified by real-time reverse transcriptase PCR. GAPDH was used as the reference gene. Data represent the mean values ± SEM (three independent experiments). *$p < 0\ 05$; **$p < 0\ 01$. An unpaired two-tailed *t*-test was used.

reagents were dissolved in sterile dimethyl sulfoxide (DMSO) to make 100 mmol/l (mM) stock. The cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated for 24 h. The cells were then treated with nutlin-3a for another 24 h. The cells were washed twice with PBS and harvested by scraping.

Real-Time Reverse Transcriptase PCR Total RNA from cultured cell lines was extracted using the FastGene RNA Basic kit (Nippon Genetics Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Semiquantitative real-time PCR was performed using the Luna Universal One-Step RT-qPCR Kit (New England BioLabs) on a LightCycler 96 (Roche) in duplicate. The gene expression of target genes was normalized to GAPDH by the differences in Ct values, and then these values were used to calculate the relative mRNA expression levels with the $2^{-\Delta\Delta Ct}$ method. The primer sequences for the genes were as previously described.[14]

Cell Viability Assay Cell viability was determined by XTT (Cell Proliferation Kit II) assay (Roche). Briefly, cells were plated in triplicate into 96-well plates and cultured for 24 h, and then incubated with irinotecan at different concentrations in the presence or absence of 5 μM nutlin-3a for 24 h. The medium was then replaced with fresh medium and the cells were incubated with the XTT reagent for 3 h. The absorbance at 450 nm (reference wavelength at 660 nm) was measured with an iMark Microplate Absorbance Reader (Bio-Rad). Best-fit IC50 values were calculated with Prism 7 (GraphPad Software Inc., San Diego, CA) and compared by an extra sum-of-square *F* test.

RESULTS AND DISCUSSION

CES2 Expression was Upregulated by p53 Activation in Gastric Cancer Cells We first asked whether p53 activation enhanced CES2 expression in gastric cancer cells. Human gastric cancer cell lines with wild-type p53 (AGS, NUGC4, and MKN74) (Table 1) were treated with the p53 activator nutlin-3a. The expression of p21, a major downstream target of p53,[15] was upregulated by nutlin-3a treatment in all p53 wild-type cell lines tested, demonstrating activation of the p53 pathway in these cells (Fig. 1A). We also observed significant upregulation of CES2 expression in these cells (Fig. 1B). In contrast, nutlin-3a treatment did not significantly affect the expression of these genes in gastric cancer cells with p53 mutation (Table 1, Fig. 2). These results indicate that nutlin-3a enhances CES2 expression by activating functional p53 in human gastric cancer cells.

Synergistic Antitumor Effects of Irinotecan and Nutlin-3a in p53 Wild-Type Cells We next investigated whether nutlin-3a treatment sensitized gastric cancer cells to irinotecan, an anticancer prodrug that is converted by CES2 to its active compound SN-38. We treated two p53 wild-type cell lines (AGS and NUGC4) and two cell lines with non-functional p53 (NUGC3 and TMK1) with various concentrations of iri-

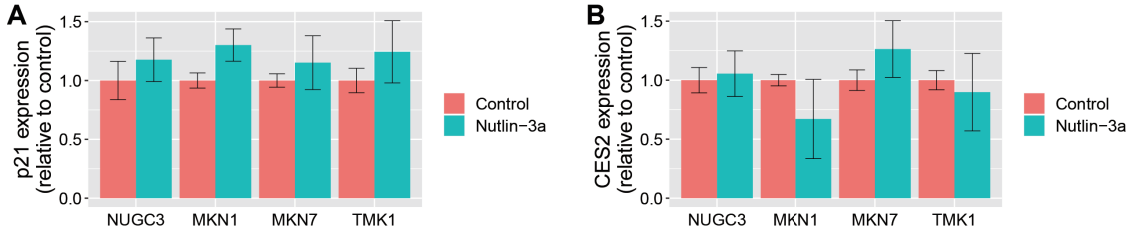

Fig. 2. Effects of Nutlin-3a on CES2 Expression in Gastric Cancer Cells with p53 Mutation.

Human gastric cancer cell lines with p53 mutation (NUGC3, MKN1, MKN7, and TMK1) were treated with 5 μM nutlin-3a for 24 h. The expression of p21 (A) and CES2 (B) was quantified by real-time reverse transcriptase PCR. GAPDH was used as the reference gene. Data represent the mean values ± SEM (three independent experiments). There was no significance between control and nutlin-3a. An unpaired two-tailed *t*-test was used.

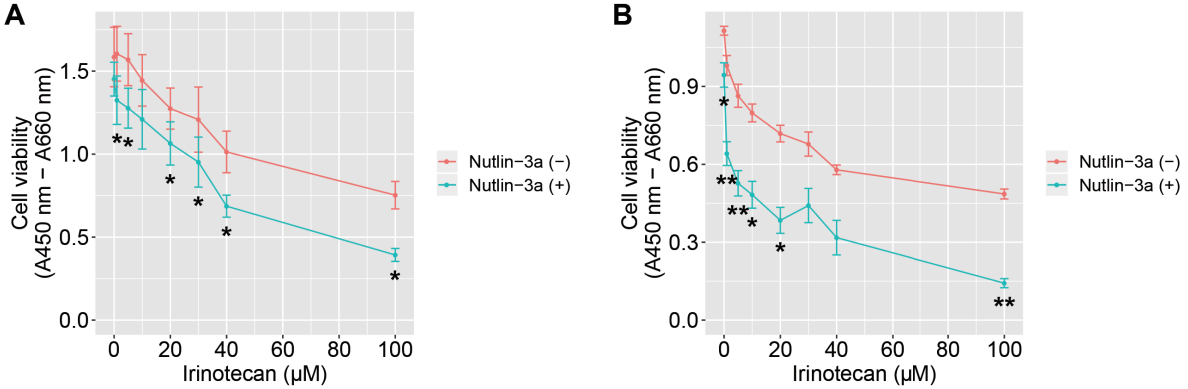

Fig. 3. Synergistic Effects of Nutlin-3a and Irinotecan in Gastric Cancer Cells with Wild-type p53.

AGS (A) and NUGC4 (B) cells were treated with various concentrations of irinotecan in the presence or absence of 5 μM nutlin-3a for 24 h. The cell viability was determined by XTT assay. Data represent the mean values ± SEM (three independent experiments). *$p < 0\ 05$; **$p < 0\ 01$. A paired two-tailed *t*-test was used.

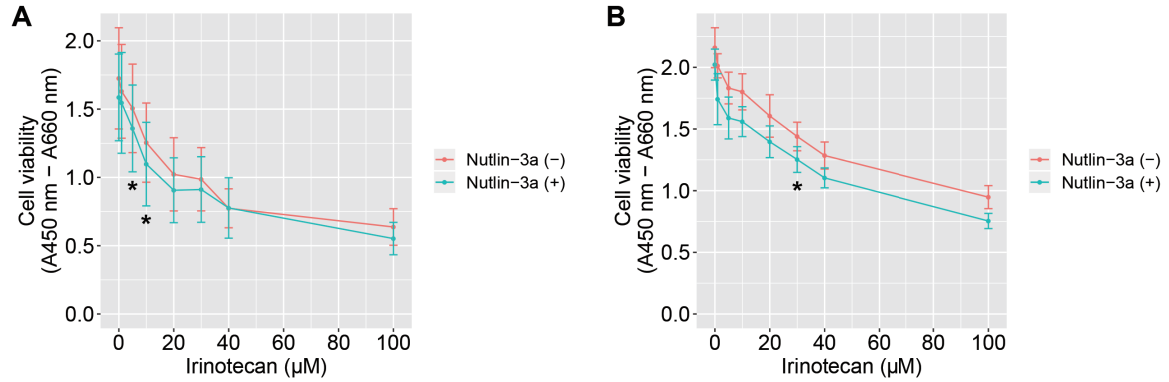

Fig. 4. Effects of Nutlin-3a and Irinotecan in Gastric Cancer Cells with p53 Mutation.

NUGC3 (A) and TMK1 (B) cells were treated with various concentrations of irinotecan in the presence or absence of 5 μM nutlin-3a for 24 h. The cell viability was determined by XTT assay. Data represent the mean values ± SEM (three independent experiments). *$p < 0\ 05$. A paired two-tailed *t*-test was used.

notecan with or without nutlin-3a. In p53 wild-type AGS and NUGC4 cells, the cell viability was not significantly or only slightly affected by single treatment with nutlin-3a, respectively (Fig. 3A and 3B). We observed strong synergistic effects of irinotecan and nutlin-3a in AGS and NUGC4 cells (Fig. 3A and 3B). The IC50 value of irinotecan was decreased by nutlin-3a by 2-fold, from 82.94 μM (95% CI [confidence interval]: 71.47–99.43 μM) to 42.94 μM (95% CI: 35.99–52.86 μM) ($p < 0.0001$) in AGS cells, and 7-fold from 59.59 μM (95% CI: 47.99–78.01 μM) to 8.731 μM (95% CI: 5.058–13.52 μM) ($p < 0.0001$) in NUGC4 cells, respectively. In contrast, nutlin-3a had almost no effect on the sensitivity to irinotecan in cells with non-functional p53 (Fig. 4A and 4B; the IC50 value from 38.69 μM [95% CI: 32.35–47.51 μM] to 35.79 μM [95% CI: 29.19–45.23 μM] in NUGC3 cells [$p = 0.5735$], from 74.25 μM [95% CI: 62.01–93.09 μM] to 56.71 μM [95% CI: 43.49–81.59 μM] in TMK1 cells [$p = 0.1328$]). These results suggest that p53 activation in gastric cancer cells leads to increased conversion of irinotecan to its active compound and thereby enhances the sensitivity to irinotecan.

Although a recent study has shown the survival benefit of irinotecan monotherapy as third-line or later treatment

in advanced gastric cancer,[16] irinotecan has been mostly used in combination with other anticancer drugs such as 5-fluorouracil (5-FU), which also activates p53.[17] Thus, the beneficial effects of these regimens in gastric cancer may be in part attributed to activation of p53 and upregulation of CES2, leading to efficient conversion of irinotecan. In this context, we used nutlin-3a to investigate the role of p53 because it directly activates p53 signaling pathway without untoward genotoxic side effects that may compromise the interpretation of the results. Consequently, we found that nutlin-3a upregulated CES2 expression only in gastric cancer cells with functional p53. Thus, p53 plays an important role in regulating CES2 expression in gastric cancer cells. p53 signaling is frequently dysregulated in many types of cancer including gastric cancer. Indeed, a genomic analysis of gastric adenocarcinomas has found p53 to be the most frequently mutated gene, accounting for 46% of total tumors.[18] In addition, irinotecan exhibits gastrointestinal toxicity and often causes severe diarrhea. Thus, understanding *TP53* gene status of gastric cancer may be useful to predict the efficacy of irinotecan-containing regimens.

In addition to irinotecan, several other anticancer prodrugs are also activated by CES2.[19–21] Capecitabine is an orally administered prodrug of 5-FU, which is effective and well tolerated in the treatment of gastric cancer. Various capecitabine-based chemotherapies have been shown to extend survival in advanced gastric cancer.[22] LY2334737 is an oral prodrug of the clinically efficacious anticancer agent gemcitabine. Gemcitabine is widely used in the treatment of pancreatic cancer[23] and advanced gastric cancer.[24] CES2 also converts Pentyl PABC-Doxaz to the active compound doxazolidine, a formaldehyde conjugate of doxorubicin that exhibits enhanced toxicity against a wide variety of tumor cell lines including cell lines resistant to doxorubicin.[25] Thus, the sensitivity of gastric cancer cells to these prodrugs may also be enhanced by p53 activation.

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Conflict of interest The authors declare no conflict of interest.

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