Am J Cancer Res 2019;9(1):79-93
www.ajcr.us /ISSN:2156-6976/ajcr0089596

Original Article

A p53-stabilizing agent, CP-31398, induces p21 expression with increased G2/M phase through the YY1 transcription factor in esophageal carcinoma defective of the p53 pathway

Boya Zhong[1,2], Masato Shingyoji[3], Michiko Hanazono[1], Thảo Thi Thanh Nguyễn[1,2], Takao Morinaga[1], Yuji Tada[4], Kenzo Hiroshima[5], Hideaki Shimada[6], Masatoshi Tagawa[1,2]

[1]Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ku, Chiba 260-8717, Japan; [2]Department of Molecular Biology and Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan; [3]Division of Respirology, Chiba Cancer Center, 666-2 Nitona, Chuo-ku, Chiba 260-8717, Japan; [4]Department of Respirology, Graduate School of Medicine, Chiba University, Chiba, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan; [5]Department of Pathology, Tokyo Women's Medical University Yachiyo Medical Center, 477-96 Ohwadashinden, Yachiyo, Chiba 276-8524, Japan; [6]Department of Surgery, School of Medicine, Toho University, 6-11-1 Oomori-nishi, Oota-ku, Tokyo 143-8541, Japan

Received December 10, 2018; Accepted December 20, 2018; Epub January 1, 2019; Published January 15, 2019

Abstract: Restoration of p53 functions is one of the therapeutic strategies for esophageal carcinoma which is often defective of the p53 pathway. We examined effects of CP-31398 which potentially increased expression of wild-type p53 or converted mutated p53 to the wild-type. We used 9 kinds of human squamous esophageal carcinoma cells with different *p53* genotypes and examined expression of p53 and the related molecules in CP-31398-treated cells. Cisplatin, a DNA damaging agent, induced cleavages of PARP and caspase-3 without increase of p53 levels, indicating that the p53 down-stream pathway was disrupted in these cells. CP-31398 induced growth retardation but the cytotoxic effects were irrelevant to *p53* genotype. CP-31398 influenced expression of p53 and the downstream molecules in a cell-dependent manner, but constantly increased p21 expression at the transcriptional level with decreased YY1 expression. Knockdown experiments with siRNA demonstrated that the CP-31398-mediated p21 up-regulation was unrelated with p53 expression but was associated with YY1 expression. We also showed that CP-31398-induced cell cycle changes including increase of G2/M populations was attributable to the up-regulated p21. These data collectively indicated that CP-31398 augmented endogenous p21 levels and induced cell cycle changes through regulation of YY1, and that YY1 was a novel target of CP-31398 in p53 dysfunctional cells.

Keywords: CP-31398, esophageal carcinoma, p53, p21, YY1, G2/M arrest

Introduction

An advanced case of esophageal carcinoma remains intractable because of the frequent invasion into vital organs in the vicinity [1]. A combination of chemotherapy and radiation is applicable to the patients and they can be subjected to surgical procedures thereafter. The current chemotherapeutic agents for esophageal carcinoma are primarily DNA-damaging agents such as cisplatin (CDDP) and DNA synthesis inhibitors. Recent genome-wide sequencing data however demonstrated that the major genetic abnormality was associated with gene mutations in the p53 pathway, which resulted in p53 dysfunction and consequently in resistance to the DNA damaging anti-cancer agents [2, 3]. Reconstitution of the authentic p53 pathway is therefore one of strategies for esophageal carcinoma to enhance anti-tumor effects by the chemotherapeutic agents.

A number of agents acting on p53 expression levels are now being investigated for the therapeutic efficacy and some of them have been examined for the possible clinical feasibility [4]. An agent to inhibit a binding between p53 and MDM2 molecules which ubiquitinate and degrade p53 is a candidate to increase p53 stability [5]. An MDM2 inhibitor can increase p53 levels but the effectiveness is restricted only in cells with the wild-type *p53* genotype. A differ-

ent type of an agent is needed to activate the p53 pathway in cells with mutated *p53* genotype. CP-31398 and PRIMA-1 belong to a functionally different group from MDM2 inhibitors and augment or activate the p53 downstream pathway irrespective of the *p53* genotypes [6, 7]. The agents can not only increase expression of the wild-type p53 also convert specific mutated p53 such as codon 248, 249 and 273 to the wild-type [8]. Nevertheless, the agents with the p53-converting activity or MDM2 inhibitors have not yet examined for the cytotoxicity in esophageal carcinoma except a report dealing with nutlin-3a, one of the MDM2 inhibitors [9].

We previously showed that adenoviruses expressing the wild-type p53 (Ad-p53) induced cell death in esophageal carcinoma and increased susceptibility to chemotherapeutic agents [10]. These data suggested that activation of the p53 pathway with exogenously expressed p53 was another therapeutic strategy for esophageal carcinoma despite the whole exome sequencing data which indicated that the majority was defective of the p53-mediated signaling [2, 3]. We further conducted a clinical study to intratumorally administer Ad-p53 into esophageal carcinoma and demonstrated the safety and clinical efficacy [11]. These data collectively suggested that stimulation of the p53 pathway with transduced p53 or up-regulated endogenous p53 produced cytotoxic effects on esophageal carcinoma and indicated that restoration of the p53 pathway played an important role in the treatment.

In the present study, we investigated a possible therapeutic efficacy of CP-31398, an agent capable to convert mutated p53 into the wild-type and to augment wild-type p53 level [8]. We analyzed how 9 kinds of esophageal carcinoma cells responded to a DNA damaging agent in terms of the p53 pathway and examined whether CP-31398 activated the p53 pathway in the esophageal carcinoma cells. The present study also analyzed a mechanism of CP-31398-mediated induction of p21 in a p53-independent manner.

Materials and methods

Cells and agents

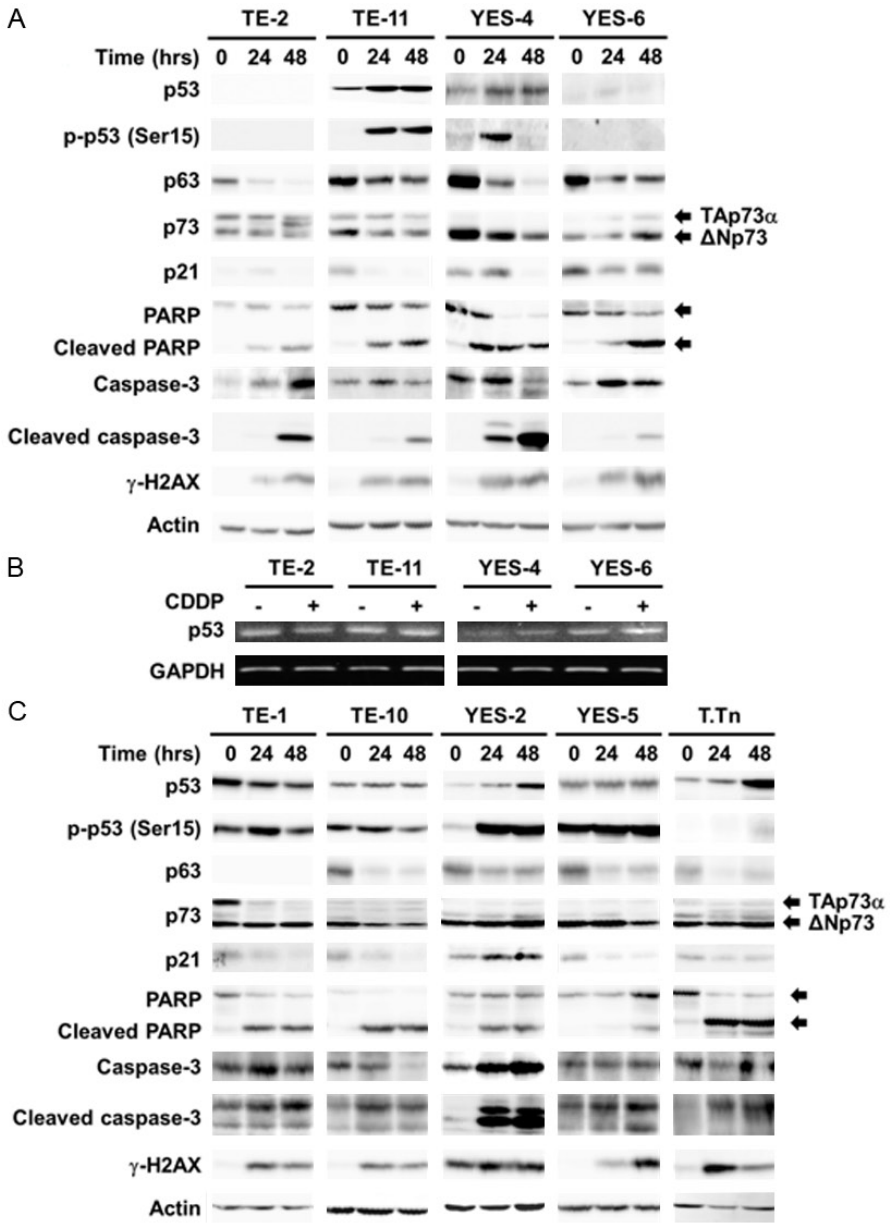
Human esophageal squamous cell carcinoma, TE-1 (mutated *p53*; at codon 272 Val to Met), TE-2 (wild-type *p53*), TE-10 (mutated at 242 Cys to Tyr), TE-11 (wild-type), YES-2 (mutated at 236 Tyr to Asn), YES-4 (wild-type), YES-5 (mutated at 280 Arg to Gly), YES-6 (wild-type) and T.Tn (mutated at 214 His to Arg and at 258 Glu to stop) cells, were from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Human mesothelioma, MSTO-211H (wild-type) and NCI-H28 (wild-type) cells, were purchased from American Type Culture Collection (Manassas, VA, USA), and JMN-1B (mutated) and EHMES-1 (mutated) which were established from Japanese patients, were kindly provided by Dr. Hironobu Hamada (Hiroshima University, Hiroshima, Japan) [12]. All cells were cultured with RPMI-1640 medium supplemented with 10% fetal calf serum. CP-31398 and nutlin-3a were purchased from Tocris Bioscience (Bristol, UK), and ChemieTek (Indianapolis, IN, USA), respectively.

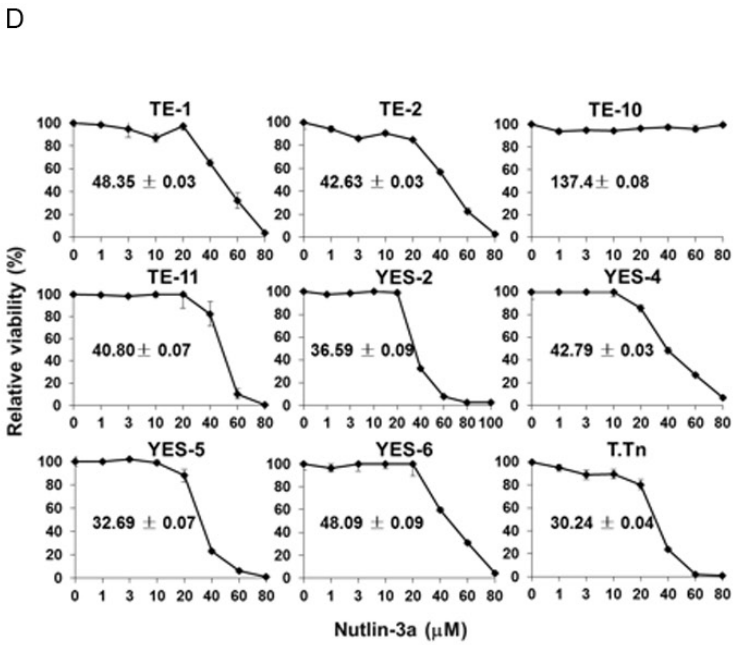
In vitro cytotoxicity and cell proliferation

Cells (2×10^3/well) were seeded in 96-well plates and were cultured for 4 days with different concentrations of an agent. Cell viability was determined with a cell-counting WST kit (Wako, Osaka, Japan) (WST assay). The amount of formazan produced from a WST-8 reagent was determined with the absorbance at 450 nm and the relative viability was calculated based on the absorbance without any treatments. Live cell numbers were also counted with the trypan blue dye (dye exclusion assay). Half maximal inhibitory concentration (IC_{50}) values were also estimated with the CalcuSyn software (Biosoft, Cambridge, UK).

Western blot analysis

Cell lysate was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein was transferred to a nylon filter and was hybridized with antibody against phosphorylated p53 at Ser 15 (catalog number: #9284) or 46 (#2521), p21 (#2947), caspase-3 (#9668), cleaved caspase-3 (#9661), poly ADP ribose polymerase (PARP) (which also detected cleaved PARP) (#4108), AMPKα (#2532), phosphorylated AMPKα (Thr172) (#2535), 4E-BP1 (#9452), phosphorylated 4E-BP1 (Thr37/46) (#9459), p70S6K (#9202), phosphorylated p70S6K (Thr389) (#9205) (Cell Signaling, Danvers, MA, USA), p53 (Ab-6, Clone DO-1) (#MS-187-P0), phosphorylated p21 (Thr145) (#PA5-36677) (Thermo Fisher Scientific, Fremont, CA, USA),


A
TE-2
TE-11
YES-4
YES-6
Time (hrs)
0 24 48 0 24 48 0 24 48 0 24 48
p53
p-p53 (Ser15)
p63
p73
TAp73α
ΔNp73
p21
PARP
Cleaved PARP
Caspase-3
Cleaved caspase-3
γ-H2AX
Actin
B
TE-2
TE-11
YES-4
YES-6
CDDP
- + - + - + - +
p53
GAPDH
C
TE-1
TE-10
YES-2
YES-5
T.Tn
Time (hrs)
0 24 48 0 24 48 0 24 48 0 24 48 0 24 48
p53
p-p53 (Ser15)
p63
p73
TAp73α
ΔNp73
p21
PARP
Cleaved PARP
Caspase-3
Cleaved caspase-3
γ-H2AX
Actin


D
TE-1
48.35 ± 0.03
TE-2
42.63 ± 0.03
TE-10
137.4 ± 0.08
TE-11
40.80 ± 0.07
YES-2
36.59 ± 0.09
YES-4
42.79 ± 0.03
YES-5
32.69 ± 0.07
YES-6
48.09 ± 0.09
T.Tn
30.24 ± 0.04
Relative viability (%)
Nutlin-3a (μM)

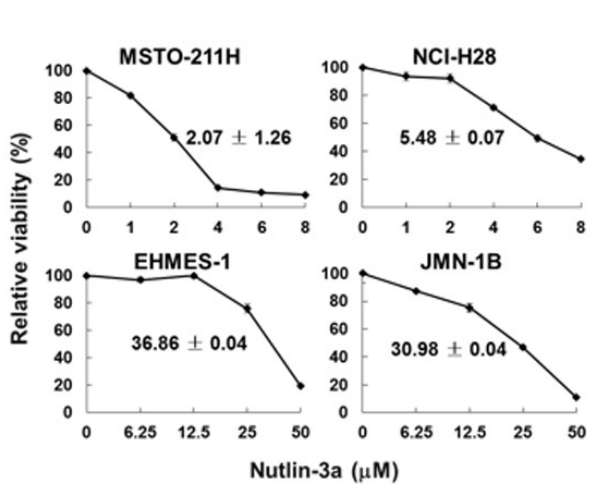

E
MSTO-211H
2.07 ± 1.26
NCI-H28
5.48 ± 0.07
EHMES-1
36.86 ± 0.04
JMN-1B
30.98 ± 0.04
Relative viability (%)
Nutlin-3a (μM)

Figure 1. Human esophageal carcinoma cells were defective of p53 activation. (A, C) Esophageal carcinoma with the wild-type *p53* (A) and mutated *p53* genotype (C) were treated with CDDP at 20 μM for 24 or 48 hrs, and expression levels of p53 and the relevant molecules were examined with Western blot analysis. Actin was used as a loading control. (B) Expression of *p53* mRNA in CDDP-treated cells. Cells which were untreated or treated with 20 μM of CDDP for 24 hrs were examined for expression of p53 and GAPDH transcripts as a loading control with RT-PCR. (D) Cells were treated with nutlin-3a at various concentrations and the relative viabilities were measured with the WST assay. IC_{50} values were calculated with CalcuSyn software. Averages and SE bars are shown (n=3). (E) Growth inhibitory effects of nutlin-3a on mesothelioma cells with different *p53* genotype. Cells were treated with nutlin-3a at various concentrations and the relative viabilities were measured with the WST assay. IC_{50} values were calculated with CalcuSyn software. Averages and SE bars are shown (n=3).

MDM2 (sc-965), YY1 (sc-7341) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p63 (ab735), p73 (ab40658), YY2 (ab116507) (Abcam, Cambridge, UK), γ-H2AX (#613401, BioLegend, San Diego, CA, USA), Kip1/p27 (#610241) (BD biosciences, San Jose, CA, USA) and actin (#4970) (Cell Signaling) as a loading control followed by appropriate second antibody. The membranes were developed with the ECL system (GE Healthcare, Buckinghamshire, UK) and imaged with ImageQuant LAS 4000 (GE Healthcare).

RNA interference

Cells were transfected with small interfering RNA (siRNA) duplex targeting YY1 (#sc-36863), p21 (#sc-29427) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 (#TP53-VHS40367) or with non-coding siRNA (#12935-114) as a control (Thermo Fisher Scientific, Fremont, CA, USA) using Lipofectamine RNAiMAX according to the manufacturer's protocol (Thermo Fisher Scientific).

Cell cycle analysis

Cells treated with an agent were fixed in ice-cold 100% ethanol, incubated with RNase (50 μg/ml) and stained with propidium iodide (50 μg/ml). The staining profiles were analyzed with FACSCalibur (BD Biosciences, San Jose, CA, USA) and CellQuest software (BD Biosciences).

Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and amplification of equal amounts of the cDNA was performed with the following primers and conditions: for the p53 gene, 5'-CTGCCCTCAACAAGATGTTTTG-3' (sense) and 5'-CTATCTGAGCAGCGCTCATGG-3' (anti-sense), and 30 sec at 96°C for denature/90 sec at 65°C for annealing/36 cycles; for the p21 gene, 5'-GACACCACTGGAGGGTGACT-3' (sense) and 5'-GGCGTTTGGAGTGGTAGAAA-3' (anti-sense), and 10 sec at 94°C/20 sec at 48°C/35 cycles; for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, 5'-ACCACAGTCCATGCCATCAC-3' (sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (anti-sense), and 15 sec at 94°C/15 sec at 60°C/30 cycles. The products were analyzed with gel electrophoresis.

Results

Esophageal carcinoma cells were defective of p53 activation

We examined possible activation of p53-mediated pathway with CDDP, a DNA damaging agent, in 9 kinds of human esophageal squamous cell carcinoma with different *p53* genotype (**Figure 1**). TE-11 and YES-4 cells, with wild-type *p53* genotype, temporally increased p53 levels and the phosphorylation at serine 15 after CDDP treatments, while the other p53 wild-type cells, TE-2 and YES-6 cells, hardly expressed p53 and did not increase the expression (**Figure 1A**). We also found that CDDP treatments scarcely increased p53 transcripts (**Figure 1B**), indicating that the p53 increase in TE-11 and YES-4 cells were due to a posttranscriptional regulation. Expression of p21, a target of p53 activation, was rather down-regulated in TE-11, YES-4 and to a lesser extent YES-6 cells, but slightly increased in TE-2 cells. Expression of p73, belonging to the p53 family proteins, was variable among the cells in both forms, TAp73α and ΔNp73, and that of p63, the another family protein, was suppressed by CDDP treatments. Induction of the DNA damage was evidenced by up-regulated γ-H2AX expression and all the cells showed cleavages of caspase-3 and PARP. These data collectively indicated that CDDP induced cell death without activation of p53 and the other p53 family proteins, and suggested that the p53 down-

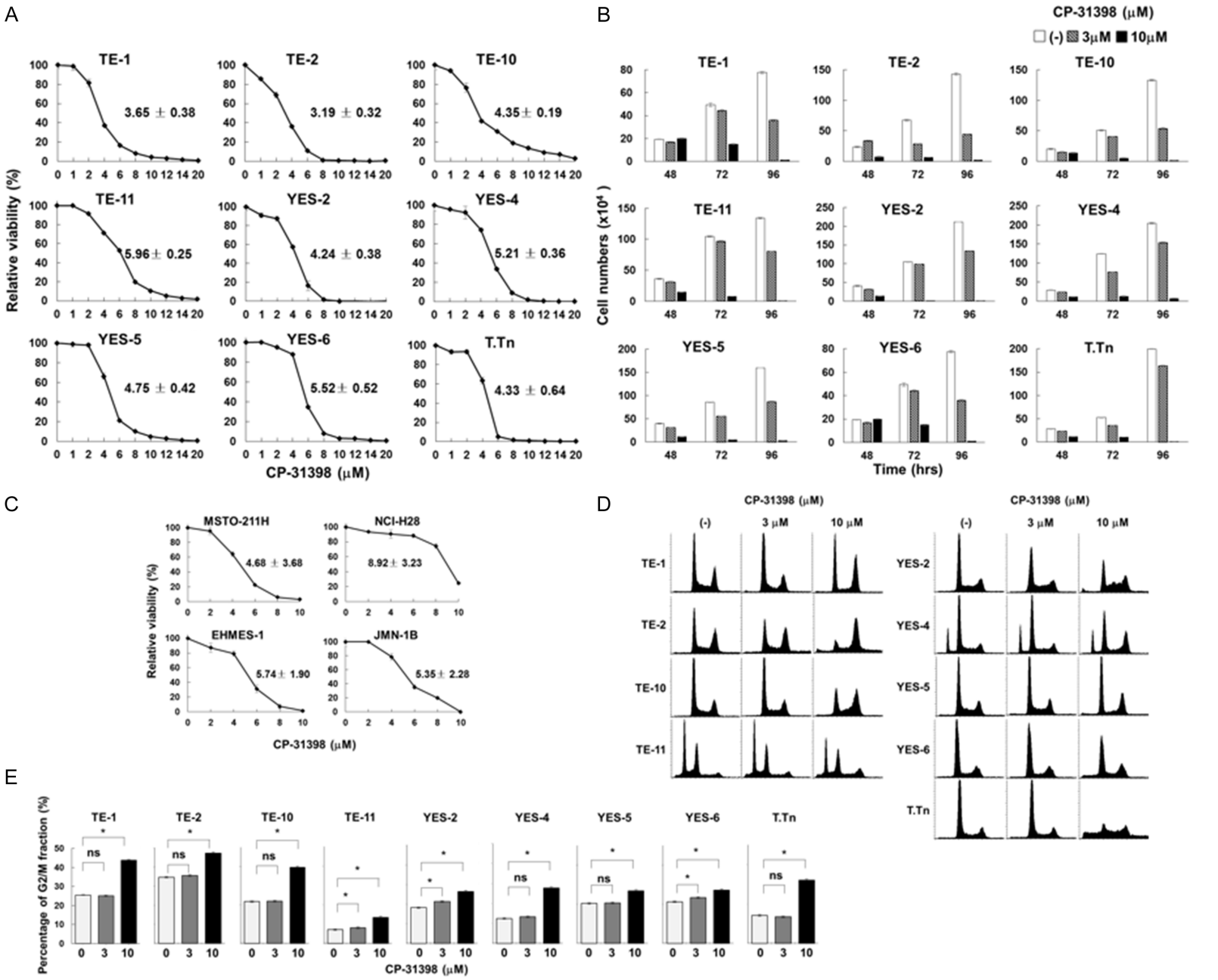

A
TE-1
3.65 ± 0.38
TE-2
3.19 ± 0.32
TE-10
4.35± 0.19
TE-11
5.96± 0.25
YES-2
4.24 ± 0.38
YES-4
5.21 ± 0.36
YES-5
4.75 ± 0.42
YES-6
5.52± 0.52
T.Tn
4.33 ± 0.64
Relative viability (%)
CP-31398 (μM)
B
CP-31398 (μM)
(-) 3μM 10μM
Cell numbers (x10⁴)
Time (hrs)
C
MSTO-211H
4.68 ± 3.68
NCI-H28
8.92± 3.23
EHMES-1
5.74± 1.90
JMN-1B
5.35± 2.28
Relative viability (%)
CP-31398 (μM)
D
CP-31398 (μM)
(-)
3 μM
10 μM
E
Percentage of G2/M fraction (%)
ns
CP-31398 (μM)

Figure 2. CP-31398-mediated effects on esophageal carcinoma. (A, B) Esophageal carcinoma (A) and mesothelioma (B) cells were treated with CP-31398 as indicated and the relative viabilities were measured with the WST assay. IC_{50} values were calculated with CalcuSyn software. Averages and SE bars are shown (n=3). (C) Cells were treated with CP-31398 as indicated and live cell numbers were counted with a trypan blue dye exclusion assay. Averages and SE bars are shown (n=3). (D) Representative cell cycle profiles of esophageal carcinoma cells which were treated with CP-31398 for 48 hrs were analyzed with a flow cytometry. Percentages of each fraction was shown in **Table 1**. (E) Percentages of G2/M phase populations in esophageal carcinoma cells treated with CP-31398 as indicated for 48 hrs. SE bars are also shown (n=3). *P<0.05. ns: not significant. The percentages are also shown in **Table 1**.

stream pathway was non-functional in esophageal carcinoma despite the wild-type *p53* genotype.

Esophageal carcinoma cells with mutated *p53* genotypes showed differential expression levels of p53 and the phosphorylation after CDDP treatments (**Figure 1C**). TE-1 and TE-10 cells decreased p53 and the phosphorylation levels, whereas YES-2 and T.Tn cells increased both p53 levels and YES-5 cells did not influence the levels. Expression levels of p21 and p63 were down-regulated except p21 in YES-2 cells, but those of TAp73α and ΔNp73 remained unchanged except TAp73α in TE-1. CDDP-treated cells showed increased γ-H2AX and cleavages of PARP and caspase-3 in these cells. These data therefore indicated that a DNA damaging agent activated apoptotic pathway in a p53-independent manner in esophageal carcinoma cells.

We also examined whether the p53 downstream pathway in the esophageal carcinoma cells functioned by analyzing relative sensitivity to nutlin-3a (**Figure 1D**). Nutlin-3a, an inhibitor for interaction between MDM2 and p53, induced growth inhibition in cells with the wild-type *p53* genotype, whereas cells with mutated *p53* genotype were insensitive to nutlin-3a [13]. We used mesothelioma cells with the wild-type and with mutated *p53* genotype, and showed that those with the wild-type *p53* were sensitive to nutlin-3a-mediated growth inhibition (average IC_{50} ± SE: 3.77 ± 1.71), whereas those with mutated *p53* were insensitive (33.92 ± 2.94) (**Figure 1E**). Susceptibility of esophageal carcinoma cells to nutlin-3a showed that the IC_{50} values were all similar to that of mesothelioma with mutated *p53* and the sensitivity was not influenced by the *p53* genotype. These data collectively indicated that the p53 downstream pathway in esophageal carcinoma were non-functional irrespective of the *p53* genotype and suggested that cell death by a DNA damage was irrelevant to the p53 pathways.

Growth suppression produced by CP-31398 was irrelevant to the p53 genotype

We then examined growth suppressive activity of CP-31398 with the esophageal carcinoma (**Figure 2A**). Sensitivity of cells with the wild-type *p53* to CP-31398 (IC_{50}=4.97 ± 1.23) was not different from that of cells with mutated *p53* (4.27 ± 0.40), indicating that the susceptibility was not associated with the *p53* genotype (P=0.26). We also tested susceptibility of mesothelioma to CP-31398 and found that CP-31398 inhibited the cell growth in a p53-independent manner (IC_{50} of wild-type *p53*: 6.80 ± 2.12, mutated *p53*: 5.55 ± 0.20) (**Figure 2B**). We examined live cell numbers of esophageal carcinoma cells treated with CP-31398 (**Figure 2C**). CP-31398 treatments induced growth retardation and decreased live cell numbers, indicating that CP-31398 produced cytostatic and cytotoxic effects depending on the agent dose and the effects were not associated with the *p53* genotype. These data collective indicated that mechanism of CP-31398-mediated cytotoxicity was irrelevant to the p53 pathway.

Cell cycle progression by CP-31398

We tested cell cycle progression of cells treated with CP-31398 (**Figure 2D**; **Table 1**). We found that a small percentage of TE-1, TE-2 and YES-5 cells showed an over-4N population (hyperploidy), and TE-4 and TE-11 cells constantly had a sub-G1 population which was different from a cell death-linked sub-G1 fraction. The hyperploidy fractions and the persistent sub-G1 population could be attributable to an abnormal cell division process in nucleoli, which was pertinent to malignant transformation. CP-31398-treated cells showed increase in G2/M populations in all the cells tested (**Figure 2E**), hyperploidy fractions in TE-2, TE-10, YES-2 and T.Tn cells, and sub-G1 populations in YES-2, YES-5, YES-6 and T.Tn cells.

Table 1. Cell cycle progression of esophageal carcinoma cells treated with CP-31398

Cells	Time (hrs)	CP-31398 (μM)	Cell cycle distribution (%) (Average ± SE)				
			Sub-G1	G1	S	G2/M	Hyperploidy
TE-1	24	(-)	1.43 ± 0.03	43.57 ± 1.13	22.02 ± 0.53	27.80 ± 0.35	5.19 ± 0.03
		3	2.20 ± 0.19	62.86 ± 0.69	15.50 ± 0.67	16.98 ± 2.16	2.46 ± 0.01
		10	2.83 ± 0.12	38.22 ± 0.38	11.57 ± 0.43	41.60 ± 0.38	5.77 ± 0.05
	48	(-)	1.30 ± 0.03	57.30 ± 0.29	10.45 ± 0.22	25.57 ± 0.10	5.38 ± 0.10
		3	1.05 ± 0.03	54.08 ± 0.69	14.00 ± 0.30	25.14 ± 0.17	5.74 ± 0.07
		10	3.87 ± 0.17	37.94 ± 0.07	8.07 ± 0.10	43.82 ± 0.30	6.30 ± 0.03
TE-2	24	(-)	4.13 ± 0.05	31.95 ± 0.44	22.68 ± 0.20	35.23 ± 0.22	6.01 ± 0.02
		3	3.31 ± 0.13	20.29 ± 0.20	26.84 ± 0.20	37.79 ± 0.26	11.77 ± 0.05
		10	3.31 ± 0.14	14.93 ± 0.21	20.12 ± 0.17	47.48 ± 0.35	14.16 ± 0.07
	48	(-)	3.32 ± 0.11	32.32 ± 0.41	23.80 ± 0.09	34.64 ± 0.33	5.92 ± 0.12
		3	4.54 ± 0.13	26.01 ± 0.20	22.68 ± 0.19	35.55 ± 0.23	11.22 ± 0.04
		10	6.97 ± 0.19	11.39 ± 0.61	15.71 ± 0.24	47.30 ± 0.15	18.64 ± 0.15
TE-10	24	(-)	1.09 ± 0.02	44.26 ± 0.50	23.00 ± 0.45	29.86 ± 0.42	1.79 ± 0.02
		3	1.67 ± 0.14	45.46 ± 0.40	25.54 ± 0.33	26.23 ± 0.23	1.00 ± 0.06
		10	3.81 ± 0.20	34.58 ± 0.46	35.04 ± 0.13	24.67 ± 0.55	1.90 ± 0.05
	48	(-)	1.35 ± 0.02	55.77 ± 0.20	20.26 ± 0.24	21.68 ± 0.16	0.94 ± 0.02
		3	1.82 ± 0.16	52.90 ± 0.40	22.03 ± 0.09	21.92 ± 0.20	1.33 ± 0.01
		10	4.68 ± 0.13	27.84 ± 0.23	23.86 ± 0.61	39.54 ± 0.33	4.08 ± 0.15
TE-11	24	(-)	42.47 ± 0.19	33.65 ± 0.20	14.77 ± 0.28	8.89 ± 0.37	0.23 ± 0.02
		3	48.38 ± 0.41	28.21 ± 0.30	15.89 ± 0.32	7.33 ± 0.48	0.19 ± 0.03
		10	39.56 ± 0.11	29.15 ± 0.25	18.17 ± 0.12	12.19 ± 0.09	0.93 ± 0.01
	48	(-)	48.54 ± 0.44	33.17 ± 0.47	10.86 ± 0.28	7.10 ± 0.29	0.32 ± 0.01
		3	48.99 ± 0.27	29.93 ± 0.30	12.42 ± 0.10	8.05 ± 0.06	0.61 ± 0.01
		10	39.56 ± 0.14	28.76 ± 0.46	16.68 ± 0.13	13.37 ± 0.22	1.63 ± 0.04
YES-2	24	(-)	1.93 ± 0.06	45.75 ± 0.27	25.96 ± 0.20	25.96 ± 0.25	0.40 ± 0.01
		3	1.95 ± 0.20	47.74 ± 0.73	23.13 ± 0.52	26.56 ± 0.84	0.62 ± 0.03
		10	2.88 ± 0.10	36.64 ± 0.44	32.74 ± 0.29	23.61 ± 0.38	4.13 ± 0.05
	48	(-)	2.87 ± 0.01	52.52 ± 0.32	25.79 ± 0.24	18.20 ± 0.15	0.62 ± 0.02
		3	2.36 ± 0.37	46.14 ± 0.73	29.03 ± 0.12	21.38 ± 1.08	1.09 ± 0.03
		10	6.39 ± 0.23	26.08 ± 0.82	35.64 ± 0.24	26.37 ± 0.27	5.52 ± 0.04
YES-4	24	(-)	16.40 ± 0.79	48.61 ± 0.36	22.06 ± 0.20	12.43 ± 0.80	0.50 ± 0.01
		3	14.95 ± 0.29	45.48 ± 0.24	23.33 ± 0.51	15.86 ± 0.04	0.38 ± 0.05
		10	14.64 ± 0.19	39.63 ± 0.09	22.11 ± 0.17	22.12 ± 0.22	1.49 ± 0.05
	48	(-)	15.29 ± 0.12	49.45 ± 0.42	21.84 ± 0.26	12.39 ± 0.38	1.03 ± 0.04
		3	16.54 ± 0.05	48.06 ± 0.24	21.08 ± 0.17	13.20 ± 0.03	1.13 ± 0.01
		10	15.41 ± 0.10	35.16 ± 0.46	19.48 ± 0.01	27.65 ± 0.34	2.29 ± 0.04
YES-5	24	(-)	2.25 ± 0.07	44.09 ± 0.57	25.03 ± 0.47	23.45 ± 0.33	5.19 ± 0.03
		3	3.49 ± 0.07	49.18 ± 0.06	21.21 ± 0.14	19.57 ± 0.28	6.55 ± 0.12
		10	9.83 ± 0.27	31.51 ± 0.83	22.01 ± 0.73	32.24 ± 0.42	4.41 ± 0.03
	48	(-)	3.39 ± 0.14	47.04 ± 0.37	24.38 ± 0.17	20.42 ± 0.03	4.77 ± 0.02
		3	3.30 ± 0.09	48.79 ± 0.06	23.09 ± 0.12	20.57 ± 0.08	4.24 ± 0.10
		10	5.21 ± 0.04	47.38 ± 0.11	12.71 ± 0.11	26.80 ± 0.29	7.90 ± 0.05
YES-6	24	(-)	2.25 ± 0.03	48.63 ± 0.31	21.38 ± 0.13	27.10 ± 0.14	0.64 ± 0.02
		3	1.69 ± 0.13	48.32 ± 0.18	20.02 ± 0.20	29.17 ± 0.49	0.81 ± 0.04
		10	1.90 ± 0.10	48.07 ± 0.21	18.11 ± 0.29	30.70 ± 0.37	1.22 ± 0.03

	48	(-)	2.69 ± 0.09	64.27 ± 0.28	11.16 ± 0.04	21.20 ± 0.22	0.68 ± 0.03
		3	2.75 ± 0.06	61.41 ± 0.18	11.11 ± 0.19	23.42 ± 0.02	1.32 ± 0.10
		10	9.29 ± 0.15	49.34 ± 0.52	12.47 ± 0.04	27.20 ± 0.37	1.69 ± 0.05
T.Tn	24	(-)	2.48 ± 0.07	57.91 ± 0.09	20.06 ± 0.13	17.83 ± 0.09	1.73 ± 0.02
		3	3.78 ± 0.08	62.78 ± 0.15	19.22 ± 0.42	12.70 ± 0.31	1.52 ± 0.04
		10	12.37 ± 0.23	27.16 ± 0.04	23.59 ± 0.39	25.33 ± 0.28	11.55 ± 0.03
	48	(-)	2.61 ± 0.04	62.33 ± 0.33	19.24 ± 0.30	14.19 ± 0.11	1.64 ± 0.03
		3	4.12 ± 0.06	61.50 ± 0.15	18.69 ± 0.13	13.54 ± 0.04	2.16 ± 0.10
		10	22.34 ± 0.16	17.82 ± 0.32	27.85 ± 0.21	15.77 ± 0.52	16.23 ± 0.05

Cells treated with CP-31398 was analyzed for the cell cycle progression with a flow cytometry.

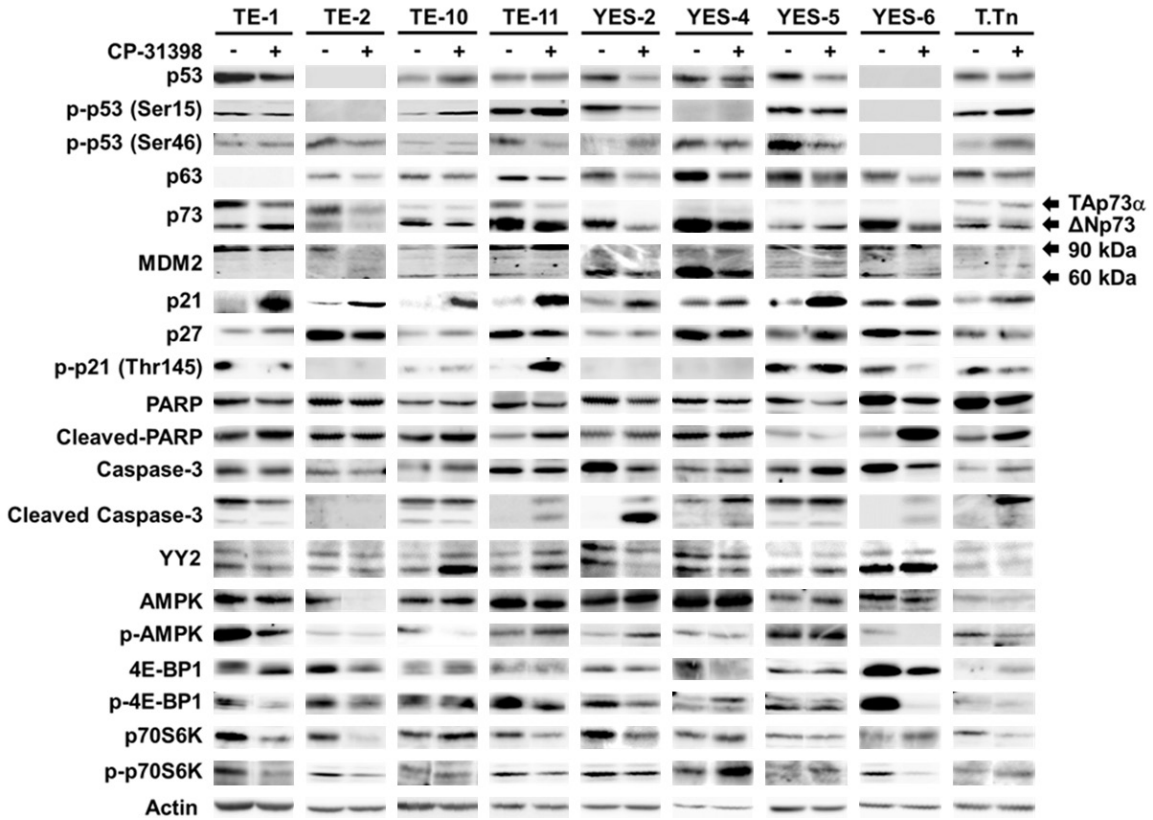


Figure 3. Expression of the p53 family and the related molecules in CP-31398-treated cells. Esophageal carcinoma cells were treated with CP-31398 at 10 μM for 48 hrs and subjected to Western blot analysis as indicated. Actin was used as a loading control.

Expression of p53 and the relevant molecules induced by CP-31398

We investigated expression of p53 and the relevant molecules in esophageal carcinoma cells treated with CP-31398 (**Figure 3**). CP-31398 increased p53 expression in TE-10 cells but decreased the levels in TE-1, YES-2 and YES-5 cells. Phosphorylated p53 levels at Ser 15 matched with changes of p53 expression in TE-10 and YES-2 cells, but those at Ser 46 in these cells were not associated with the p53 changes. TE-1 cells showed no change of the phosphorylated p53 levels and YES-5 cells decreased only Ser 46 phosphorylation. T.Tn cells up-regulated p53 phosphorylation in both Ser 15 and 46 but the p53 levels remained the same, and TE-11 cells increased the phosphorylation at Ser 15 but decreased at Ser 46 with constant p53 levels. Expression of p53 and the phosphorylation remained unchanged in YES-4, but TE-2 and YES-6 scarcely expressed p53 and the phosphorylation at Ser 15 as shown in CDDP-treated cells. CP-31398 did not

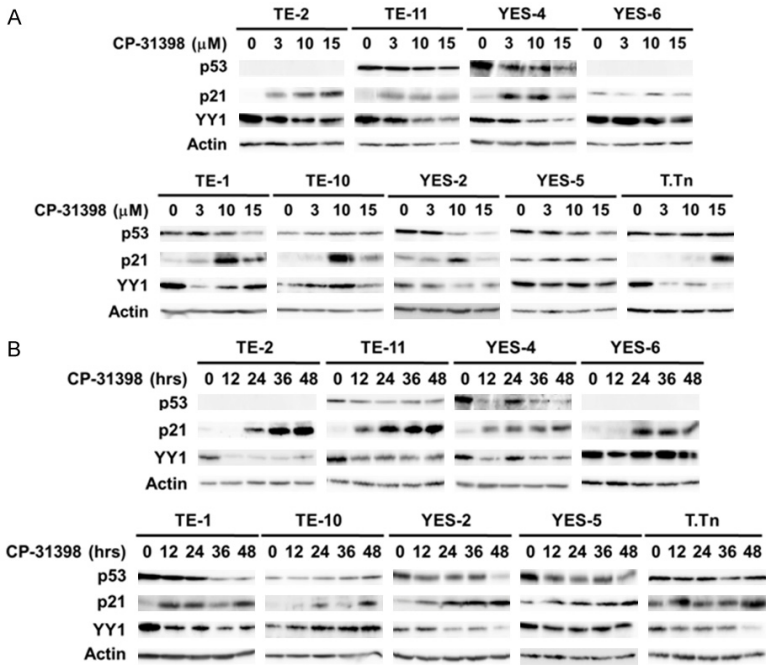


Figure 4. CP-31398-mediated augmentation of p21 and suppression of YY1 expression. Esophageal carcinoma cells treated with CP-31398 (A) at different concentrations as indicated for 48 hrs or (B) at 10 μM for different times as indicated, were subjected to Western blot analysis. Actin was used as a loading control.

increase p53 levels not only in *p53* wild-type cells but in mutated *p53* cells, suggesting that CP-31398 did not increase stability of wild-type p53 or probably conversion from mutated p53 to the wild-type p53. As for other p53 family proteins, esophageal carcinoma cells decreased p63 and/or p73 isoform expression levels except TE-1 and TE-10 cells, and consequently CP-31398-mediated changes of the p53 family proteins were not linked with the *p53* genotypes. Expression of MDM2, a target of the p53 pathway, was constant except in TE-2 and YES-4 cells which down-regulated the expression, whereas expression of p21, also the p53 target molecules, was up-regulated in all the cells. Phosphorylated p21 at Thr 145, a marker of p21 stability, was however not correlated with increased p21 levels, suggesting that augmented p21 expression was attributable to the transcriptional activation. CP-31398-induced changes of p27 expression were variable among the cells and the expressional changes were inconsistent with the p53 changes. Increased cleavage of PARP or caspase-3 was detected in all the cells except TE-2 cells, indicating that CP-31398 induced the apoptotic pathway in a p53-independent manner.

We also examined expression of YY2, p53-bindindg molecules involved in p21 transcriptional activation [14], and that of AMPK which was in an upstream pathway of 4E-BP1 which played a role in p21 stability [15]. Expression of YY2 increased in TE-10 and to a lesser extent TE-11 cells, but decreased in YES-2 cells. Other cells treated with CP-31398 did not show any changes of YY2 expression. Expression of AMPK or the phosphorylation was down-regulated in the majority of cells, but TE-11 and YES-2 cells increased the phosphorylation levels. YES-4 cells was not influenced in both expression levels and YES-5 minimally increased AMPK expression. Expression levels of 4E-BP1, p70S6K and respective phosphorylated molecules was in general down-regulated with CP-31398 except increased levels of phosphorylated 4E-BP1 and p70S6K in YES-4, marginally increased p70S6K in YES-6 and 4E-BP1 in T.Tn cells. These data collectively indicated that YY2 or 4E-BP1 expression profiles did not matched with the p21 up-regulation by CP-31398 and suggested that both molecules were not responsible for the augmented expression.

We further examined expression of p53 and the related molecules in CP-31398-treated cells with different doses and incubation periods (**Figure 4**). Expression of p53 was down-regulated in the majority in dose- and time-dependent manners except TE-10 which increased the level, T.Tn with unchanged levels, TE-2 and TE-6 which did not expressed p53. The responses to CP-31398 in respective cells were thus different from those to CDDP, indicating that CP-31398 did not induce the same DNA damage as CDDP did. The dose and time course experiments showed that expression of p21 was up-regulated with CP-31398 compared with the expression in untreated cells. Expression of YY1, a transcriptional factor which negatively regulates p21 transcripts [16, 17], decreased with CP-31398 treatments except

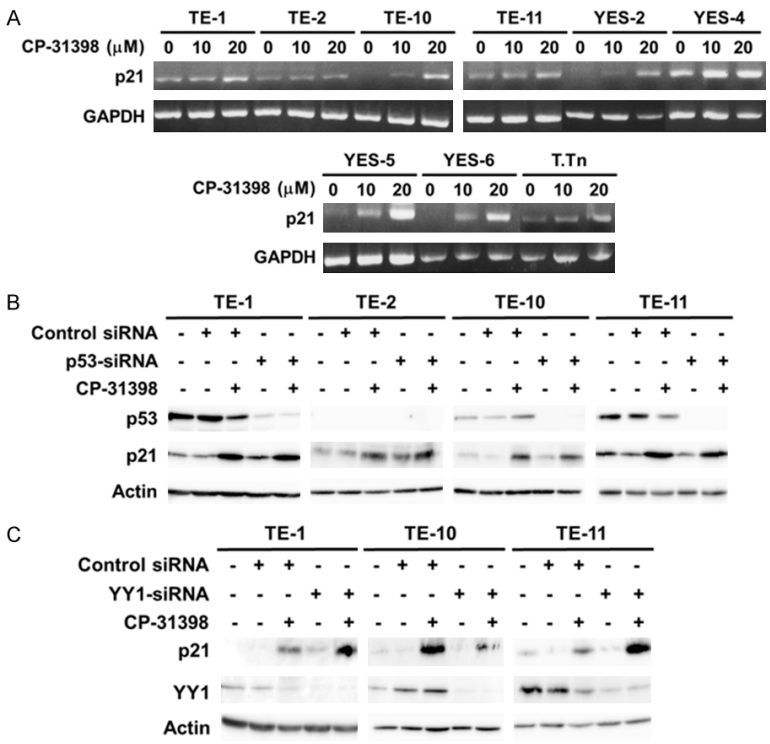


Figure 5. CP-31398-mediated up-regulation of p21 was independent of p53 but associated with YY1 expression. (A) Esophageal carcinoma cells treated with CP-31398 as indicated for 48 hrs and amounts of *p21* transcripts were analyzed with RT-PCR. GAPDH transcripts are shown as a control. (B, C) Cells transfected with either (B) p53-siRNA or (C) YY1-siRNA were treated with CP-31398 at 10 μM for 48 hrs and subjected to Western blot analysis. Cells transfected with control siRNA were also used as a reference. Actin was used as a loading control.

TE-10 cells that increased the level. These data collectively suggested that CP-31398-mediated p21 increase was independent of the p53 pathway and was well associated with YY1 expression.

CP-31398 activated p21 transcription and YY1 involvement in the p21 induction

We investigated whether the CP-31398 regulated p21 expression at the transcriptional level with RT-PCR (**Figure 5A**). An amount of p21 transcripts increased in esophageal carcinoma cells treated with CP-31398 and the up-regulation was dependent on CP-31398 concentrations used. All the esophageal carcinoma showed increase of p21 transcripts but the mRNA expression levels were not matched with the protein levels, suggesting that p21 expression was also regulated at the post-transcriptional level.

We examined a possible involvement of p53 in the CP-31398-mediated p21 up-regulation with siRNA in 4 representative cells regarding *p53* genotype (**Figure 5B**). Expression of p53 was down-regulated with p53-siRNA but not with control siRNA irrespective of the genotype. Knockdown of p53 scarcely influenced endogenous p21 expression or the CP-31398-induced augmented p21 levels. Cells treated with control siRNA augmented p21 expression after CP-31398 treatments. These data with the siRNA indicated that the CP-mediated p21 augmentation was irrelevant to p53 expression. We next investigated how YY1 regulated the CP-31398-mediated p21 up-regulation in YY1 down-regulated cells (**Figure 5C**). TE-1, TE-10 and TE-11 cells decreased YY1 expression with YY1-siRNA but not with control siRNA. Down-regulation of YY1 increased p21 expression in TE-1 and TE-11 cells but decreased the expression in TE-10 cells when they were treated with CP-31398. TE-10 cells treated with CP-31398 exceptionally increased YY1 expression in contrast with the other cells and showed p21 augmentation like the others (**Figure 4A** and **4B**). These data indicated that YY1 was a negative regulator for p21 expression in CP-31398-treated cells except TE-10 cells in which p21 was positively controlled by YY1. We noticed that knockdown of YY1 did not increase p21 expression in CP-31398-untreated cells and TE-10 cells treated with control siRNA by itself increased YY1 expression but did not influence p21 expression. These data suggested that p21 expression was not totally dependent on YY1 and that regulation of YY1 in TE-10 cells was different from that in the other cells.

Up-regulated p21 mediated CP-31398-induced cell cycle changes

We examined how the up-regulated p21 expression contributed to cell cycle changes induced in CP-31398-treated cells with siRNA-mediated

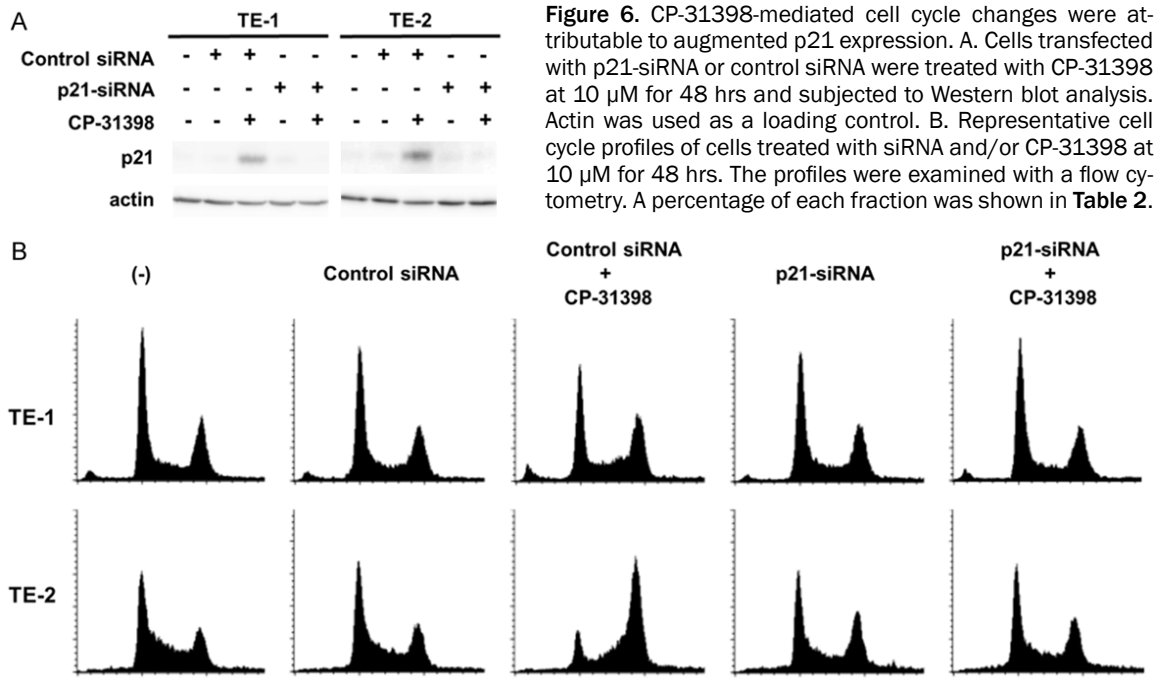


Figure 6. CP-31398-mediated cell cycle changes were attributable to augmented p21 expression. A. Cells transfected with p21-siRNA or control siRNA were treated with CP-31398 at 10 μM for 48 hrs and subjected to Western blot analysis. Actin was used as a loading control. B. Representative cell cycle profiles of cells treated with siRNA and/or CP-31398 at 10 μM for 48 hrs. The profiles were examined with a flow cytometry. A percentage of each fraction was shown in **Table 2**.

p21 knockdown (**Figure 6**). Expression of p21 became completely negative with the siRNA even in CP-31398-treated cells (**Figure 6A**). Cell cycle progression induced by CP-31398 was examined in cells treated with the siRNA (**Figure 6B**; **Table 2**). Increased G2/M fractions observed in TE-1 cells treated with CP-31398 for 48 hrs were not detected in cells treated with p21-siRNA. Likewise, TE-2 cells increased G2/M population with CP31398 treatments but p21-siRNA treated cells decreased the population to the level of untreated cells. TE-2 cells showed increased hyperploidy with CP-31398 treatments and the up-regulation was disappeared with p21-siRNA treatments. Decreased G0/G1 populations induced by CP-31398, probably accompanied by increased G2/M and hyperploidy fractions, was returned to the level of untreated cells. These data collectively indicated that cell cycle changes caused by CP-31398 was mainly due to increased p21 expression.

Discussion

The present study examined effects of a p53-stabilizing CP-31398 on p53 relevant pathways in human esophageal squamous cell carcinoma cells bearing the wild-type *p53* or mutated *p53* of which conformation was not corrected by CP-31398 [8]. We showed that CP-31398 did not activated the p53 pathway but up-regulated p21 expression at the transcription level in a p53-independent manner, and demonstrated firstly to our knowledge that the CP-31398-mediated augmentation of p21 expression was regulated by a transcriptional factor YY1. We also showed that the p21 up-regulation was responsible for cell cycle changes induced by CP-31398 in the esophageal carcinoma cells.

Previous studies showed that CP-31398 augmented p53 expression by stabilizing p53 through inhibiting p53 ubiquitination but not inhibiting the p53-MDM2 interactions in *p53* wild-type cells [18]. The agent also converted a few types of mutated p53 to wild-type p53 due to a conformational change at the DNA binding domain of p53 [6, 8]. Nevertheless, subsequent studies demonstrated that CP-31398-mediated effects were complex [19] and were dependent on tumors tested and on the genetic backgrounds [20]. The present study investigated CP-31398-mediated effects in p53-dyfunctional but not p53-deleted tumors on cell growth inhibition, and demonstrated that CP-31398-mediated p53 and the phosphorylation levels, and the cytotoxicity were irrelevant to the *p53* genotype, and furthermore cleavages of PARP and caspase-3 were not associated with p53 up-regulations. These data th-

Table 2. Cell cycle progression of esophageal carcinoma cells which were treated with CP-31398 and siRNA

Cells	Time (hrs)	Conditions	Cell cycle distribution (%) (Average ± SE)				
			Sub-G1	G1	S	G2/M	Hyperploidy
TE-1	24	(-)	5.21 ± 0.14	44.96 ± 0.68	14.41 ± 0.25	28.11 ± 0.29	7.32 ± 0.01
		Control siRNA	5.45 ± 0.12	43.70 ± 0.37	14.40 ± 0.11	29.03 ± 0.47	7.41 ± 0.03
		Control siRNA + CP-31398	6.14 ± 0.04	40.95 ± 0.37	12.88 ± 0.10	32.35 ± 0.30	7.69 ± 0.14
		p21-siRNA	5.00 ± 0.10	44.01 ± 0.31	14.56 ± 0.05	29.01 ± 0.30	7.42 ± 0.30
		p21-siRNA + CP-31398	4.17 ± 0.03	44.98 ± 0.14	14.49 ± 0.11	28.82 ± 0.07	7.55 ± 0.14
	48	(-)	2.65 ± 0.23	42.29 ± 0.82	16.96 ± 0.58	27.10 ± 0.06	11.00 ± 0.01
		Control siRNA	2.81 ± 0.14	40.93 ± 0.41	17.77 ± 0.17	26.57 ± 0.24	11.92 ± 0.11
		Control siRNA + CP-31398	4.06 ± 0.02	31.51 ± 0.26	19.40 ± 0.51	31.71 ± 0.67	13.34 ± 0.01
		p21-siRNA	1.92 ± 0.05	39.90 ± 0.35	17.99 ± 0.36	27.18 ± 0.11	13.01 ± 0.20
		p21-siRNA + CP-31398	2.66 ± 0.08	41.11 ± 0.30	17.54 ± 0.33	25.70 ± 0.35	12.99 ± 0.03
TE-2	24	(-)	2.59 ± 0.04	29.66 ± 0.17	29.81 ± 0.14	34.11 ± 0.16	3.83 ± 0.02
		Control siRNA	2.20 ± 0.08	33.53 ± 0.35	30.00 ± 0.04	31.37 ± 0.41	2.90 ± 0.07
		Control siRNA + CP-31398	4.35 ± 0.03	30.88 ± 0.69	15.88 ± 0.30	38.73 ± 0.17	10.16 ± 0.07
		p21-siRNA	4.26 ± 0.28	32.26 ± 0.44	27.07 ± 0.62	30.69 ± 0.38	5.72 ± 0.05
		p21-siRNA + CP-31398	5.01 ± 0.26	33.10 ± 1.13	25.94 ± 0.23	30.38 ± 0.40	5.57 ± 0.07
	48	(-)	2.72 ± 0.04	32.21 ± 0.21	39.47 ± 0.06	20.33 ± 0.39	5.27 ± 0.12
		Control siRNA	3.67 ± 0.34	38.03 ± 2.58	34.26 ± 0.37	20.36 ± 1.57	3.68 ± 0.07
		Control siRNA + CP-31398	3.00 ± 0.42	13.08 ± 1.26	36.56 ± 1.42	39.16 ± 1.65	7.48 ± 0.07
		p21-siRNA	4.48 ± 0.07	32.04 ± 1.09	29.38 ± 0.27	25.42 ± 0.64	8.68 ± 0.04
		p21-siRNA + CP-31398	4.61 ± 0.03	33.36 ± 0.35	29.68 ± 0.06	23.26 ± 0.04	9.09 ± 0.15

Cells transfected with p21-siRNA or control siRNA were treated with CP-31398 at 10 mM. The cell cycle profiles was analyzed with a flow cytometry.

erefore indicated that these esophageal carcinoma cells were defective of p53 up-stream pathways as well as p53 down-stream pathway, and suggested that CP-31398 induced cell death and cell cycle changes were at least partly attributable to non-p53 pathways. Expression profiles of p53 induced by CP-31398 was different from those by CDDP, but the profile changes in p63 and p73 were similar to those by CDDP. The CP-31398-mediated DNA damage responses might thereby not be totally different with CDDP in these cells.

We demonstrated in the present study that p21 expression was up-regulated by CP-31398 in contrast to CDDP-induced p21 down-regulation. The up-regulation was irrelevant to activation of the p53 pathway and knockdown of p53 with siRNA confirmed the p53-independence. We therefore investigated a possible mechanism involved in non-p53-mediated p21 augmentation. Previous studies showed that CP-31398 induced p21 expression in a p53-independent manner [21] but this is the first report to indicate that CP-31398 influenced YY1 expression and regulated p21 expression. YY1 is a transcriptional factor ubiquitously expressed and regulates a number of genes positively or negatively depending on co-factors involved in the regulation [22, 23]. In addition, the over-expression was often associated with tumorigenesis and tumor progression including esophageal carcinoma [24]. YY1 promoted functions and expression of oncogenes [23], and inhibition of YY1 suppressed cell invasion and metastatic potentials [25]. We showed with CP-31398 treated cells that YY1 expression was reversely correlated with p21 expression and furthermore demonstrated that knockdown of YY1 expression augmented the p21 levels. Up-regulation of p21 contributed to cell growth suppression and YY1 down-regulation therefore played a role in inhibiting tumorigenic potentials. Previous studies showed that YY1 inhibited p21 transcription by blocking an access of other factors to the regulatory sequences in p53 wild-type cells [16. 17], but the present study also demonstrated the YY1-mediated p21 regulation in p53 dysfunctional cells. TE-10 cells were exception in the CP-31398- and YY1-

siRNA-treated studies, which showed that YY1 expression was positively associated with p21 expression and knockdown of YY1 rather decreased p21 expression. Contradictory effects of YY1 on p21 expression found between TE-10 cells and the other cells can be due to differential co-factors engagement in the p21 transcriptions. Nevertheless, these results of TE-10 cells also showed that YY1 regulated p21 expression in CP-31398-treated cells.

We examined other possible factors involved in p53-independent p21 regulation. YY2, a transcription factor belonging to the YY family, had similarity to YY1 at the DNA sequence level and reciprocally regulated target gene expression of YY1 by competing a binding site at the transcriptional regulatory region [26]. YY2 therefore struck a balance of the gene expression with YY1. The present data however showed that profiles of YY2 expression by CP-31398 were unrelated with those of YY1, indicating that YY2 played a minor role in the p21 regulation. A recent study however showed an example of YY2-mediated gene regulation without YY1 involvement, and demonstrated that YY2 increased p53 transcripts through regulation at non-YY1 binding site, and consequently augmented p21 expression irrespective of YY1 [14]. YY1 also regulated p53 expression in a different manner from YY2, which included facilitation of p53 ubiquitination by increasing binding between p53 and MDM2 [27-29]. The current study however did not showed any correlation between p53 and YY1 or YY2 expression levels in the esophageal carcinoma cells. Interestingly, TE-10 cells exceptionally increased YY1 with CP-31398, and also greatly up-regulated YY2 expression in comparison with other cells that showed constant YY2 expression after the CP-31398 treatment. It is however currently unknown how the YY2 increase in TE-10 cells contributed the p21 augmentation under increased YY1 expression.

We also investigated a possible contribution of mechanistic target of rapamycin complex 1 (mTORC1) pathway to p21 expression in a p53-independent manner. Phosphorylation of 4E-BP1, a down-stream molecule of mTORC1, stabilized p21, but the non-phosphorylated form degraded p21 [15]. On the contrary, activated mTORC1 inhibited MDM2 functions through p70S6K, and increased p21 levels in a p53-dependent manner [30]. The present study examined AMPK, an up-stream pathway of mTORC1, and 4E-BP1 and p70S6K after CP-31398 stimulation. Phosphorylated AMPK induced phosphorylation of 4E-BP1 and p70S6K in cells which had intact AMPK-mTORC1-4E-BP1/p70S6K pathways, but a majority of the esophageal carcinoma cells showed different phosphorylation profiles regarding respective molecules. These data indicated that the majority had distorted signaling in the pathways and the p21 up-regulation was not attributable to phosphorylated 4E-BP1 in these cells.

Esophageal carcinoma cells treated with CP-31398 showed increased G2/M populations to a lesser extent sub-G1 and hyperploidy fractions. We firstly reported that CP-31398 augmented G2/M fractions although increased sub-G1 and G0/G1 populations by CP-31398 were previously reported [21, 31]. These cell cycle changes induced by CP-31398 in the previous studies were primarily due to augmented p53 levels, but the present study also showed that the changes were generated without p53 involvement. We also firstly showed that CP-31398 increased hyperploidy fractions in several cell types, which was caused by an aberrant cell division process and might be precedent for cell death by CP-31398. Cell cycle arrest at G2/M was often associated with increased p27, but the present study showed that increased p27 was irrelevant. Inhibition of cyclin B1-Cdk1 complex and degradation of cyclins A2 and B1 induced by p21 promoted cell cycle arrest at G2 and G2/M phase [32, 33]. We however did not analyze a mechanism of p21-induced G2/M arrest by CP-31398, but p21-siRNA released the cell cycle arrest and decreased sub-G1 fraction. The data indicated that CP-31398-mediated cell cycle changes were at least partly due to the up-regulated p21 expression.

In conclusions, we investigated CP-31398-mediated effects on p53 dysfunctional tumors and showed that the agent activated p21 expression at the transcriptional levels. The present study demonstrated that the p21 augmented expression was linked with the YY1 level without involvement of p53, YY2 or mTORC signaling. We also showed that cell cycle changes induced by CP-31398 was attributable to the YY1-mediated increased p21 levels. The present study firstly demonstrated that a cytotoxic agent, CP-31398, targeted YY1 which was

often over-expressed in tumors, and suggested that the CP-31398-mediated effects including the cytotoxicity were attributable to decreased YY1 levels in p53 dysfunctional tumors.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (KAKENHI: 16K09598, 17K10617, 18K15937) and Grant-in-aid from the Nichias Corporation. The fund bodies were not involved in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Masatoshi Tagawa, Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ku, Chiba 260-8717, Japan. Tel: +81-43-264-5431 Ext. 5101; Fax: +81-43-265-4459; E-mail: mtagawa@chiba-cc.jp

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