CANCER GENOMICS & PROTEOMICS *16*: 543-552 (2019)
doi:10.21873/cgp.20156

PRIMA-1^{MET} Induces Cellular Senescence and Apoptotic Cell Death in Cholangiocarcinoma Cells

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Abstract. *Background/Aim: This study examined the in vitro effects of the bile duct cancer drug PRIMA-1^{MET} on cholangiocarcinoma (CCA) cell growth to determine its potential usefulness in CCA therapy. Materials and Methods: The effect of this drug on the expression of senescent markers (p16^{INK4A} and p21) and the phosphorylation of p53 was investigated, as was the association between senescent markers and the patients' clinicopathological data. Results: PRIMA-1^{MET} inhibited CCA cell growth with the half maximal-inhibitory concentration (IC_{50}) values of 21.9-40.8 μM. PRIMA-1^{MET} induced phospho-p53, p16^{INK4A} and p21 triggering cellular senescence and apoptosis. High expressions of p16^{INK4A} and p21 were associated with a high survival rate of patients with CCA. Conclusion: PRIMA-1^{MET} may potentially be an alternative anticancer agent that might lead to a better prognosis in patients with CCA.*

Cholangiocarcinoma (CCA) is described as a malignant tumor arising from the bile duct epithelia (1). Although rare in Western countries, it has high incidences in Asia, with the highest rate occurring in the Northeast Thailand (2). In these countries, CCA develops along the biliary trees after repeated infection with liver fluke (*Opisthorchis viverrini*) followed by chronic inflammation. This is defined as a major risk factor of CCA (3-5). This cancer is resistant to common chemotherapy, leading to a poor prognosis, with low 5-year survival and high mortality rates. Most patients receive treatment when the disease is already advanced, when surgical resection, which is prospectively curative for early-stage disease, is usually ineffective (6, 7). Therefore, the development of targeted cancer therapies leading to the replacement of conventional cancer chemotherapy could be a better strategy for CCA treatment.

Recently, targeting the cellular senescence pathway became a new strategy for cancer therapy (8). Cellular senescence is an irreversible growth arrest in response to various stresses by activating p53-dependent and -independent pathways (9). Currently, increased levels of lysosomal β-galactosidase activity and tumor-suppressor proteins such as p16^{INK4A} and p21 are commonly used as biomarkers of senescence (10, 11). Moreover, cellular senescence also acts as a barrier to tumorigenesis. Environmental stresses can force cancer cells to become responsive to cellular senescence *via* the activation of the p53-dependent and -independent pathways. The accumulation of p16^{INK4A} and p21 represents a good prognostic factor for cancer treatment (12). This leads us to focus more on using pro-senescent agents for CCA treatment. PRIMA-1^{MET} is a methylated derivative and structural analog of PRIMA-1 (p53 re-activation and induction of massive apoptosis). PRIMA-1^{MET} has been reported to restore p53 activity leading to the induction of cellular senescence and apoptosis (13). PRIMA-1^{MET} has been approved for treatment of acute myeloid leukemia and other hematological malignancies, and cancer of the lung, prostate, colorectum, ovary, esophagus, and breast in several different countries (14-20). However, the effect of PRIMA-1^{MET} on CCA is still unknown. In the present study, we aimed to investigate whether PRIMA-1^{MET} inhibits CCA progression and has any value for the therapy of CCA.

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Key Words: Cellular senescence, PRIMA-1^{MET}, apoptosis, cholangiocarcinoma.

Materials and Methods

Human CCA tissues. Human CCA tissues were collected from patients with CCA who were admitted to Srinagarind Hospital, Khon Kaen University, and were kept at the Specimen Bank, Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University. The study protocol was approved by the Ethics Committee for Human Research, Khon Kaen University (HE571283 and HE611390).

Human CCA cell lines. Three human CCA cell lines which possess mutant TP53 gene (21) namely poorly differentiated adenocarcinoma KKU-100 cells and well-differentiated adenocarcinoma KKU-213 and KKU-G023 cells were obtained from the tumor tissues of patients with CCA established at the Cholangiocarcinoma Research Institute Khon Kaen University. Human CCA cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) augmented with 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Grand Island, NY, USA), 2 mg/ml sodium bicarbonate and 1% antibiotic solution (Life Technologies Inc., Grand Island, NY, USA). All cell lines were cultured at 37˚C in a humidified incubator maintained with an atmosphere of 5% CO_2.

Cell viability assay. CCA cells (2×10^3) suspended in 100 μl media were seeded in 96-well plates in triplicate. Cells were treated with different concentrations of PRIMA-1^{MET} ranging from 1.563 to 100 μM for 48 h. A cell viability assay was performed using sulforhodamine B (SRB; Sigma-Aldrich, St. Louis, MO, USA) as previously described (22). Absorbance was measured at 540 nm using a microplate reader (Tecan Austria GmbH, Grodig, Austria). Two cell lines with low sensitive and highly sensitive to PRIMA-1^{MET} were selected to further molecular analyses.

Western blotting. CCA cells (1×10^5 cells/ml) were seeded in 6-well plates and left to adhere on plates overnight prior to start of treatment with PRIMA-1^{MET} at 3.125 to 50 μM for 24 h. CCA cells were lysed with radioimmuno-precipitation assay buffer. The amount of protein was quantified using the Pierce BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Forty micrograms of protein were electrophoresed using a 4-12% (w/v) sodium dodecyl sulfate–polyacrylamide gel before transfer to a polyvinylidene fluoride membrane (Whatman, Dassel, Germany). The membrane was incubated with 5% skim milk in tris-buffered saline (TBS) to block nonspecific binding at room temperature for 1 h then incubated with primary antibodies including anti-cyclin dependent kinase inhibitor 2A (CDKN2A/$p16^{INK4A}$), anti-p21, anti-phospho-p53 (Ser15), anti-p53 (Abcam, Cambridge, MA, USA), anti-BCL2-associated X (BAX) (BD Biosciences, San Jose, CA, USA), anti-B-cell lymphoma 2 (BCL2) (Cell Signaling Technology, Danvers, MA, USA) at 4˚C overnight. After rinsing with 0.1% Tween-20 in TBS, the membrane was incubated with horseradish peroxidase-conjugated rabbit or mouse IgG at room temperature for 1 h. After rinsing the membrane with Tween-20 in TBS, immunoreactive materials were developed by Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Life Science, Buckinghamshire, UK) for chemiluminescent detection. β-Actin was used as an internal loading control. Intensity of each protein bands was semi-quantitatively assessed by densitometry using ImageJ software 1.48v (Wayne Rasband, USA). Each experiment was performed in triplicate.

Senescence associated β-galactosidase staining. CCA cell lines (200 cell/ml) were seeded in 12-well plates and allowed to adhere overnight. Cells were then treated with DMEM as a control or 3.0 μM of PRIMA-1^{MET} and incubated at 37˚C for 24 h. After that, the treated cells were continued in DMEM for 9 days post-treatment. Senescence-associated β-galactosidase staining was performed using senescence-associated β-galactosidase staining kit (Cell Signaling Technology). The cells were checked under a microscope (×200 magnification) for the development of blue color.

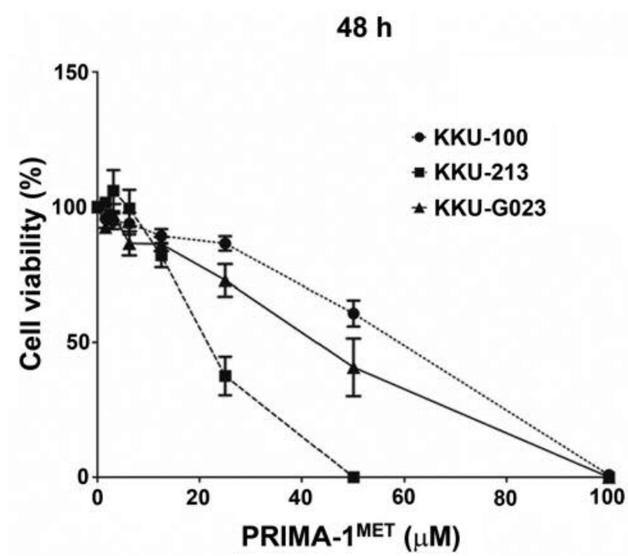

Figure 1. *Cytotoxic effect of PRIMA-1^{MET} on cholangiocarcinoma (CCA) cell growth. PRIMA-1^{MET} treated CCA cell lines showed a decrease in cell proliferation at 48 h. Data are shown as mean±SD obtained from three independent experiments.*

Flow cytometric analysis. CCA cell lines (1×10^5 cells/ml) were seeded in a 6-well plate and treated with different concentrations of PRIMA-1^{MET} ranging from 12.5 to 100 μM for 24 h. After suspending using trypsin they were washed twice with cold phosphate-buffered saline. Each sample was resuspended in 100 μl of incubation buffer, 2 μl of annexin-V-fluorescein and propidium iodide, and incubated on ice for 15 min in the dark. Each sample was mixed with 400 μl of incubation buffer and put into a BD Falcon tube (BD Biosciences, Tewsbury, MA, USA). Apoptosis cells were detected using a flow cytometer (BD FACSCanto™ II; BD Biosciences, San Jose, CA, USA).

Immunohistochemical analysis. Immunohistochemical analysis was performed to investigate the expression pattern of $p16^{INK4A}$ and p21 in human CCA tissues using the standard protocol (23). Antigen retrieval was performed using a pressure cooker with 1x Tris-EDTA+0.05% Tween20. Rabbit anti-CDKN2A/$p16^{INK4A}$ (dilution 1:100) and Rabbit anti-p21 (dilution 1:50) were used as primary antibodies to react antigens in the tissue sections at 4˚C overnight. After washing, the tissue sections were incubated with a horse radish peroxidase-conjugated secondary antibody (DakoEnvision+System-HRP Labelled Polymer Anti-Rabbit antibody; Dako, Santa Clara, CA, USA) for 1 h at room temperature. The stained tissue sections were reviewed under a light microscope. Tumor tissues were given a score according to the intensity of nuclear staining (negative=0, weak=1, moderate=2, and strong=3) and percentage of stained cells. The staining score was calculated by multiplying the intensity and frequency for each case. The expression of $p16^{INK4A}$ and p21

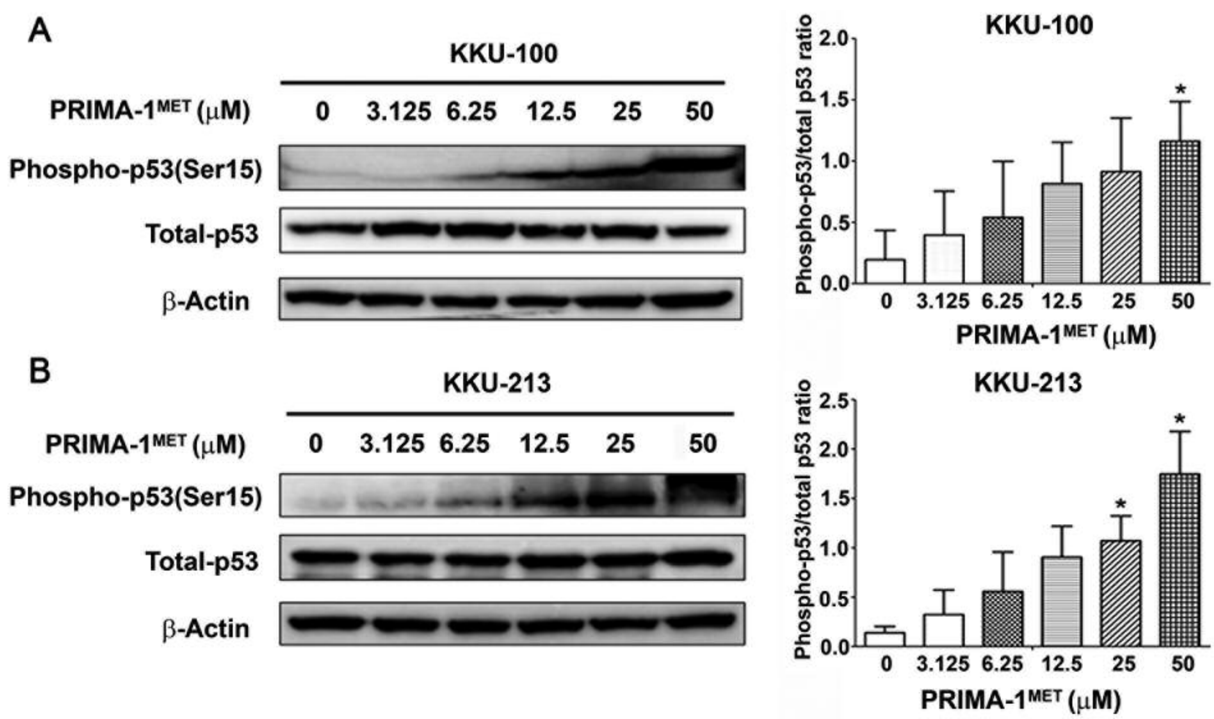

Figure 2. *Reactivation of p53 by PRIMA-1^{MET} in KKU-100 and KKU-213 cells. Western blot analysis showed phospho-p53(Ser15) protein expression in KKU-100 (A) and KKU-213 (B) after PRIMA-1^{MET} treatment. The relative level of phospho-p53(Ser15) protein expression was quantified for PRIMA-1^{MET}-treated cholangiocarcinoma cells. The phospho-p53(Ser15)/total-p53 ratio was analyzed. Data represent the mean±SD of protein band intensities taken from three independent experiments. These were normalized to the intensity of β-actin. *Significantly different from untreated cells at p<0.05.*

proteins was categorized into low and high expression groups using the mean of p16^{INK4A} and p21 scores as a cut-off value.

Statistical analysis. Statistical analysis was performed using the Statistical Package for the Social Science; SPSS software v.16 (SPSS, Chicago, IL, USA). The correlation between the expression of p16^{INK4A} and p21 with the clinicopathological data of CCA patients was analyzed by Fisher's exact test. Survival analysis was performed using Kaplan–Meier and the log-rank test. Protein expression levels and growth were compared using mean±S.D. of treated and untreated cells by Student *t*-test. The results were considered to be significant at *p*-values of less than 0.05.

Results

PRIMA-1^{MET} inhibits CCA cell proliferation. The cytotoxic effect of PRIMA-1^{MET} on the growth of the human CCA cell lines KKU-100, KKU-213 and KKU-G023 was determined using an SRB assay. The three CCA cell lines were treated with PRIMA-1^{MET} for 48 h at different concentrations (0-100 μM). The results show that PRIMA-1^{MET} effectively inhibited CCA cell proliferation in a dose-dependent manner (Figure 1). Our results show that at the end of 48 h, PRIMA-1^{MET} inhibited KKU-100, KKU-213 and KKU-G023 with average the half maximal-inhibitory concentration values of 58.5±5.4, 21.9±3.4 and 44.9±11.0 μM, respectively. Two cell lines with low sensitive (KKU-100) and highly sensitive (KKU-213) to PRIMA-1^{MET} were selected for further molecular analyses.

Reactivation of p53 by PRIMA-1^{MET}. Two cell lines, KKU-100 (low sensitivity) and KKU213 (highly sensitivity) were treated with PRIMA-1^{MET} at different concentrations (0-50 μM) for 24 h. Western blot analysis demonstrated that the phospho-p53 (Ser15)/total p53 ratio was markedly increased in KKU-100 at 50 μM (Figure 2A) and in KKU-213 at 25 and 50 μM (Figure 2B) cells as compared with untreated cells.

Induction of cellular senescence by PRIMA-1^{MET} in CCA cell lines. Two cell lines, KKU-100 and KKU213 treated with 3.125 μM of PRIMA-1^{MET} exhibited a senescent phenotype as visualized under a light microscope, including enlarged size, flattened cell shape, induced nuclear granularity and increased volume of cytoplasm (Figure 3). PRIMA-1^{MET} treatment of cells at 3.125 μM showed that the

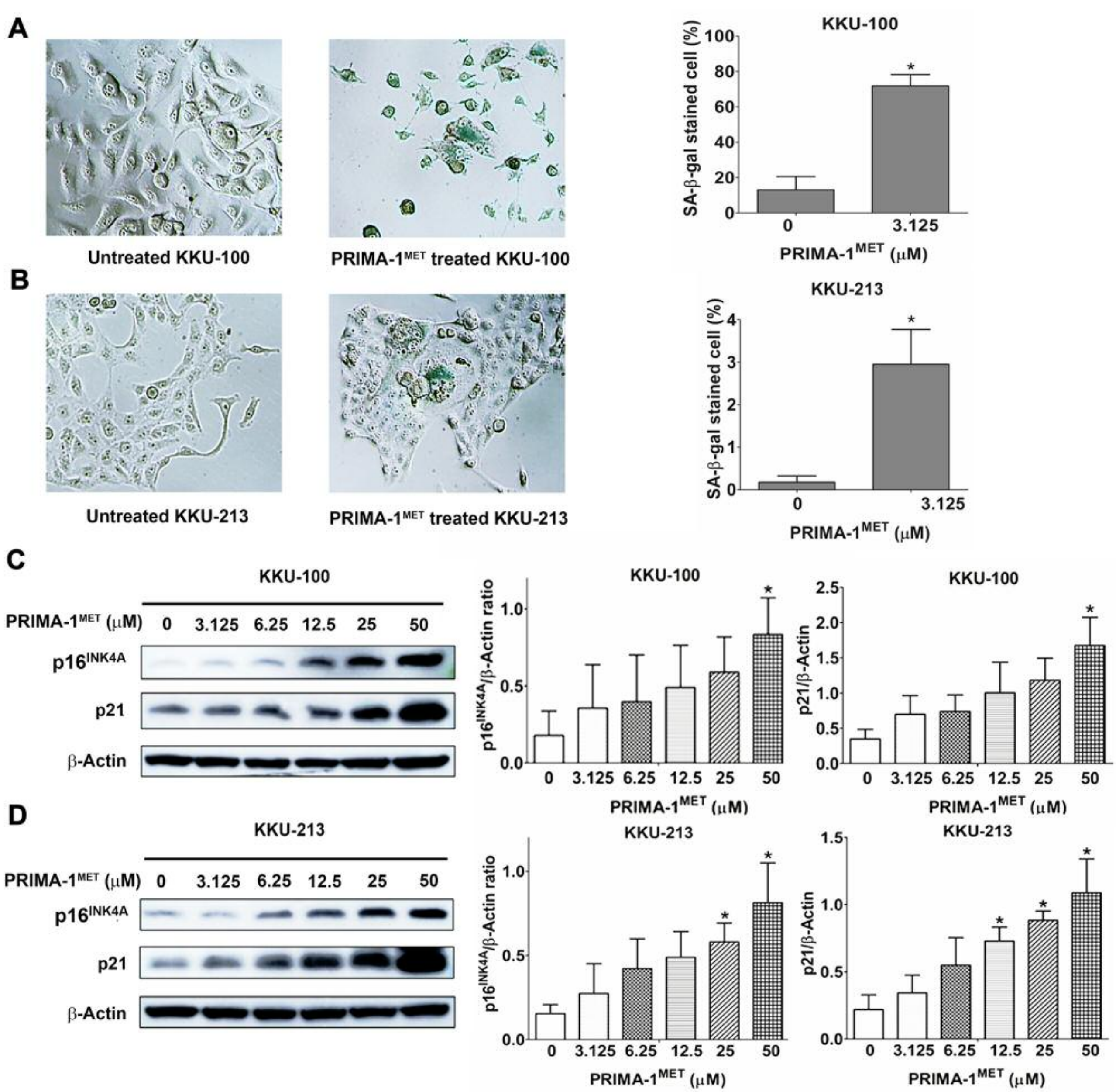

Figure 3. *The induction of cellular senescence by PRIMA-1MET in KKU-100 and KKU-213 cells. A and B: The left panel shows senescence associated (SA)-β-galactosidase staining in KKU-100 (A) and KKU-213 (B) for 9 days after the initiation of treatment with 3.125 μM PRIMA-1MET (original magnification 100×). The right panel shows quantification of the mean±SD proportion of SA-β-gal-positive CCA cells taken from three independent experiments. C and D: The left panel shows western blot analysis of p16INK4A and p21 protein expression in KKU-100 (C) and KKU-213 (D) cells after PRIMA-1MET treatment. The right panel shows quantification of the mean±SD protein band intensity taken from three independent experiments. These were normalized to the intensity of β-actin. *Significantly different from untreated cells at $p<0.05$.*

proportion of cells with positive staining for senescence-associated β-galactosidase was significantly higher than that of the untreated cells for both KKU-100 (Figure 3A) and KKU-213 (Figure 3B) cells. KKU-100 and KKU-213 cells were treated with PRIMA-1MET for 24 h and western blotting was performed to evaluate the expression levels of p16INK4A and p21 proteins (Figure 3C and D, left panel). Our results showed that p16INK4A expression was significantly increased in PRIMA-1MET treated KKU-100 cells at 50 μM and KKU-213 cells at 25 and 50 μM when

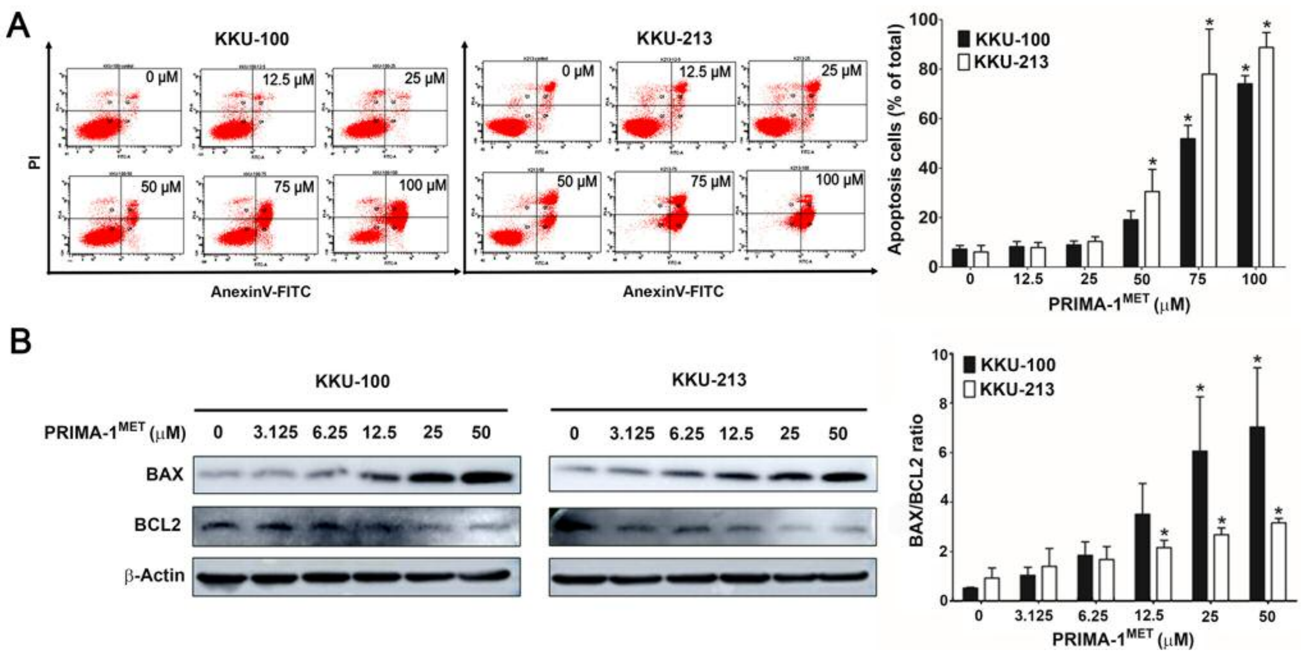

Figure 4. *Effects of PRIMA-1^{MET} on the induction of apoptosis in KKU-100 and KKU-213 cells. KKU-100 and KKU-213 cells were treated with PRIMA-1^{MET} for 24 h. Annexin-V and PI staining was analyzed by flow cytometry and expression of apoptosis-associated proteins was quantified. A: Representative histograms from flow cytometry are shown in the left panels, with data the percentage of annexin-V and propidium iodine (PI)-positive CCA cells with and without PRIMA-1^{MET} treatment in the right panel. B: The left panel shows western blot analysis of BCL2-associated X (BAX) and B-cell lymphoma 2 (BCL2) expression in CCA cell lines after PRIMA-1^{MET} treatment for 24 h. The BAX/BCL2 ratio was analyzed. The right panel shows the mean±SD protein band intensity from three independent experiments. The data were normalized to the intensity of β-actin. *Significantly different from untreated cells at p<0.05.*

compared with the untreated cells. Similarly, p21 expression was significantly increased in PRIMA-1^{MET} treated KKU-100 at 50 μM and KKU-213 cells at 12.5, 25 and 50 μM when compared with the untreated cells (Figure 3C and D, right panel).

PRIMA-1^{MET} induced apoptosis of CCA cells. KKU-100 and KKU-213 cells were treated with PRIMA-1^{MET} at different concentrations (0-100 μM) for 24 h, they were then evaluated by flow cytometry using annexin-V and propidium iodide staining. The results showed that the annexin V-positive cells were significantly increased in KKU-100 at 75 and 50 μM and KKU-213 cells at 50, 75 and 100 μM treated with PRIMA-1^{MET} in a dose-dependent manner when compared with untreated cells (Figure 4A). Western blotting was performed to evaluate the expression levels of BAX and BCL2 proteins in KKU-100 and KKU-213 cells treated with PRIMA-1^{MET} for 24 h (Figure 4B, left panel). Our results showed that the BAX/BCL2 ratio was significantly increased in PRIMA-1^{MET}-treated KKU-100 at 25 and 50 μM and KKU-213 cells at 12.5, 25 and 50 μM (Figure 4B, right panel) when compared with the untreated cells.

Expression of p16^{INK4A} and p21 protein in human CCA tissues. Of the CCA tissues from 160 patients with intrahepatic CCA, 101 (63%) were from males and 59 (37%) from females. The age of the patients ranged from 36 to 79 years (median age=60 years). For all 160 CCA cases, positive staining of p16^{INK4A} and p21 proteins was seen in the nuclei of cells in the tissue sections. Exemplary images of staining of cancerous tissues are shown in Figure 5A and B. p16^{INK4A} expression was found to be low in 45% (72/160) and high in 55% (88/160) of cases. Similarly, p21 expression was found to be low in 39% (62/160) and high in 61% (98/160) of cases.

Correlation of p16^{INK4A} and p21 protein scores with clinicopathological features of patients with CCA. As shown in Table I, Fisher's exact test demonstrated that high p16^{INK4A} and p21 expression was associated with longer patient survival (p=0.035 and p=0.034, respectively). Age, gender, histological type, overall metastasis and lymph node metastasis did not significantly differ between these two groups. The immunohistochemical scores for p16^{INK4A} and p21 proteins were analyzed for their association using bivariate analysis. The results revealed that p16^{INK4A} protein

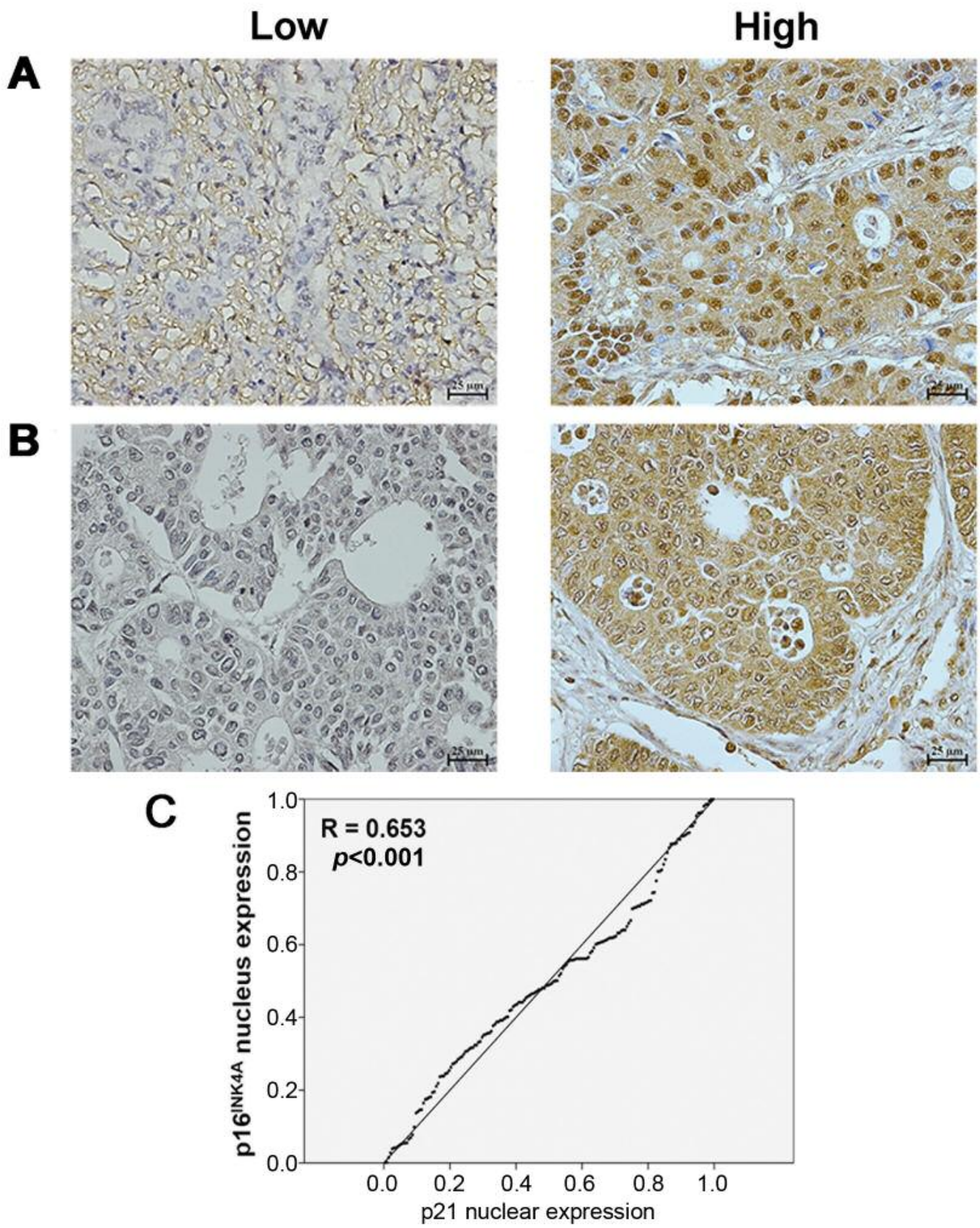

Figure 5. *Immunohistochemical staining scored as low (left) and high (right) p16^{INK4A} (A) and p21 (B) protein expression in 160 cases of human cholangiocarcinoma tissues. Magnification was ×200. C: Bivariate analysis revealed that p16^{INK4A} protein expression was significantly positively correlated with p21.*

was positively correlated with p21 (p<0.001) (Figure 5C).

Log-rank analysis was carried out to determine the cumulative survival rate of patients with CCA according to expression of p16^{INK4A} or p21. Individuals with high expression had a significantly longer survival rate than those with low expression (p<0.001) (Figure 6A and B). Moreover, patients with CCA who had high expression of both of p16^{INK4A} and p21 had significantly longer survival than those with low expression of only one or of both proteins (p<0.001) (Figure 6C).

Multivariate Cox proportional hazards regression analyses were adjusted for the effects of age, gender, histological

Table I. *Correlation between the expression of p16^{INK4A} and p21 proteins in tumor tissues and clinicopathological findings for patients with cholangiocarcinoma*.

Factor	N=160	p16^{INK4A}			p21		
		Low	High	*p*-Value	Low	High	*p*-Value*
Age							
<60 Years	75	39	36	0.095	39	36	0.635
≥60 Years	85	33	52		41	44	
Gender							
Male	101	40	61	0.073	47	54	0.251
Female	59	32	27		33	26	
Histological type							
Non-papillary	87	39	48	0.962	41	46	0.427
Papillary	73	33	40		39	34	
Overall metastasis							
No	85	33	52	0.095	42	43	0.874
Yes	75	39	36		38	37	
Stage							
Early	54	22	32	0.440	27	27	>0.99
Advanced	106	50	56		53	53	
Survival							
<545 Days	99	51	48	**0.035**	56	43	**0.034**
≥545 Days	61	21	40		24	37	

*Fisher's exact test. Statistically significant *p*-values are shown in bold.

Table II. *Multivariate Cox regression analyses of clinicopathological data of patients with cholangiocarcinoma and molecular markers for the prediction of survival*.

Factors (N=160)	N	p16^{INK4A}			p21			p16^{INK4A} and p21		
		Hazard ratio	95% CI	*p*-Value	Hazard ratio	95% CI	*p*-Value	Hazard ratio	95% CI	*p*-Value
Age										
<60 Years	75	1			1			1		
≥60 Years	85	1.148	0.832-1.585	0.401	1.029	0.746-1.419	0.862	1.107	0.802-1.526	0.537
Gender										
Male	101	1			1			1		
Female	59	1.212	0.873-1.682	0.252	1.258	0.904-1.749	0.173	1.333	0.957-1.855	0.089
p16^{INK4A}										
Low	72	1	0							
High	88	0.523	0.376-0.727	**<0.001**						
p21										
Low	62				1					
High	98				0.462	0.330-0.647	**<0.001**			
p16^{INK4A} and p21										
Other	90							1		
High	70							0.430	0.305-0.606	**<0.001**
Histological type										
Non-papillary	87	1			1			1		
Papillary	73	0.809	0.576-1.052	0.104	0.765	0.556-1.054	0.102	1.83	0.604-1.142	0.253
Overall metastasis										
No	85	1			1			1		
Yes	75	1.239	0.589-1.112	0.192	1.156	0.760-1.758	0.498	1.055	0.690-1.615	0.804
Stage										
Advanced	99	1			1			1		
Early	61	0.442	0.311-0.629	**<0.001**	0.393	0.275-0.562	**<0.001**	0.414	0.291-0.590	**<0.001**

CI: Confidence interval. Statistically significant *p*-values are shown in bold.

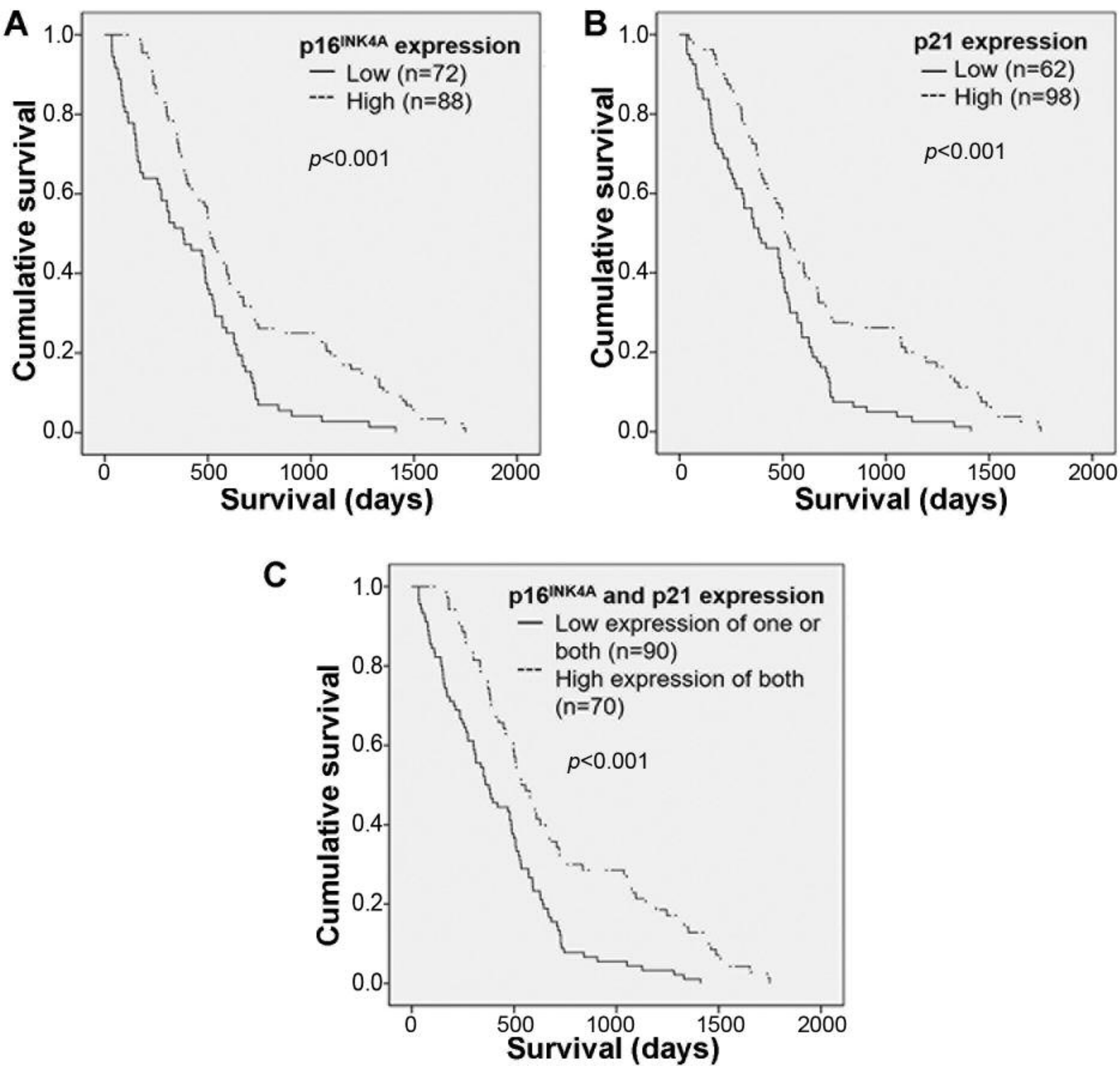

Figure 6. *Cumulative survival rate of patients with cholangiocarcinoma according to expression of p16^{INK4A} and p21 alone (A and B) and in combination (C). Patients with high expression of either protein alone had a significantly longer survival than did those with low expression. Patients who had high expression of both of p16^{INK4A} and p21 had significantly longer survival than those with low expression of one or both proteins.*

type, overall metastasis, stage, as well as p16^{INK4A} and p21 scores (Table II). The results showed that both p16^{INK4A} and p21 scores were independent prognostic factors, with higher levels conferring statistically significant protection against CCA (both $p<0.001$). Patients who had high p16^{INK4A} and p21 expression had a 1.9- and 2.1-fold reduced risk for CCA, respectively. The effects of the combination of p16^{INK4A} and p21 proteins with the clinicopathological data of CCA patients were adjusted and also analyzed using multivariate Cox proportional hazard regression method. High expression of both p16^{INK4A} and p21 remained a significant predictor for CCA patients ($p<0.001$). The high expression of p16^{INK4A}, and of p21, in CCA tissues of patients with early-stage disease was confirmed to be independently prognostic for patients with CCA as shown in Table II.

Discussion

Pro-senescent drugs can serve as a new weapon to inhibit tumor cell growth. Approximately 50% of all human cancers bear a p53 mutation, including CCA (24), resulting in resistance to conventional chemotherapy (25). Anticancer drugs such as PRIMA-1^{MET} have been shown to restore expression of p53, leading to the induction of cellular senescence and apoptosis, in both *in vivo* and *in vivo* studies (26-29). We demonstrated that PRIMA-1^{MET} significantly inhibited cell proliferation of CCA cell lines. PRIMA-1^{MET} restored p53 activity, demonstrating an increase in the phospho-p53 (Ser15) level in both cell lines. This phosphorylation activated the p53 signaling pathway, leading to the induction of cellular senescence through an increase in senescence-associated β-galactosidase activity. p16^{INK4A} and p21 protein levels were also associated with an increase in the BAX/BCL2 ratio and apoptosis. Our results are consistent with previous studies showing the effect of PRIMA-^{MET} on other types of cancer (14, 16-20, 30).

Cellular senescence is an irreversible arrest of growth that acts as a barrier to tumorigenesis by activating both p53-dependent and p53-independent pathways. We found that high expression of the senescent markers p16^{INK4A} and p21 in CCA tissues was significantly associated with longer patient survival compared to low expression. Our results are consistent with previous studies demonstrating that the p16^{INK4A} and p21 proteins can possibly serve as good prognostic markers for predicting overall survival in many types of cancer, such as adenocarcinoma, gastric cancer, Hodgkin lymphoma, and non-small cell lung cancer (10, 12, 31, 32).

Taken together, the findings of our study suggest that patients with CCA who have a high expression of p16^{INK4A} and p21 might be predicted as belonging to a good prognostic group. The induction of cellular senescence is a therapeutic strategy for inhibiting the growth of CCA cells. Our results reveal that PRIMA-1^{MET} might be a potential anticancer drug for CCA treatment by the induction of cellular senescence and apoptosis. Currently, recruitment is underway for three clinical trials with the objective of testing the safety and efficacy of PRIMA-1^{MET} treatment in advanced esophageal carcinoma (NCT02999893), high-grade serous ovarian cancer (NCT02098343), and mutant p53 hematological myeloid malignant disease (NCT03072043) (https://clinicaltrials.gov). Our results suggest that PRIMA-1^{MET} is an attractive candidate for further investigation as an anticancer agent in clinical trials for patients with CCA.

Conflicts of Interest

The Authors declare that no competing interest exists in regard to this study.

Authors' Contributions

NN and WL designed the study. AT collected patient tissues. PS evaluated the clinicopathological data of patients. CP and YK performed experiments and result analysis. CP wrote the article. NN read, edited and approved the final version of article.

Acknowledgements

This work was supported by a grant from a Mid-Career Grant (RSA5980012) of Thailand Research Fund to NN, the Faculty of Medicine, Khon Kaen University, for its support by a grant fund for the M.Sc. program, to CP (IN61322), a grant from Khon Kaen University to NN, and a grant from CASCAP Program to NN. We would like to acknowledge Prof. Trevor N. Petney, for editing the MS *via* Publication Clinic KKU, Thailand.

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Received July 10, 2019
Revised July 22, 2019
Accepted July 24, 2019