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Original Article

Aberrant Methylation of *p21* Gene in Lung Cancer and Malignant Pleural Mesothelioma

Hirotake Teramen[a], Kazunori Tsukuda[a], Norimitsu Tanaka[a], Tsuyoshi Ueno[a], Takafumi Kubo[a], Midori Ando[a], Junichi Soh[a], Hiroaki Asano[a], Harvery I. Pass[b], Shinichi Toyooka[a]*, and Shinichiro Miyoshi[a]

[a]*Department of Cancer and Thoracic Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, 700–8558 Japan, and*
[b]*Department of Thoracic Surgery, NYU Langone Medical Center, New York, NY 10016, USA*

Suppression of p21 has been implicated in the genesis and progression of many human malignancies. DNA methylation is an important mechanism of gene silencing in human malignancies. In this study, we examined the expression status and aberrant methylaion of p21 in lung cancers and malignant pleural mesotheliomas (MPM). We used 12 small cell lung cancer (SCLC) cell lines, 13 non-small cell lung cancer (NSCLC) cell lines, 50 primary NSCLCs, 6 MPM cell lines and 10 primary MPMs. The expression and methylation of p21 was examined by reverse transcription-PCR (RT-PCR), Western blotting and methylation-specific PCR (MSP) assay. Loss of p21 protein expression was observed in 7 SCLC cell lines (58.3%), 5 NSCLC cell lines (38.5%) and 3 MPM cell lines (50%) while mRNA expression was lost in 2 SCLC cell lines (16.7%), 2 NSCLC cell lines (15.4%) and none of the MPM cell lines. Aberrant methylation of *p21* was found in 8.3% of SCLC cell lines, 30.2% of NSCLCs and 6.3% of MPMs. Among primary NSCLCs, methylation in adenocarcinomas was significantly more frequent than in squamous cell carcinomas. Loss of p21 expression was frequently observed in lung cancers and MPMs and aberrant methylation was one of the mechanisms of suppression of p21, especially in NSCLCs.

Key words: p21, methylation, lung cancer, mesothelioma

The cell cycle progresses through sequential activation and inactivation of cyclin-dependent kinases (CDKs). The transition from the G1 to the S phase is known to be regulated by a family of negative cell cycle regulators known as, CDK inhibitors. p21 (CDKN1A/CIP1/WAF1) is one of the CDK inhibitors and plays a key role in preventing the progression to the S phase of the cell cycle [1, 2]. p21 has been shown to have a pro-apoptotic role under p53-mediated apoptosis and to be activated by p53, which was shown to be a tumor suppressor gene and to have been inactivated in several human malignancies.

Recently, epigenetic alterations including promoter DNA methylation and histone deacetylation have been established as one of the crucial mechanisms of carcinogenesis. Among them, transcriptional inactivation by promoter methylation in various tumor suppressor genes plays an important role in human malignancies including lung cancers and malignant pleural mesotheliomas (MPMs) [3, 4]. Loss of p21 has been shown

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*Corresponding author. Phone:+81–86–235–7265; Fax:+81–86–235–7269
E-mail:toyooka@md.okayama-u.ac.jp (S. Toyooka)

to occur in colorectal cancer, but promoter methylation does not appear to play a key role in the loss of function [5]. Additionally, the *p21* gene is frequently methylated and is an important factor in predicting the clinical outcome of acute lymphoblastic leukemia patients [6, 7]. On the other hand, almost no mutation in the *p21* gene has been found in malignancies [8].

Several tumor suppressor genes are silenced by promoter methylation in lung cancers and MPM, but the subsets of genes that are affected by methylation in these 2 kinds of malignancies are quite different [4]. In lung cancers, the profile of gene methylation is affected by clinicopathological features such as smoking status and histological subtype [3]. Low frequency of *p21* methylation was previously reported in a study that examined a small number of lung cancer cell lines [9]. However, no information of regarding primary tumors has been documented. In addition, no study has shown *p21* methylation in MPMs. In this study, we examined the DNA methylation of the *p21* gene in lung cancers and MPMs and assessed the extent to which it correlated with the expression of p21 as well as that of p53. We also investigated the relationship between *p21* methylation and the clinicopathological features of primary lung cancers.

Materials and Methods

Clinical samples and cell lines. Twelve SCLC, 13 NSCLC and 6 MPM cell lines were used in this study. The HP1 cell line was a kind gift from Dr. Harvey I. Pass; the SBC-3, SBC-4 and SBC-5 cell lines were from Dr. Katsuyuki Kiura (Okayama University) and other cell lines were from Dr. Adi F. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX, USA). The human bronchial epithelial cell (HBEC) 5KT cell established from non-malignant human bronchial cells was also provided by Dr. Adi F. Gazdar. The cells were maintained in RPMI-1640 medium (Sigma Chemical Co., Saint Louis, MO, USA) supplemented with 10% FBS and incubated in 5% CO_2. Surgically resected specimens of 50 NSCLCs were obtained from Okayama University Hospital, and 10 MPMs were obtained from Karmanos Cancer Center, Detroit, MI, USA. Ten non-neoplastic pleura and 10 non-malignant lung tissues from lung cancer patients were obtained from surgically resected pulmonary specimens and used for the methylation assay. All tissues were frozen with liquid nitrogen immediately after surgery and stored at −80℃ after the acquisition of informed consent from each patient.

Western blot analysis. Cells were grown to 80% confluency and harvested in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/ml leupeptin] (Cell Signaling Technology, Beverly, MA, USA) supplemented with Complete, Mini (Roche, Basel, Switzerland) to extract protein. A total 30 μg of protein was separated by SDS-PAGE and transferred to PVDF membranes. The proteins on membranes were incubated overnight at 4℃ with the primary antibodies. The primary antibodies of anti-p21 and anti-p53 (Cat-No. #2946 and #9282; Cell Signaling Technology, Danvers, MA, USA) were used for Western blotting. The following secondary antibodies were used: goat antirabbit or antimouse IgG-conjugated horseradish peroxidase (HRP) (Santa Cruz, CA, USA). To detect specific signals, the membranes were detected by ECL plus Western Blotting Detection Reagents (Amersham Biosciences UK Limited, Buckinghamshire, UK).

Expression of p21 mRNA. Total cellular RNA was isolated from cell lines using an RNeasy minikit (Qiagen Sciences, Maryland) according to the manufacturer's instructions and then treated with 2 units/μL of DNase I (Ambion, Austin, TX, USA) for 30 min at 37℃. RT reaction was performed for 2 μg of total RNA with the SuperScript II First-strand Synthesis using the Oligo (dT) primer system (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) in 20 μl of reaction mixture. Expression of *p21* was confirmed by RT-PCR. The primers designed by primer 3 targeting *p21* cDNA were as follows (sense and antisense, respectively): 5'-GCTCTGCTGCA-GGGGACAGC-3', and 5'-TCTGCCGCCGTTTTCG-ACCC-3'. *GAPDH* was used as a control. The 25-μl reaction mixtures contained the forward and reverse primers at 300 nM each, 1 unit of HotStar Taq DNA polymerase (Qiagen Science) and 5 μl cDNA from the 20-μl volume of RT reaction mixture. PCR was performed under the following conditions: 50℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of 95℃

for 15 sec and 60℃ for 1 min. RT-PCR products were analyzed in 2% agarose gels and stained with ethidium bromide.

5-aza-2'-deoxycytidine treatment. Two cell lines, H522 and H841, were treated with 5-aza-2'-deoxycytidine (Sigma-Aldrich Co.) at a concentration of 5μM for 6 days to restore gene expression.

Methylation specific PCR (MSP) assay and bisulfate DNA sequencing. Genomic DNA was extracted from cell lines and tissues by standard phenol-chloroform (1 : 1) extraction or using a DNeasy Tissue Kit (QIAGEN, Valencia, CA, USA). DNA was subjected to bisulfate treatment using the EZ DNA Methylation Gold kit (Zymo Research Corp., Orange, CA, USA) according to the manufacturer's protocol. The methylation statuses of *p21* were determined by MSP assay using specific primers for the methylated and unmethylated forms in accordance with a previous report [10]. The DNA from HBEC 5KT was treated with Sss1 methyltransferase (New England BioLabs, Beverly, MA, USA) and was used as a positive control for methylated alleles. PCR products were analyzed in 2% agarose gels and stained with ethidium bromide.

Statistics. Univariate analysis of the methylation rate was carried out using the χ^2 test and multivariate analysis was done using multiple logistic regression analysis. All data were analyzed with JMP for Windows (SAS Institute, Tokyo, Japan).

Results

The expression of p21 in cell lines was examined using RT-PCR and Western blotting (Fig. 1). The expression of the p53 protein as determined by Western blotting and the genetic alteration of *p53* status which has been reported in databases of Trust Sanger Institute (www.sanger.ac.uk) are shown in Table 1. The p53 expression was preserved in 7 SCLC cell lines (58.3%), 7 NSCLC cell lines (53.8%) and 5 MPM cell lines (83.3%). The loss of *p21* mRNA expression was observed in 2 SCLC cell lines (16.7%), 2 NSCLC cell lines (15.4%) and none of the MPM cell lines (0%), while a loss of protein expression was observed in 7 SCLC cell lines (58.3%), 5 NSCLC cell lines (38.5%) and 3 MPM cell lines (50.0%). In 3 cell lines namely SBC-4, H358 and H1299, negative p53 expression but positive p21 expression were shown, which conflicted with the fact that p21 was mediated by p53. However, p21

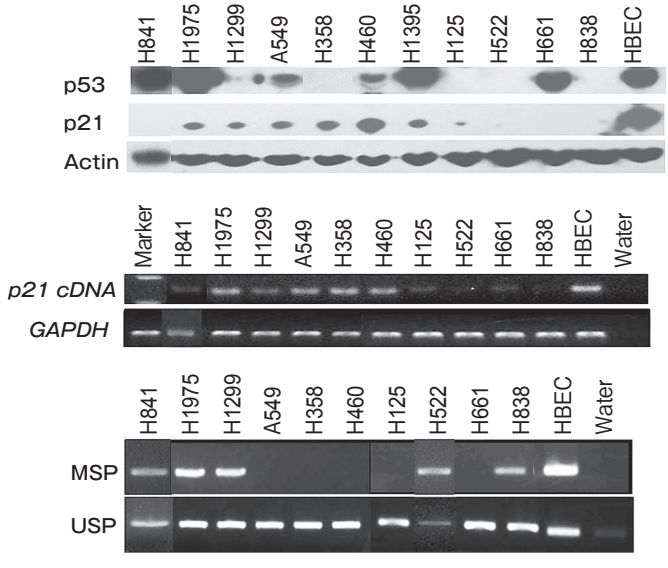

Fig. 1 Representative examples of the expression and methylation status of p21. The upper panel shows the protein expression of p53 and p21 protein. Actin is used as a control. The middle panel shows the mRNA expression of *p21*. *GAPDH* is used as an expression control. The lower panel shows the methylation status of the *p21* gene. HBEC cells are used as a normal control. MSP, methylated; USP, unmethylated.

Table 1 Expression and methylation profiles of cell lines

Cell line	Histology	p21			p53	
		Gene methy.	mRNA exp.	Protein exp.	Gene mut.	Protein exp.
H82	SCLC	U	+	−	Splice mutation	−
H211	SCLC	U	−	−	P248E	+
H249	SCLC	U	+	−	Wt	+
H524	SCLC	U	+	−	T155N	+
H841	SCLC	M/U	−	−	C242S	+
H1084	SCLC	U	+	+	S46F, R273C	+
H1780	SCLC	U	+	+	*	+
H1963	SCLC	U	+	+	V147D, H214R	ND
H2141	SCLC	U	+	−	R209Stop	−
SBC-3	SCLC	U	+	+	Wt	+
SBC-4	SCLC	U	+	+	*	−
SBC-5	SCLC	U	+	−	R245L	−
A549	NSCLC (AD)	U	+	+	Wt	+
H125	NSCLC (ADSQ)	U	+	−	239 frame shift	−
H358	NSCLC (AD)	U	+	+	HD	−
H460	NSCLC (LC)	U	+	+	Wt	+
H522	NSCLC (AD)	M/U	−	−	191 frame shift	−
H661	NSCLC (LC)	U	+	−	S215I	+
H838	NSCLC (AD)	M/U	−	−	E62stop	−
H1299	NSCLC (LC)	M/U	+	+	HD	−
H1395	NSCLC (AD)	U	+	+	Wt	+
H1975	NSCLC (AD)	M/U	+	+	R273H	+
H3255	NSCLC(AD)	U	+	+	*	ND
HCC827	NSCLC (AD)	U	+	−	*	+
PC-9	NSCLC (AD)	U	+	+	R24Q	+
H28	MPM	U	+	+	*	+
H290	MPM	U	+	−	*	+
H513	MPM	U	+	−	*	−
H2052	MPM	U	+	+	*	+
H2452	MPM	U	+	+	*	+
HP1	MPM	U	+	−	*	+

* There is no information about p53 gene mutation.
Wt, wild type; HD, homozygous deletion; ND, not determined; U, unmethylated; M, methylated; M/U, both methylated and unmethylated alleles were present indicating partial methylation. AD, adenocarcinoma; ADSQ, adenosquamous cell carcinoma; LC, large cell carcinoma.

expression was very weak in these cells.

A conventional MSP assay was performed, and representative examples are shown. Aberrant methylation of *p21* was found in 1 [H841] out of 12 (8.3%) SCLC cell lines and 4 [H522, H838, H1299 and H1975] out of 13 (30.8%) NSCLC cell lines. All 5 cell lines with aberrant methylation showed both methylated and unmethylated alleles, which meant that the promoter of *p21* was partially methylated. Though H522, H838 and H841 lost both p21 mRNA and protein, the other 2 methylated cell lines retained the expression of p21.

Next, we tested for aberrant methylaton of *p21* in 10 primary MPMs and 50 NSCLCs, which included 20 squamous cell carcinomas, 20 adenocarcinomas and 10 adenocarcinomas with bronchioalveolar carcinoma features (BAC). The methylation of *p21* was found in 13 (26%) primary NSCLCs and only 1 (10%) primary MPM (Fig. 2 and Table 2). No methylation was found in non-malignant lung tissues, non-neoplastic pleura specimens or non-malignant mesothelial cells. Among primary NSCLCs, methylation in adenocarcinomas was significantly more frequent than in squamous cell carcinomas ($p = 0.03$), but there was no

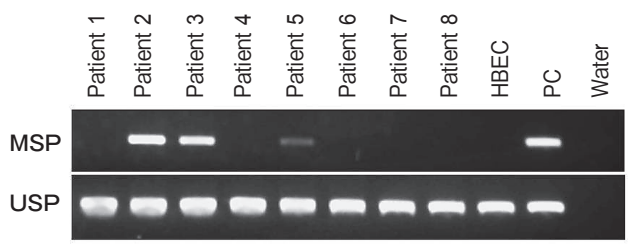

Fig. 2 Representative results of MSP in primary tumors. MSP, methylated; USP, unmethylated; PC, positive control.

Table 2A Frequency of DNA methylation of *p21*

Histology	Sample kinds		Number	Methylated	%
SCLC	Cell line		12	1	8.3
NSCLC	Cell line		13	4	30.8
	Primary	SQ	20	1	5
		Ad	20	9	45
		BAC	10	3	30
MPM	Cell line		6	0	0
	Primary		10	1	10

Ad, adenocarcinoma; SQ, squamous cell carcinoma; BAC, adenocarcinomas with bronchioalveolar carcinoma features

Table 2B Patient characteristics and frequency of DNA methylation in primary NSCLCs

Variables	Number	Methylated	%
Age (year-old)			
<70	27	8	30
70≤	23	5	22
Gender			
Male	29	7	24
Female	21	6	29
Smoking history			
Never smoker	17	7	41
Ever smoker	33	6	18
Stage			
IA	32	11	34
IB	11	0	0
II, III	7	2	29

correlation between *p21* methylation and smoking history or other characteristics (Table 2).

Two methylated cell lines, H522 and H841, were treated with 5-aza-2'-deoxycytidine and p21 protein and mRNA expression was restored, which indicated that methylation was responsible for *p21* silencing (Fig. 3).

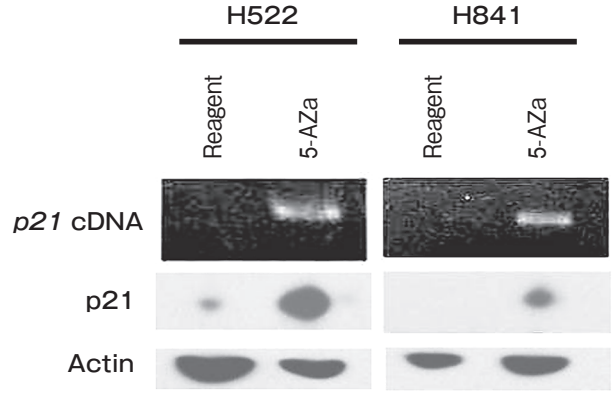

Fig. 3 Protein and mRNA expression after 5-Aza treatment in methylated cell lines.

Discussion

In the present study, we demonstrated that epigenetic modification of *p21* via hypermethylation represents a critical mechanism for inactivation in lung cancers. A previous report showed the hypermethylation of *p21* using the digestion of CG-containing enzymes after bisulfate modification in 4 out of 13 lung cancer cell lines [9]. Though the methods were different, the frequency of *p21* methylation observed in thas study is similar to our result. However, the hypermethylation of *p21* was not the main mechanism of p21 suppression. As we have shown in this study, a loss of p21 protein expression was frequently observed in lung cancer cell lines and MPM cell lines, while DNA methylation was observed to be less frequent and partial in both lung cancer and MPM. We showed the presence of methylation in primary tumors, and the frequency of methylation varied by histology. The suppression of p21 in MPMs was shown by immunohistochemistry in a previous report [11]. We showed loss of p21 protein expression by Western blotting in 3 cell lines, but DNA methylation was not the mechanism of this loss.

Most cell lines which lost p53 expression also lost p21 expression, and even in cells in which p53 expression was preserved, the *p53* mutation may ruin its transcriptional potency and cause p21 expression to be lost. Additionally, discrepancies between mRNA and protein expression were seen in 8 cell lines, which may indicate a difference of sensitivity between PCR and blotting or the possibility of post-transcriptional

modification. The mechanisms of p21 suppression vary in each cell line. Still, DNA methylation is a part of the mechanisms, especially in NSCLCs.

The ARF/p53/p21 tumor suppression pathway acts as a molecular sensor and regulator of cellular stress, senescence, and immortalization [12]. The *p21* promoter contains five p53 binding sites, and its expression is regulated in manners both dependent and independent of p53. The p53-dependent induction of p21 causes cell cycle arrest after DNA damage. Also, p21 can be regulated independently from p53 in several situations including cellular differentiation and normal tissue development. The fact that a basal level of p21 can be found in some p53-null cell lines suggests that p21 can also be induced by pathways independent of p53. Understanding the molecular regulation of this pathway by intrinsic and extrinsic signals is extremely important in order to address unsolved questions regarding senescence and cancer. In tumor cells, in the case of the loss of the p53 protein or the presence of an altered form of p53, p21 expression is dramatically reduced or totally absent. Additionally, the deletion and methylation of p21 itself, have been shown in several malignancies. The expression of p21 has been reported as a prognostic factor in malignancies. The high expression of p21 was reported to be an independent prognostic factor associated with longer survival in NSCLC patients, but some reports have shown a controversial result [13, 14]. A reduction of p21 expression has been reported to have significant positive relationship with poor survival in MPM patients [11]. Moreover, SV40 protein which can inactivate p53, may be involved in the silencing of p21 in MPMs.

In conclusion, our results show that a loss of p21 expression was frequently observed in lung cancers and MPMs and that aberrant methylation was one of the mechanisms by which p21 was suppressed, especially in lung adenocaricnomas.

References

1. Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inihibitor of G1 cyclin-dependent kinases. Cell (1993) 75: 805–816.
2. Garrel AL, Serfas MS and Tyner AL: p21-negative regulator of the cell cycle. Pros Soc Exp Biol Med (1996) 213: 138–149.
3. Toyooka S, Toyooka KO, Maruyama R, Virmani AK, Girard L, Miyajima K, Harada K, Ariyoshi Y, Takahashi T, Sugio K, Brambilla E, Gilcrease M, Minna JD and Gazdar AF: DNA methylation profiles of lung tumors. Mol Cancer Ther (2001) 1: 61–67.
4. Toyooka S, Pass HI, Shivapurkar N, Fukuyama Y, Maruyama R, Toyooka KO, Gilcrease M, Farinas A, Minna JD and Gazdar AF: Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma. Cancer Res (2001) 61: 5727–5730.
5. Ogino S, Kawasaki T, Kirkner GJ, Ogawa A, Dorfman I, Loda M and Fuchs CS: Down-regulation of p21(CDKN1A/CIP1) is inversely associated with microsatellite instability and CpG island methylator phenotype (CIMP) in colorectal cancer. J Pathol (2006) 210: 147–154.
6. Ying J, Srivastava G, Gao Z, Zhang X, Murray P, Ambinder R and Tao Q: Promoter hypermethylation of the cyclin-dependent kinase inhibitor (CDKI) gene p21WAF1/CIP1/SDI1 is rare in various lymphomas and carcinomas. Blood (2004) 103: 743–746.
7. Roman-Gomez J, Castillejo JA, Jimenez A, Gonzalez MG, Moreno F, Rodriguez Mdel C, Barrios M, Maldonado J and Torres A: 5'CpG island hypermethylation is associated with transcriptional silencing of the p21(CIP1/WAF1/SDI1) gene and confers poor prognosis in acute lymphoblastic leukemia. Blood (2002) 99: 2291–2296.
8. Fang JY and Lu YY: Effects of histone acetylation and DNA methylation on p21WAF1 regulation. World J Gastroenterol (2002) 8: 400–405.
9. Zhu WG, Srinivasan K, Dai Z Duan, W, Druhan LJ, Ding H, Yee L, Villalona-Calero MA, Plass C and Otterson GA: Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21CIP1 promoter. Mol Cell Biol (2003) 23: 4056–4065.
10. Brakensiek K, Langer F, Kreipe H and Lehmann U: Absence of p21CIP1, p27KIP1 and p57KIP2 methylation in MDS and AML. Leuk Res (2005) 29: 1357–1360.
11. Baldi A, Groeger AM, Esposito V, Cassandro R, Tonini G, Battista T, Di Marino MP, Vincenzi B, Santini M, Angelini A, Rossiello R, Baldi F and Paggi MG: Expression of p21 in SV40 large T antigen positive human pleural mesothelioma: relationship with survival. Thorax (2002) 57: 353–356.
12. Cheung CT, Hasan MK, Widodo N, Kaul SC and Wadhwa R: CARF: An emerging regulator of p53 tumor suppressor and senescence pathway. Mech Ageing Dev (2009) 130: 18–23.
13. Dworakowska D, Jassem E, Jassem J, Boltze C, Wiedorn KH, Dworakowski R, Skokowski J, Jaśkiewicz K and Czestochowska E: Absence of prognostic significance of p21(WAF1/CIP1) protein expression in non-small cell lung cancer. Acta Oncol (2005) 44: 75–79.
14. Kwon MS, Lee YI and Lee KY: p21 as a prognostic factor in non-small cell lung carcinomas. Pathol Res Pract (2006) 202: 846–856.