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Oncogenic phosphatase Wip1 is a novel prognostic marker for lung adenocarcinoma patient survival

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DNA damage response pathways are important for maintaining genomic stability. The oncogenic phosphatase Wip1 plays a crucial role in DNA damage response by inhibiting several cell cycle proteins, including p53. Although *Wip1* gene amplification has been reported in various primary tumors, including lung cancer, its biological significance for survival of primary lung tumor patients remains unclear. We investigated the expression of Wip1 in cancer epithelial cells immunohistochemically in 84 consecutive resected cases of lung adenocarcinoma. Increased Wip1 expression was observed in 54 (64.3%) of the 84 cases. Wip1 expression was found to be correlated significantly with two clinicopathological factors: γ-H2AX expression, and invasion to the pulmonary vein. A univariate analysis and log–rank test indicated a significant association between Wip1 expression and lower overall survival rate ($P = 0.019$ and $P = 0.0099$, respectively). A multivariate analysis also indicated a statistically significant association between increased Wip1 expression and lower overall survival rate (hazard ratio, 4.3; $P = 0.026$). The Ki67 index level was higher in the Wip1-positive group than in the negative group ($P < 0.04$, Mann–Whitney *U*-test). Moreover, in a subgroup analysis of only stage I patients, increased Wip1 expression was also significantly associated with a lower overall survival rate ($P = 0.023$, log–rank test). These results indicate that the increased expression of Wip1 in cancer epithelial cells has significant value for tumor progression and the clinical prognosis of patients with primary lung adenocarcinoma. (*Cancer Sci* 2011; 102: 1101–1106)

Cellular DNA is constantly exposed to various environmental and endogenous mutagenic insults. To maintain genomic integrity and prevent cancers caused by these potentially mutagenic events, a sophisticated array of damage sensors, signaling molecules, and repair functions have evolved. Among the key sensors of DNA damage are the phosphoinositide-3-kinase-related kinase family, which includes ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia and Rad3-related), and DNA-PK_{cs} (DNA-dependent protein kinase catalytic subunit).[1,2] A direct role for the ATM/ATR-initiated damage response pathways in cancer prevention has been recently determined.[3,4] Human pre-neoplastic lesions from a variety of different human cancers were shown to express various markers reflecting responses to DNA damage response, including activated and phosphorylated ATM, Chk2, p53, and H2AX.[3,4] In particular, phosphorylated H2AX (called γ-H2AX) plays a crucial role in recruiting DNA damage response factors to damage sites for accurate DNA repair and is considered a specific and sensitive molecular marker of DNA damage and repair.[5–7] Interestingly, late-stage tumors often show loss of these DNA damage response markers, suggesting that a decrease in the activity of DNA damage response pathways may contribute to cancer progression.[3,4]

Wild-type p53-induced phosphatase 1 (Wip1), also called PPM1D, is a member of the magnesium-dependent serine/threonine protein phosphatase (PPM) family.[8] These proteins, whose defining member is PP2Cα, are present in both prokaryotes and eukaryotes.[9] The human *Wip1* gene was first identified as a transcript induced by ultraviolet and ionizing radiation in a p53-dependent manner.[10] To date, Wip1 has been shown to dephosphorylate at least six proteins, ATM, Chk1, Chk2, p53, p38, and Mdm2.[11] A number of studies have shown that the Wip1 phosphatase is a key integrator of a response that attenuates signaling through the ATM and ATR pathways and negatively regulates the stress-responsive p38 MAPK pathway.[11] Furthermore, several reports recently showed that Wip1 directly dephosphorylated γ-H2AX, which might result in attenuating the DNA damage response.[12,13] Thus, Wip1 is considered to be an inhibitor or homeostatic regulator of the DNA damage response that facilitates the return of cells to a normal pre-stress state following DNA damage repair.

In addition, Wip1 is regarded as an oncogenic phosphatase because of the above noted functions. Indeed, amplified levels of *Wip1* have been found in cancer cell lines of the lung, breast, pancreas, bladder, liver, and meninges, and neuroblastomas.[14,15] Moreover, a number of human primary tumors (e.g., breast adenocarcinoma, ovarian clear cell adenocarcinoma, neuroblastoma, and pancreatic adenocarcinoma) contain amplified *Wip1* gene and high levels of Wip1 protein, which appear to correlate with poor prognosis for cancer patients.[16–19] However, it is still unknown whether Wip1 overexpression affects the survival of primary lung carcinoma patients. In this study, we analyzed the expression of Wip1 by immunohistochemistry in surgically resected human primary pulmonary adenocarcinoma tissue from 84 patients. We also investigated whether Wip1 expression in tumor tissues influenced the outcome of these patients.

Materials and Methods

Collection of samples and patient data. Eighty-four patients (46 males, 38 females) examined and treated at Kobe University Hospital (Kobe City, Japan) between 2001 and 2003 for lung adenocarcinoma were evaluated for this study. The study was approved by the Regional Ethics Committee for Clinical Research of Kobe University and conducted according to the principles in the Declaration of Helsinki. All patients gave dated and written informed consent. Primary tumors and adjacent non-neoplastic lung tissue were obtained at the time of surgery. Peripheral portions of resected lung carcinomas were sectioned, evaluated by a pathologist, and used for immunohistochemistry (IHC).

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All patients were consecutively enrolled in this study. Detailed clinical and demographic information, prognostic factors, and disease progression were collected retrospectively.

Immunohistochemistry. Formalin-fixed paraffin-embedded specimens were sectioned in 5 μm-thick slices and sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was carried out by placing specimens in Dako REAL Target Retrieval Solution (Dako, Glostrup, Denmark) at 98°C for 20 min. Rabbit anti-human Wip1 polyclonal antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-human phospho-histone H2AX (S139) polyclonal antibodies (5 μg/mL; R&D Systems, Minneapolis, MN, USA) were used as the primary antibodies for detection of Wip1 and γ-H2AX, respectively. The Dako EnVision/HRP Universal (DAB) kit (Dako) was used for endogenous peroxidase blocking, treatment with a secondary antibody against anti-rabbit and anti-mouse immunoglobulin antibody, and the visualization of HRP. Hematoxylin staining was used as the counterstain. Photographs of immunohistochemical stained sections were taken by a camera mounted on a Keyence BZ-8000 digital microscope (Keyence, Osaka, Japan).

Detection of *EGFR* gene mutation. Genomic DNA of tumor cells was successfully extracted from 19 paraffin-embedded tissue specimens.[20] *EGFR* gene (exons 18–21) of the DNA samples was investigated by the peptide nucleic acid-locked nucleic acid PCR clamp method.[21]

Classification of immunohistochemically stained patterns. Immunochemically stained sections were classified by light microscopy. For the assessment of the protein expression of Wip1, samples were classified as Wip1-positive if the ratio of stained cells in total epithelial cancer cells of a tumor tissue was more than 10%; if samples contained <10% stained cells, they were classified as Wip1-negative. Ten percent was used as the cut-off value because of the statistical advantage in this study. For evaluation of γ-H2AX expression, the cut-off value (the ratio of stained cells in total epithelial cancer cells) was set at 3% to obtain high sensitivity for detecting DNA damage. Sample classification was done independently by two pathologists (C.O. and Y.H.) in a blind manner. Ki67 (MIB-1) index (Ki67 expression ratio) and tumor protein p53 (TP53) expression were determined by the Division of Diagnostic Pathology, Kobe University.

Statistical analysis. All statistical analyses were carried out using Stata software version 10.1 (Stata, College Station, TX, USA). Baseline characteristics were reported as percentages for categorical variables and means for ±SD for continuous variables. Fisher's exact or Student's *t*-test were used to examine the association between Wip1 expression and various clinicopathological parameters. For survival analyses, we used the Kaplan–Meier method, and statistical significance between survival curves was assessed by the log–rank test. Overall survival (OS) and relapse-free interval (RFI) were determined from the date of surgery to the time of death or relapse, respectively. The Cox proportional hazards model was used to examine the association between the OS and the RFI and potential prognostic factors. Data were censored at the time of last visit. Significant variables from the univariate analysis were entered into the Cox hazard model analysis. Probability values <0.05 were considered statistically significant in all analyses.

Results

Wip1 expression in epithelial cancer cells of human lung adenocarcinoma. The expression of Wip1 was examined in 84 lung adenocarcinomas and the adjacent normal lung tissues by IHC using anti-human Wip1 polyclonal antibodies. In normal lung tissues, the expression of Wip1 was not detected (Fig. 1A). In some tumor tissues, Wip1 expression was observed in cancer cells (Fig. 1B–D). The frequency of Wip1-stained samples was 64.3% of all samples examined (54/84).

Relationship between Wip1 expression and clinicopathological characteristics of patients. For assessment purposes, we regarded specimens as Wip1 positive if 10% or more cancer cells within a tumor were strongly stained; all other specimens were regarded as negative. Based on this, 54 specimens were classified as Wip1 positive (64.3%) and 30 specimens as Wip1 negative (35.7%).

The relationships between Wip1-positive cases and various clinicopathological characteristics at the time of surgery are shown in Table 1. Expression of γ-H2AX was observed in 38 of 84 specimens (45.2%). Increased expression of Wip1 was significantly associated with γ-H2AX expression ($P < 0.001$) and cancer invasion to the pulmonary vein ($P = 0.019$). Wip1 expression was not significantly related to age ($P = 0.59$),

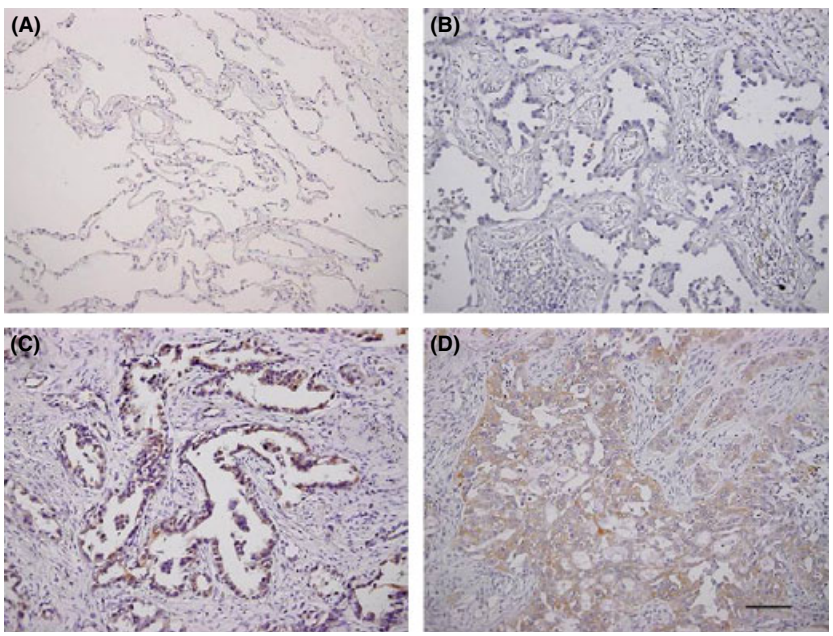

Fig. 1. Immunohistochemical analysis of expression of oncogenic phosphatase Wip1 in epithelial cancer cells of human primary lung adenocarcinoma. (A) Wip1-negative normal lung tissue. (B) Wip1-negative tumor tissue. Cancer cells were not stained. (C,D) Wip1-positive tumor tissues. Cancer cells were diffusely stained. Scale line = 100 μm (magnification, ×200).

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Table 1. Association between increased expression of oncogenic phosphatase Wip1 and clinicopathologic characteristics in 84 patients with lung adenocarcinoma

Variable	Total	Wip1		*P*-value
		Negative	Positive	
No. patients (%)	84	30 (35.7)	54 (64.3)	NA
Age in years, mean ± SD (range)	67.3 ± 9.1 (42–81)	68.0 ± 8.5 (49–80)	66.9 ± 9.5 (42–81)	0.59*
Gender				
Male/female	46/38	19/11	27/27	0.26
T factor				
T1/T2/T3/T4	45/31/3/4†	20/10/0/0	25/21/3/4	0.17
N factor				
N0/N1/N2/N3	59/8/15/1†	24/3/3/0	35/5/12/1	0.49
M factor				
M0/M1	82/1†	30/0	52/1	1.0
Stage				
I/II/III, IV	56/10/17†	24/3/3	32/7/14	0.14
P factor				
0/1/2/3	56/14/10/4	23/4/3/0	33/10/7/4	0.43
PA invasion				
Negative/positive	67/15‡	26/3	41/12	0.24
PV invasion				
Negative/positive	47/35‡	22/7	25/28	0.019
LY invasion				
Negative/positive	50/32‡	22/7	28/25	0.058
TP53 expression				
Negative/positive	45/39	16/14	29/25	1.0
γ-H2AX expression				
Negative/positive	46/38	25/5	21/33	<0.001

**P*-value by Student's *t*-test. Fisher's exact test was used for statistical analysis. †One sample missing. ‡Two samples missing. LY, lymphatic duct; NA, not applicable; PA, pulmonary artery; PV, pulmonary vein.

gender ($P = 0.26$), TNM stage ($P = 0.14$), T factor ($P = 0.17$), P factor ($P = 0.43$) according to the criteria of the International Staging System for Lung Cancer, lymph node metastasis (0.49), distant metastasis ($P = 1.0$), cancer invasion to the pulmonary artery ($P = 0.24$) or the lymphatic ducts ($P = 0.058$), or TP53 expression ($P = 1.0$). *EGFR* mutation was detected in 6 of 19 samples (31.6%). Wip1 expression was not significantly related to *EGFR* mutation (three samples with *EGFR* mutation in nine Wip1-negative samples and 3 in 10 Wip1-positive samples; 33.3% and 30.0%, respectively; $P = 1.0$).

Increased expression of Wip1 related to poor patient prognosis and proliferation of cancer cells. Using the data collected from 84 study patients, we evaluated their prognosis and its relationship to the expression of Wip1. We examined the OS of Wip1-negative and Wip1-positive groups and found a statistically significant difference between the two groups using the log–rank test ($P = 0.0099$). As shown, survival of Wip1-negative patients was greater than that observed for Wip1-positive patients (Fig. 2). Moreover, using the Mann–Whitney *U*-test, the Ki67 index level was higher in the Wip1-positive group than in the negative group (Fig. 3). The median Ki67 index was 6% and 10% in Wip1-negative and Wip1-positive tumors, respectively. A univariate analysis indicated that among clinicopathological factors, tumor classification, lymph node metastasis, and increased Wip1 expression correlated with outcome (Table 2). Further assessment using the Cox multivariate analysis indicated that gender (male), lymph node metastasis, and increased Wip1 expression were statistically significant predictors for OS (Table 2).

We also analyzed the RFI rate for increased Wip1 expression. In our study, the RFI rate in patients positive for increased Wip1 expression was notably lower than that in the negative group ($P = 0.013$, log–rank test; data not shown). Univariate analysis of RFI also indicated that increased Wip1 expression correlated with outcome ($P = 0.018$, hazard ratio; 2.9, 95% CI; 1.2–7.2).

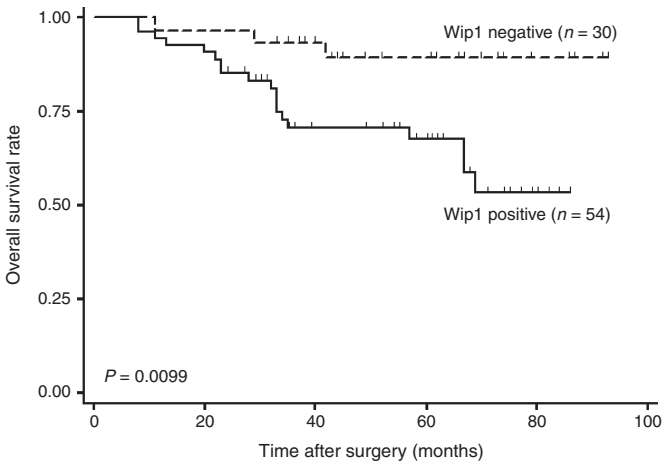

Fig. 2. Kaplan–Meier plot of the overall survival rate in 84 patients with lung adenocarcinoma, and its relationship to expression of oncogenic phosphatase Wip1. *P*-value determined using the log–rank test.

Increased expression of Wip1 also related to poor patient prognosis in stage I lung adenocarcinoma. In the stage I cases, 32 (57.1%) and 24 (42.9%) patients were classified as Wip1 positive and Wip1 negative, respectively (Table 1). A survival analysis that included only stage I patients revealed that the overall survival curve for the Wip1-positive group was lower than the Wip1-negative group. The log–rank test showed that the difference was statistically significant ($P = 0.023$) (Fig. 4).

Discussion

In the present study, we carried out IHC staining of human primary adenocarcinoma tissue specimens to detect the protein

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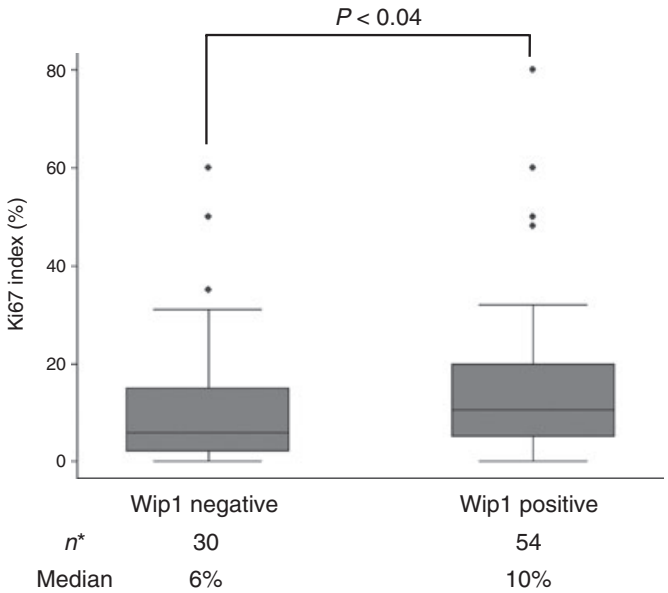

Fig. 3. Ki67 index (%) in lung adenocarcinoma samples and its relationship to the expression of oncogenic phosphatase Wip1. *P*-value determined using the Mann–Whitney *U*-test. **n*, number of lung tumors.

Table 2. Univariate and multivariate analysis of the association between the overall survival of 84 patients with lung adenocarcinoma and prognostic factors, by Cox proportional hazard models

Variable	Hazard ratio	95% Confidence interval	*P*-value
Univariate			
Age	1.0	1.3–14.6	0.93
Gender (male *versus* female)	0.51	0.21–1.3	0.14
T factor (T1<)	2.9	1.2–7.1	0.021
LN (negative *versus* positive)	3.8	1.6–8.9	0.002
PV invasion (negative *versus* positive)	2.2	0.92–5.1	0.077
Wip1 (negative *versus* positive)	4.3	1.3–14.6	0.019
Multivariate			
Age	1.0	0.97–1.1	0.42
Gender (male *versus* female)	0.31	0.11–0.84	0.031
T factor (T1<)	2.2	0.81–5.9	0.12
LN (negative *versus* positive)	3.4	1.3–9.2	0.015
PV (negative *versus* positive)	0.63	0.21–1.9	0.42
Wip1 (negative *versus* positive)	4.3	1.2–15.6	0.026

LN, lymph node metastasis; PV, invasion to pulmonary vein.

expression of oncogenic phosphatase Wip1 and observed the increased expression of Wip1 in tumor tissues, but not in normal lung tissues. The increased Wip1 expression was associated significantly with lower overall survival rate of lung adenocarcinoma patients. To our knowledge, this is the first study to detect protein expression of Wip1 in lung adenocarcinoma and to report that Wip1 expression might be a useful prognostic marker for lung adenocarcinoma patient survival.

Using IHC staining, increased Wip1 protein expression was observed in 64.3% (54/84) of lung adenocarcinoma specimens but was not detected in adjacent non-neoplastic lung tissues (Fig. 1). In order to define the effects of increased Wip1 expression on the prognosis of patients with lung cancer, a prognostic analysis was carried out on follow-up data. The results of the

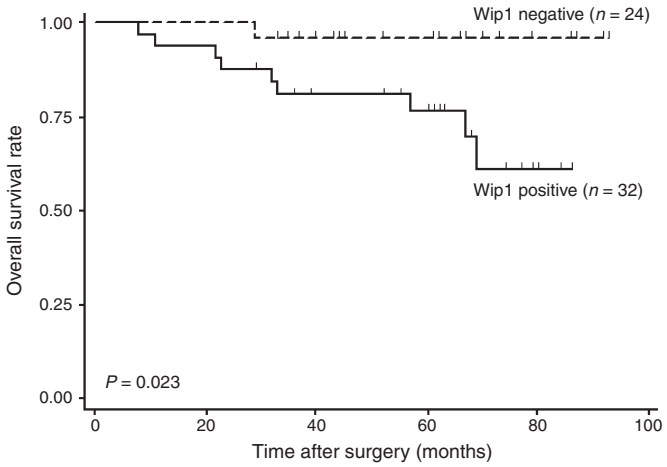

Fig. 4. Kaplan–Meier plot of the overall survival rate in 84 patients with lung adenocarcinoma and its relationship to expression of oncogenic phosphatase Wip1 in stage I patients. *P*-value determined using the log–rank test.

survival analysis showed that the OS rate in patients positive for increased Wip1 expression was notably lower than that of the Wip1-negative group (Fig. 2). These findings indicate that increased Wip1 expression negatively affects the clinical course and that increased Wip1 expression is correlated with malignant behavior of tumors. Our Cox multivariate analysis indicated that increased Wip1 expression, gender (male), and lymph node metastasis were significant prognostic predictors. It has been reported that once lung adenocarcinoma was resected completely, women survived longer than male patients.[22] Furthermore, a prognostic analysis that included only stage I cases revealed that the OS rate of the Wip1-positive group was significantly lower than that of the Wip1-negative group. These findings suggest that increased Wip1 expression may be used as a reference index for molecular staging of patients with a high risk of death who are likely to benefit from intensive adjuvant therapy.

A number of recent reports indicate that Wip1 overexpression in mouse embryonic fibloblasts and transgenic mice promotes cell transformation and accelerated cancer progression.[14,23,24] Furthermore, *Wip1*-disrupted mice are resistant to mammary cancer, and even when tumors form in such mice, their tumor cells have a lower proliferation potential.[15] It has been suggested that the effects of Wip1 overexpression might be due to its dephosphorylation of p38, p53, and regulators of p53 (ATM, Chk1, Chk2).[11] Although the direct downstream effector of Wip1 leading to tumor progression is still unclear, we consider it more likely that increased Wip1 expression contributes to cell proliferation. For this reason, we examined the relationship between increased Wip1 expression and cell proliferation. As an indicator of cell proliferation, we used the Ki67 (MIB-1) expression index (determined by pathologists in the Division of Diagnostic Pathology, Kobe University). Using the Mann–Whitney *U*-test, the Ki67 index level was higher in the Wip1-positive group than in the negative group (Fig. 3). Moreover, the size of tumors (mm^3) was slightly greater in the Wip1-positive group than in the negative group ($P = 0.062$, Mann–Whitney *U*-test, median; 12.0 *vs* 8.4 mm^3, data not shown). In the stage I patients, the Ki67 index levels tended to be higher in the Wip1-positive group than in the negative group ($P = 0.084$, Mann–Whitney *U*-test, data not shown). In our study, increased expression of Wip1 was significantly associated with cancer invasion to the pulmonary vein ($P = 0.019$) and tended to be related to cancer invasion to the pulmonary lymphatic vessel ($P = 0.058$; Table 2). These

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results suggest that increased Wip1 expression may enhance cancer cell proliferation and tumor progression, resulted in cancer invasion to the tumor vessels.

Multiple studies showed that continuous formation of DNA double-strand breaks might contribute to increased genomic instability, leading to tumorigenesis, because of breach of a barrier (such as DNA damage response including *p53* activation).[3,4,25,26] In this study, IHC staining of γ-H2AX protein was carried out to detect presence of DNA damage in the tumor tissues and γ-H2AX expression was observed in 38 of 84 samples (45.2%; Table 1). Interestingly, our result showed that increased expression of Wip1 was significantly associated with γ-H2AX expression ($P < 0.001$). In the presence of DNA damage (indicated by γ-H2AX expression), Wip1 expression might be activated in the process of DNA damage response.[11] It is still unknown whether increased Wip1 expression results from genomic instability or not, and further studies will be required to substantiate these notions.

Alterations of the p53 tumor suppressor gene are the most common genetic changes found in human malignancies, including lung cancer.[27] Although a number of clinical prognostic studies of p53 mutations in lung cancer have been reported, using either IHC or molecular analysis, their effects on survival are unclear. Most studies suggest that the prognosis of patients with mutations in p53 are poorer than those devoid of such alterations,[28] however, others have reported an opposite relationship.[29,30] In our study, overexpression of mutated p53 was observed in 39 of 84 (46.4%) lung adenocarcinoma specimens (Table 1). However, the presence of mutated p53 did not significantly affect the overall survival rate ($P = 0.85$, data not shown). It was previously reported that only one of eight primary breast tumors with elevated levels of Wip1 showed p53 mutations and that Wip1 overexpression correlated with a poor prognosis despite the absence of p53 mutations in the same tumor.[14] In our studies (using IHC staining) we did not observe any association between increased Wip1 expression and p53 mutations in lung adenocarcinoma (Table 1). Recently, it has been reported that activating mutations of *EGFR* are present in a subset of pulmonary adenocarcinomas and also prognostic for survival benefit.[31,32] In this study, *EGFR* mutation was detected in 6 of 19 lung adenocarcinomas (31.6%) and increased Wip1 expression was not significantly related to *EGFR* mutation (three samples with *EGFR* mutation of nine Wip1-negative samples and 3 of 10 Wip1-positive samples; 33.3% and 30.0%, respectively; $P = 1.0$). These results suggest that Wip1 expression itself was not directly related to development of EGFR mutation.

It has been recently reported that p38α MAPK is essential for both proliferation and differentiation of lung stem and progenitor cells, and that the downregulation of p38α might result in human lung tumorigenesis.[33] According to these results, p38 MAPK that is dephosphorylated by Wip1 can negatively regulate the action of EGFR in the proliferation and self-renewal of lung stem and progenitor cells. Interestingly, p38 protein expression was approximately three times lower in human lung tumor samples than that found in normal lung tissues. Thus, p38 dephosphorylation, resulting in upregulation of EGFR, might explain why Wip1 enhances the progression and malignancy of lung adenocarcinoma. Although the downstream factor(s) in the Wip1 pathway that can explain the relationship between increased Wip1 expression and poor prognosis of lung adenocarcinoma patients is presently unknown, dephosphorylation of p38, p53, and γ-H2AX by Wip1 may contribute importantly to tumorigenesis and tumor progression. Thus, Wip1 might be a new lung cancer therapy target.

In conclusion, our results suggest that increased Wip1 expression in cancer cells in primary lung adenocarcinoma plays an important role in the progression of lung adenocarcinoma and acts as a negative factor for the prognosis of patients. These results suggest that increased expression of Wip1 can be used as a reference index of molecular staging to select patients at high risk of death as well as relapsed patients who may benefit from intensive adjuvant therapy.

Disclosure Statement

None of the authors have any interests which may be perceived as posing a conflict or bias.

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