Overexpression of Cytochrome P450 1B1 in Advanced Non-small Cell Lung Cancer: A Potential Therapeutic Target

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Abstract. *Background: The association of aryl hydrocarbon receptor (AhR) overexpression with cytochrome P450 1B1 (CYP1B1) expression is commonly detected in non-small cell lung cancer (NSCLC). In vitro and animal studies have shown an interaction between AhR and p53, or the epidermal growth factor receptor (EGFR). The clinical importance of AhR or CYP1B1 overexpression, however, as well as the relationship between AhR and p53 or EGFR in lung carcinomas, remains unclear. Patients and Methods: Immunohistochemistry for AhR, CYP1B1, EGFR and/or p53 expression was performed on two tissue microarrays containing 152 NSCLC specimens. Results: High levels of CYP1B1, EGFR and p53 expression were more prevalent in stage-IV disease than in earlier stages (OR, 6.0; 95% C.I., 2.25-15.90), whereas AhR was not. The AhR expression, but not CYP1B1, was associated with both the EGFR (p=0.040) and p53 expression (p=0.026). The expression of AhR and CYP1B1 did not affect the survival of NSCLC patients. Conclusion: CYP1B1, EGFR and p53 overexpression are considered to be aggressive biomarkers for NSCLC, indicating that early-staged patients warrant an aggressive treatment when these factors are overexpressed in the cancer cells.*

Lung cancer is the major cause of cancer death in many countries, including Taiwan. The tumor histology of lung cancer is clinically categorized into non-small cell lung cancer (NSCLC) and small cell lung cancer. About 80 to 85% of lung carcinomas are NSCLC, of which adenocarcinoma (AD) and squamous cell carcinoma (SQ) are the two major types (1). Because of its high mortality and poor response to chemotherapy, it is critical to evaluate the clinical significance of tumor-associated biomarkers.

Cytochrome P450 1B1 (CYP1B1) is a major member of the extrahepatic xenobiotic-metabolizing CYP enzymes, which is transactivated by the liganded aryl hydrocarbon receptor (AhR) (2) and has been shown to activate several human promutagens and procarcinogens (3, 4). Our previous studies have shown that AhR overexpression was positively associated with CYP1B1 expression in NSCLC (5, 6). To the best of our knowledge, the underlying mechanisms in the relationship between AhR/CYP1B1 expression and other clinical features and tumor biomarkers, including various cancer stages, are not well understood.

Epidermal growth factor receptors (EGFR) facilitate the intrinsic tyrosine kinase activity which is involved in the behavior of malignant cells when they are mutated or phosphorylated (7, 8). Evidence from animal studies has shown that toxicities induced by AhR ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and hexachlorobenzene were associated with AhR activation and EGFR phosphorylation (9-11). P53, a tumor suppressor, regulates various signaling pathways of cell-cycle arrest, DNA repair and apoptosis (12). It has been demonstrated that benzo[a]pyrene-induced apoptosis was associated with p53 accumulation in hepatoma cells (13, 14). Thus, animal and *in vitro* studies have suggested the possibility of an interaction between AhR and EGFR, or p53.

Although we have reported that AhR and CYP1B1 appeared to be overexpressed in NSCLC, the AhR/CYP1B1-related clinical features and survival effects for NSCLC patients remain to be determined. Thus, the clinical role of AhR/CYP1B1 expression, as well as their association with EGFR and p53 expression in lung carcinomas was investigated in this study.

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Key Words: Lung cancer, aryl hydrocarbon receptor, CYP1B1, p53, epidermal growth factor receptor.

Patients and Methods

Study subjects and tissue microarray constructs. Retrospectively, 174 paraffin tissue blocks, obtained from 174 NSCLC patients (stage I to IV) treated in the Chung Shan Medical University Hospital, were collected to construct two tissue microarrays (TMA). These lung cancer tissues were obtained during surgery or by core biopsy and were then immediately fixed with 10% buffered neutral formalin and embedded in paraffin. For this study, only SQ and AD as diagnosed according to the World Health Organization classification were selected. Other tumor types were excluded because of the small number of cases. One of the Authors reviewed all the studied cases and selected the area of the tumor for sampling. Such areas were cored (2 mm in diameter) and placed in two separate array blocks. This study was approved by the Institutional Review Board of the Chung Shan Medical University Hospital.

Clinical features. The clinical features of gender, age, cancer stage and smoking status at diagnosis verified from the hospital medical records or telephone interview. The samples were divided into stages I to IV according to the TNM (tumor, node, metastasis) criteria for NSCLC outlined in the cancer staging manual (15). Smoking status was assessed as either smoker or nonsmoker. Nonsmokers were defined as those who had never smoked previously. Individuals who currently, or had ever smoked were counted as smokers.

Immunohistochemistry. The TMA sections were dewaxed with xylene and rehydrated with decreasing ethanol concentrations ending with distilled water. For the AhR, CYP1B1 and p53 immunohistochemistry, the TMA sections were autoclaved (TM-327; Tomin Medical Equipment Co., Ltd., Taipei, Taiwan, ROC) in a 0.01 M citrate buffer (pH=6.0) for 20 min. For the EGFR immunohistochemistry, proteinase K (20 μg/ml) was used to digest the tissue sections for 30 min at 37˚C. After cooling to room temperature, all the tissue sections were incubated with 3% H_2O_2 for 15 min so as to block endogenous peroxidase activity. Subsequently, the tissue sections were incubated with primary antibodies including anti-AhR (1:200 dilution; Biomol, Plymouth Meeting, PA, USA), anti-CYP1B1 (1:3,000 dilution, clone WB-1B1; Gentest Corp., Woburn, MA, USA), anti-EGFR (1:50 dilution, Clone 31G7; Zymed Laboratories Inc., San Francisco, CA, USA) or anti-p53 (1:100 dilution; DakoCytomation, Glostrup, Denmark) for various intervals (8-16 h) at room temperature. The biotinylated secondary antibody and the streptoavidin-peroxidase conjugate (Universal LSAB2 kit; DakoCytomation) were then used according to the manufacturer's instructions. 3',3'-Diaminobenzidine (DakoCytomation) was used as a peroxidase substrate for developing the brown color and, subsequently, hematoxylin (Merck Ltd., Taipei, Taiwan, ROC) was used as a counterstain. The negative controls were prepared using normal serum or phosphate-buffered saline instead of the primary antibody. A total of twenty-two tissue cores lost tissues or retained insufficient tumor cells for interpretation during the immunohistochemical processing. Therefore, only 152 tissue cores, in total, were available for the TMA study.

Assessment of AhR, CYP1B1, EGFR and p53 immunohistochemistry. The immunostaining of AhR or CYP1B1 revealed reactivity in the cytoplasm of the tumor cells (Figure 1, A and B). The stromal cells were AhR/CYP1B1-negative. In order to quantify the AhR and CYP1B1 staining intensity of the tumor cells, each TMA section was interpreted by two separate investigators. Prostate basal cells and vascular walls of lung tissues were used as positive controls for the AhR and CYP1B1 immunostaining, respectively (6, 16). In addition, their mean staining intensities as quantified by MetaMorph imaging software (Molecular Devices Corp., Downington, PA, USA) were used as a reference to define the intensity strength of AhR and CYP1B1 expression of lung tumors, essentially, either as high or low. When more than 10% of the tumor cells exhibited cytoplasmic staining and there was a staining intensity which was equal to or lower than the reference, the tumor was rated as low expression, whereas higher staining intensity was graded as high expression. The staining assessments of each independent investigator were compared before the final rating was agreed. All the representative images were taken by a Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a CoolSNAP CCD camera (Photometrics, Pleasanton, CA, USA) at a magnification of ×200 for each tissue core.

The EGFR immunohistochemistry showed both cytoplasmic and membranous expression of the tumor cells. High EGFR expression was defined as completely membranous (but not cytoplasmic) staining of more than 10% of the tumor cells (Figure 1, C). Negative and incomplete membranous staining was rated as low EGFR expression. The p53 immunoreactivity was demonstrated in the nuclei of the tumor cells and was interpreted as high expression when more than 10% of the tumor cells displayed positive nuclear staining (Figure 1, D). The percentage of positive-expressing cells for all the tumor cells (at least 300 cells) from each tissue core of each TMA section was calculated.

Statistical analysis. Descriptive statistics were used to calculate the frequency of all the clinical features, consisting of age, gender, smoking history, tumor histology type and TNM cancer stage at diagnosis. Pearson Chi-square testing was performed in order to examine the association between the biomarkers of AhR, CYP1B1, EGFR and/or p53 and the clinical features. Multivariate logistic regression testing was performed to determine the association of high expression with the dichotomous dependent variables when adjusted for covariates. The product-limit life-table method and log-rank test were carried out to determine the cumulative survival rate and median survival time in relation to AhR/CYP1B1 expression. The follow-up period was defined as the time from cancer diagnosis to patient death or the last contact. A p-value <0.05 was considered significant. All the statistical operations were performed using SPSS 10.0 statistical software package (SPSS Taiwan Corp.).

Results

AhR, CYP1B1, EGFR and p53 expression and clinical features. Table I shows the correlation between the expression of AhR, CYP1B1, EGFR and p53 and the clinical features in the 103 AD cases and 49 SQ cases. The frequency of AhR overexpression was 52% . High CYP1B1 expression was more common in females, AD and nonsmokers than it was for males, SQ or smokers ($p<0.05$). Furthermore, high CYP1B1 expression was more prevalent in the cases with stage IV tumors than it was in the cases with earlier stages ($p=0.004$). A similar association was observed between the various cancer stages and both the EGFR ($p=0.025$) and the p53 expression ($p=0.001$). No association was demonstrated

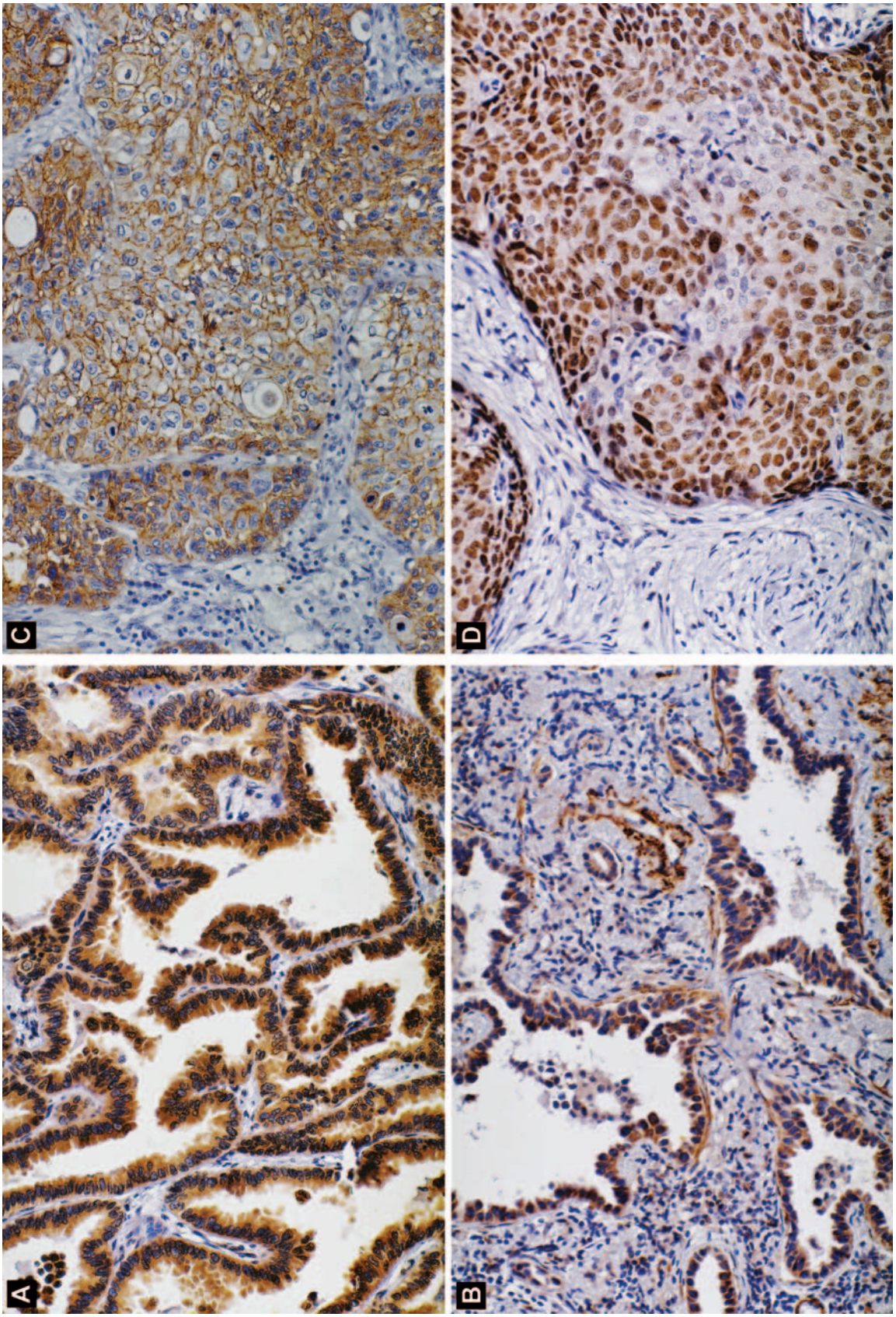

Figure 1. *Immunohistochemistry of non-small cell lung cancer for AhR, CYP1B1, EGFR and p53 on tissue microarray sections. Adenocarcinoma showing diffuse and strong cytoplasmic AhR (A) and CYP1B1 (B) staining. Squamous cell carcinoma showing diffuse and complete membranous EGFR (C) and nuclear p53 (D) staining. AhR, aryl hydrocarbon receptor; CYP1B1, cytochrome P450 1B1; EGFR, epidermal growth factor receptor. Original magnification, ×200.*

Table I. *Association of AhR, CYP1B1, EGFR and p53 expression with clinical features in non-small cell lung cancer.*

		Number (%) of high-expression cases							
	All cases	AhR	*p*-Value	CYP1B1	*p*-Value	EGFR	*p*-Value	p53	*p*-Value
Total	152	79 (52)		95 (62)		96 (63)		91 (60)	
Gender									
Male	92	43 (47)	0.110	51 (55)	0.026*	59 (64)	0.758	52 (56)	0.297
Female	60	36 (60)		44 (73)		37 (62)		39 (65)	
Age (years)									
<60	35	19 (54)	0.755	19 (54)	0.253	23 (66)	0.721	21 (60)	0.986
≥60	117	60 (51)		76 (65)		73 (62)		70 (60)	
Tumor histology									
AD	103	60 (58)	0.025*	79 (77)	<0.001*	63 (61)	0.460	60 (58)	0.556
SQ	49	19 (39)		16 (33)		33 (67)		31 (63)	
Smoking status									
Smoker	78	35 (45)	0.072	39 (50)	0.001*	49 (63)	0.929	45 (58)	0.574
Nonsmoker	74	44 (59)		56 (76)		47 (64)		46 (62)	
TNM stage									
I/II	53	28 (53)	0.165	26 (49)	0.004**	26 (49)	0.025**	25 (47)	0.001**
III	38	15 (39)		21 (55)		25 (66)		18 (47)	
IV	61	36 (59)		48 (79)		45 (74)		48 (79)	

*Statistically significant *p*-value for Pearson's Chi-square test used to compare categorical variables. **Statistically significant *p*-value for logistic regression for trend test used to examine the linear trend of the TNM stage. AD, adenocarcinoma; SQ, squamous cell carcinoma.

between EGFR or p53 expression and the other clinical features. Only CYP1B1 overexpression was associated with a number of clinical factors, including gender, tumor histology, smoking status and TNM cancer stage.

Major clinical features associated with high CYP1B1 expression. To further clarify the relationship between the clinical factors and CYP1B1 expression, multivariate logistic regression testing was carried out (Table II). After adjustment, the cases with AD tumor histology were found to be 8 times more likely to have high CYP1B1 expression when compared with SQ (OR, 8.0; CI, 2.93-21.71). Furthermore, the cases with stage IV tumors were more likely to express high levels of CYP1B1 than were those in the earlier stages of disease (OR, 6.0; 95% C.I., 2.25-15.90). Thus, the data showed that the risk of stage IV disease was also significantly and independently associated with high CYP1B1 expression. Gender and smoking status were not associated with CYP1B1 expression after this adjustment.

Association of AhR expression with EGFR or p53 expression. Among the high AhR expression samples, 78% (62/79) expressed high levels of CYP1B1 with a significant association between expression levels of AhR and CYP1B1 ($p<0.001$, data not shown). There was a significant association between AhR and both EGFR (Table III, $p=0.040$) and p53 expression ($p=0.026$); however, CYP1B1 expression was not correlated with either EGFR or p53 expression.

Table II. *Multivariate logistic regression to identify the independent factors associated with high-CYP1B1 expression in non-small cell lung cancer.*

Clinical feature	High-CYP1B1 expression	
	Odds ratio (95% CI)	*p*-Value
Gender		
Female *vs*. Male	0.6 (0.17-1.88)	0.348
Age		
≥60 *vs*. <60 years old	2.0 (0.82-5.05)	0.124
Tumor histology		
AD *vs*. SQ	8.0 (2.93-21.71)	<0.001*
Smoking status		
Nonsmoker *vs*. Smoker	1.9 (0.53-7.00)	0.318
TNM stage		
Stage III *vs*. I/II	1.5 (0.57-3.88)	0.414
Stage IV *vs*. I/II	6.0 (2.25-15.90)	<0.001*

AD, Adenocarcinoma; SQ, squamous cell carcinoma; CI, confidence interval. *Statistically significant *p*-value when adjusted for the covariates.

Effect of AhR and CYP1B1 expression on survival. Among the 152 studied cases, the median follow-up period was 14.03 months, ranging from 0.10 to 93.77 months. Among the high AhR expressors, the 1-year and 5-year survival rates were 71% and 32%, respectively, in comparison with 71% and 33% among the high CYP1B1 expressors. The overall survival time of the high and low AhR expressors was 22.93 months and

Table III. *Association between AhR, CYP1B1 and EGFR or p53 expression in NSCLC.*

	EGFR			p53		
	Low	High	*p*-Value	Low	High	*p*-Value
AhR						
Low	33	40	0.040*	36	37	0.026*
High	23	56		25	54	
CYP1B1						
Low	21	36	1.000	25	32	0.468
High	35	60		36	59	

*Statistically significant *p*-value for Pearson's Chi-square test used to compare categorical variables.

18.80 months, respectively. The overall survival time of the high and low CYP1B1 expressors was 21.80 months and 26.33 months, respectively. The AhR or CYP1B1 expression levels showed no significant association with patient survival.

Discussion

In this study, the important finding was that the overexpression of CYP1B1 as well as EGFR and p53, but not AhR, was more frequent in the advanced stages (stage III and IV) than in the early stages (stage I and II) of NSCLC. CYP1B1, EGFR and p53 expression appeared to be associated with tumor progression, while AhR overexpression was only associated with specific tumor histology. Since advanced lung carcinomas are characterized by extensive tumor invasion and metastasis (17), this result suggests that CYP1B1 overexpression could be a biomarker for aggressive NSCLC. This observation supported a previous study of colon cancer suggesting that the frequency of CYP1B1 overexpression was higher in metastatic than in primary carcinomas (18). Therefore, we propose that CYP1B1-directed anticancer prodrugs, of which resveratrol is a well-known example with a reported antiproliferative effect against cancer cells (19), may prevent lung cancer progression. The presence of CYP1B1 may "bioactivate" resveratrol into an anticancer metabolite, namely, 3,4,5,4'-tetrahydroxystilbene (20). This stilbene could cause apoptosis of the transformed lung cells *via* a pro-apoptotic p53/Bax signaling pathway, but show no effect on the normal lung cells (21). The administration of CYP1B1-directed anticancer agents to those patients overexpressing CYP1B1 in the early stages of lung cancer may prevent cancer progression to the later stages. In addition, CYP1B1 has also been utilized as an immunotherapy target for the later stage of lung cancer. A phase I trial has shown that CYP1B1 vaccination for 17 advanced cancer patients improved their overall outcome (22). CYP1B1 overexpression could, thus, constitute a biomarker for cancer therapy.

Activated AhR regulates the expression of CYP1B1, but unlike CYP1B1, AhR overexpression was not prevalent in the advanced stages of NSCLC. A possible explanation is that CYP1B1 overexpression is induced by endogenous AhR ligands or AhR-independent pathways (23, 24), or is modulated by the methylation of the *CYP1B1* promoter (25). Further investigation is planned to clarify this inconsistent situation.

The EGFR expression was positively associated with cancer stages for NSCLC. Clinical studies have shown that EGFR expression was frequently detected in metastatic or poorly differentiated lung carcinomas (26). Furthermore, an *in vitro* study has shown that cell proliferation was inhibited in human lung cancer cells when the EGFR was blocked (26). Recently, Xue and colleagues (27) showed that EGFR overexpression enhanced tumor metastasis in a mouse model of breast cancer. These findings further support the present data that EGFR overexpression was associated with tumor progression in NSCLC. However, some studies have demonstrated that *EGFR* mutation, but not overexpression, was associated with a poorer prognosis for lung cancer patients because the presence of *EGFR* mutation could predict the response to chemotherapeutic tyrosine kinase inhibitors (28, 29). Furthermore, Suzuki and colleagues (30) reported that 76% of lung carcinomas with *EGFR* mutations (point mutation, insertions and/or deletions) revealed EGFR overexpression. Thus, mutation is one of the mechanisms involved in the expression of EGFR.

In this study, AhR overexpression, but not CYP1B1, was associated with EGFR overexpression in NSCLC, indicating that AhR signaling is involved, at least in part, in EGFR activity. It is known that TCDD is an AhR ligand and amphiregulin (AR) is an EGFR ligand. Choi and colleagues (31) have proven that the increase of *AR* mRNA expression was responsive to TCDD treatment in a wild-type hepatoma cell line, but was not in an AhR-deficient hepatoma cell line. This finding showed a cross-talk between AhR and EGFR signaling pathways. Furthermore, Wu and colleagues (32) demonstrated that the EGFR protein level decreased in AhR-deficient hepatoma cells, when compared with wild-type hepatoma cells, cultivated in the absence of exogenous AhR ligands. Taken together, the evidence seems to suggest that AhR interacts in the EGFR signaling pathway *via* an as yet unknown, specific mechanism.

An association between AhR, but not CYP1B1, overexpression and p53 accumulation was also demonstrated in the NSCLC patients. Schrenk and colleagues (33), using the characteristic of p53-dependent apoptosis triggered by various types of cellular DNA damage, demonstrated that TCDD inhibited ultraviolet-induced apoptosis in rat hepatocytes and simultaneously repressed the p53 accumulation mediated by AhR activation. However, Hoagland and colleagues (34) found no significant

difference of the endogenous p53 expression between wild-type and AhR-deficient hepatoma cells. Thus, the effect of AhR on the p53 response may be mediated by exogenous AhR ligands and involve genotoxicity.

In the present study, CYP1B1 expression showed no significant association with patient survival. It is unclear why CYP1B1 overexpression was associated with patients having advanced lung carcinomas but showed no effect on patient survival. One possible reason is that many factors affect patient survival including cancer stage and anticancer drug treatment. Further studies are needed to clarify the relationship between the overexpression of CYP1B1 and patient survival in order to plan the treatment of lung carcinomas.

Acknowledgements

This work was supported in part by the National Science Council and Chung Shan Medical University (Taiwan) under Grant numbers NSC 95-2320-B-040-013 and NSC 96-2320-B-040-016.

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Received August 11, 2008
Revised December 4, 2008
Accepted December 10, 2008