Oncogene (2011) 1–11

www.nature.com/onc

ORIGINAL ARTICLE

Aryl hydrocarbon receptor in association with RelA modulates IL-6 expression in non-smoking lung cancer

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Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is activated by cigarette smoke. Previously, we demonstrated that AhR is overexpressed in lung adenocarcinomas (ADs). In this study we observed that AhR expression is significantly correlated with nuclear RelA (a nuclear factor-κB (NFκB) subunit) and cytosolic interleukin-6 (IL-6) in 200 non-small cell lung cancer patients, especially among never smokers. Overexpression of AhR increased IL-6 expression in H1355 cells and immortalized human bronchial epithelial cells BEAS-2B. As NFκB inhibitor and knockdown RelA expression greatly reduced constitutive AhR-induced IL-6 expression, we hypothesized that AhR expression, in the absence of exogenous ligand, is able to modulate NFκB activity and subsequently upregulate IL-6 expression, thus promoting the development of lung AD. Specifically, AhR overexpression significantly increased NFκB activity, whereas interference with AhR expression significantly reduced NFκB activity and IL-6 expression in H1355 cells. We demonstrated that AhR associates with RelA in the cytosol and nucleus of human lung cells. Furthermore, AhR overexpression enhanced nuclear localization of AhR and RelA, and increased the association of AhR–RelA with the NFκB response element of the IL-6 promoter. However, p50 was not involved. Our results indicate that AhR, without exposure to a ligand, associates with RelA, which then positively modulates NFκB activity and then upregulates IL-6 expression in human lung cells. Thus we have identified a new mechanism for lung tumorigenesis in non-smokers.

Oncogene advance online publication, 26 September 2011; doi:10.1038/onc.2011.438

Keywords: AhR; NFκB; IL-6; non-smokers; lung adeno carcinoma

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Received 3 May 2011; revised 30 July 2011; accepted 24 August 2011

Introduction

Lung cancer is the most common cause of cancer death worldwide. The incidence of lung adenocarcinoma (AD) has increased recently, and AD has become the most common type of lung cancer (Devesa *et al*., 2005). Cigarette smoking is the major risk factor for lung cancer. It attributes to 90% of male and 80% of female lung cancer patients in the western countries. However, in East Asia, smoking is much less prevalent in women than men by 5% versus 50% in general population and by 11% versus 80% in lung cancer patients (Ando *et al*., 2003; Liaw *et al*., 2005; Thun *et al*., 2008; Scagliotti *et al*., 2009). Risk factors for lung cancer in Asian never-smokers include previous pulmonary diseases, family cancer history, environmental tobacco smoke, cooking fumes exposure, ingested arsenic and human papillomavirus infection (Chen *et al*., 2004a, b; Yu *et al*., 2006; Tse *et al*., 2009; Wang *et al*., 2009b). Furthermore, the abnormal molecular signatures in lung cancer tissue are very different when smokers and non-smokers are compared (Carolan *et al*., 2008; Tan *et al*., 2009). Therefore, lung cancer in non-smokers is considered to be a different disease (Bryant and Cerfolio, 2007; Subramanian *et al*., 2007), and the etiology of non-smoking lung cancer is very unclear. Thus, early prevention and treatment are important issues for lung AD, especially in non-smokers. Identifying molecular mechanisms for lung AD in non-smokers will greatly facilitate these aims.

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the Per-AhR nuclear translocator (Arnt)-Sim family. Various exogenous chemicals, such as benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), bind to AhR with high affinity and activate AhR (Denison and Nagy, 2003). Cigarette smoke also activated AhR *in vitro* and *in vivo* (Kasai *et al*., 2006). Liganded AhR translocates into the nucleus and dimerizes with the Arnt. The AhR–Arnt complex further binds to xenobiotic response elements and these regulate expression of various genes, such as members of the cytochrome P450 families and phase II enzymes (Whitlock, 1999). Activated AhR not only has a key role in tumor initiation but also in tumor progression (Dietrich and Kaina, 2010). For example, the TCDD-activated AhR pathway has been found to enhance the

invasion of gastric cancer cells, and urothelial carcinoma cells (Peng *et al*., 2009; Ishida *et al*., 2010). Matsumoto *et al*. (2007) also showed that AhR is essential for carcinogenesis of airborne particulates in mice.

Recent reports have started to elucidate the function of AhR in the absence of any exogenous ligands (Barouki *et al*., 2007). For example, Hayashibara *et al*. (2003) showed that AhR is constitutively expressed and is active in human adult T-cell leukemia. Shimba *et al*. (2002) showed that overexpression of AhR promoted human lung cancer cells' proliferation. Previously, we have shown that AhR is highly expressed in lung AD as well as in bronchioalveolar carcinoma, which is considered to be carcinoma *in situ* (Lin *et al*., 2003; Su *et al*., 2009). Furthermore, interference with AhR expression reduces colony formation by human lung AD cells (Chang *et al*., 2007a). Therefore, we propose that AhR-mediated biological responses may enhance the development of lung AD.

Interleukin-6 (IL-6) is a pleiotropic cytokine regulated by nuclear factor-κB (NFκB) and has a role in tumor progression (Smith *et al*., 2001; Trikha *et al*., 2003; Zerbini *et al*., 2003; Dijsselbloem *et al*., 2004). For example, cyclooxygenase-2-induced IL-6 expression has been found to prevent the apoptosis of non-small cell lung carcinoma (NSCLC) cells (Dalwadi *et al*., 2005). Besides, Gao *et al*. (2007) demonstrated that a reduction in IL-6 expression decreased growth of lung cancer cells. AhR ligands, such as benzo(a)pyrene (Chen *et al*., 2005; Chang *et al*., 2007b) and TCDD (Puga *et al*., 2000), are known to activate various NFκB signaling pathways. But, sometimes, the cross talk between NFκB and liganded AhR mutually suppressed downstream gene expression (Tian, 2009). Especially, the effect of AhR ligands on IL-6 expression is still controversial (Jensen *et al*., 2003; DiNatale *et al*., 2010).

By examining the expression of AhR, CYP1A1, RelA and IL-6 in lung specimen, we surprisingly found that the association between AhR, RelA and IL-6 occurred significantly in non-smoking lung cancer specimens. We thus hypothesize that in the absence of exogenous ligand AhR overexpression in lung cancer cells should increase IL-6 expression via a modulation of NFκB activation with mechanism different from ligand activated AhR (Figure 1). We demonstrated that unliganded AhR associated with RelA, without Arnt or p50, and increased NFκB activity, as well as IL-6 expression, in lung cancer cells. Here, we present a novel mechanism for AhR-enhanced IL-6 expression in non-smoking lung cancer patients. AhR may be a potential molecular target for the prevention or treatment of lung cancer in non-smokers.

Results

Association of clinical features with expression of cytosolic and nuclear AhR, CYP1A1, nuclear RelA and IL-6 in NSCLC

The expression of AhR, CYP1A1, RelA and IL-6 was examined in 127 AD and 73 squamous-cell-carcinoma patients. Both AhR and RelA are transcription factors and undergo nuclear translocation after activation. Cytosolic AhR is associated with downstream gene expression in AD (Chang *et al*., 2007a; Wang *et al*., 2009a) and therefore both cytosolic and nuclear AhR expression were measured and reported. More than 90% of lung tumor cells highly expressed cytosolic RelA, but only nuclear RelA was measured and reported in this study. CYP1A1 and IL-6 were mainly expressed in the cytosol. As shown in Table 1, the frequencies of AhR

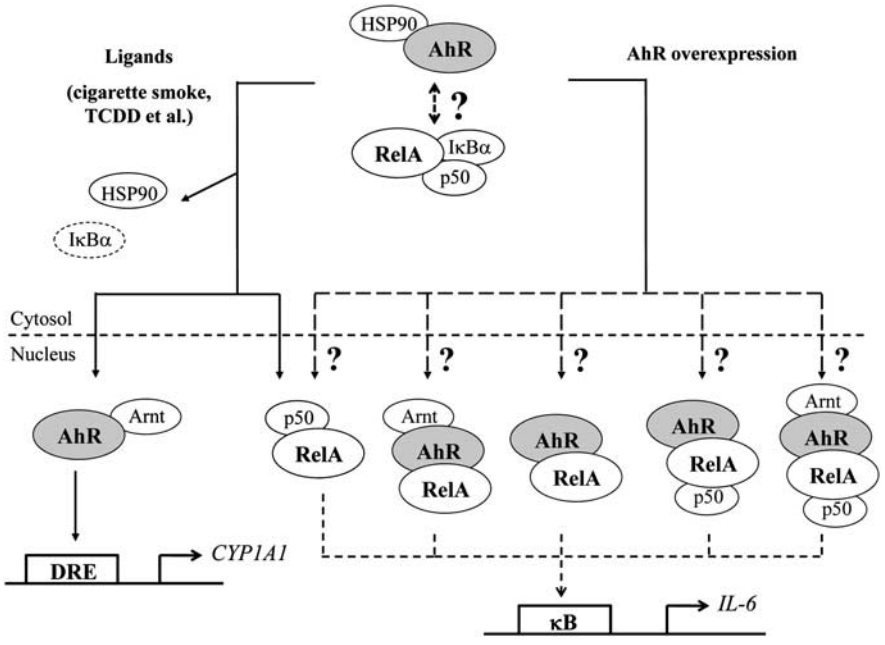

Figure 1 The possible protein complexes for AhR-regulated IL-6 expression, in the presence and absence of exogenous ligands.

Table 1 Association of clinical features with expression of cytosolic and nuclear AhR, CYP1A1, nuclear RelA and IL-6 in NSCLC

	All cases	*Number of cytosolic AhR expressers (%)*	P-*value*[a]	*Number of nuclear AhR expressers (%)*	P-*value*[b]	*Number of CYP1A1 expressers (%)*	P-*value*[b]	*Number of nuclear RelA*[c] *expressers (%)*	P-*value*[b]	*Number of IL-6 expressers (%)*	P-*value*[b]
Total	200	95 (48)		64 (32)		125 (63)	0.101	74 (37)		146 (73)	
Gender			0.600		0.756				0.545		0.100
Male	124	51 (41)		41 (33)		83 (67)		44 (35)		96 (77)	
Female	76	44 (59)		23 (30)		42 (55)		30 (39)		50 (66)	
Tumor type			0.046		0.273		0.129		0.358		0.249
AD	127	69 (54)		37 (29)		74 (58)		44 (35)		89 (70)	
SQ	73	26 (36)		27 (37)		51 (70)		30 (41)		57 (78)	
Smoking status[d]			0.733		0.538		0.046		0.297		0.198
Ever smoker	110	44 (40)		37 (34)		76 (69)		38 (35)		84 (76)	
Never smoker	86	49 (57)		25 (29)		47 (54)		36 (42)		58 (67)	

Abbreviations: AD, adenocarcinoma; AhR, aryl hydrocarbon receptor; IL-6, interleukin; NSCLC, non-small cell lung carcinoma; SQ, squamous-cell carcinoma.
[a]Logistic regression was used to compare categorical variables with frequencies; statistically significant at P-value <0.05.
[b]Fisher's χ^2-test was used to compare categorical variables with frequencies; statistically significant at P-value <0.05.
[c]Three cases did not have RelA immunohistochemistry.
[d]Four cases did not have smoking status data.

overexpression in the cytosol and nucleus occurred in 48 and 32% of the NSCLC patients. Using univariate analysis, high AhR expression was more common in females, associated with AD or with never smokers compared with in males, associated with squamous-cell carcinoma or with ever smokers (data not shown). After adjustment for gender, tumor type and smoking status, a high expression of cytosolic AhR was still more common in AD than in squamous-cell carcinoma (Table 1). High CYP1A1 expression was more prevalent in ever smokers than in never smokers (Table 1). Expression of nuclear AhR, nuclear RelA or IL-6 was not associated with any clinical feature.

Correlation between cytosolic/nuclear AhR with nuclear RelA and IL-6 in NSCLC

We hypothesized that AhR is a key regulator of *IL-6* gene expression and further analyzed the correlation of AhR expression with RelA, CYP1A1 and IL-6 expression in these specimens. We found that cytosolic and nuclear AhR correlated well with nuclear RelA and IL-6, but CYP1A1 only correlated with cytosolic AhR (Table 2). After stratification based on smoking history, nuclear AhR was still well correlated with nuclear RelA and IL-6 in both ever smokers and never smokers (Table 2). On the other hand, cytosolic AhR correlated with CYP1A1 in ever smokers, but correlated with nuclear RelA and IL-6 in never smokers (Table 2). In general, nuclear RelA and IL-6 expression correlated with both cytosol and nuclear AhR in NSCLC. However, CYP1A1 only correlated with cytosolic AhR. It is well known that AhR is constitutively expressed in the cytosol of cultivated epithelial cells and is transiently translocated into the nucleus following treatment with ligands, where it interacts with dioxin response element (DRE) and then returns to the cytosol (Pollenz, 2002). Therefore, it is reasonable that elevated AhR expression in the cytosol of tumor cells is significantly correlated with CYP1A1 expression in ever smokers who have been exposed to exogenous AhR ligands from cigarette smoke. Thus, it seems likely that the mechanism for the correlation of AhR with nuclear RelA/IL-6 expression might be different between smokers and non-smokers (Figure 1).

AhR expression modulates IL-6 expression in human lung cells

Our results for lung cancer specimens showed that AhR expression positively correlated with IL-6 expression in non-smoking lung cancer patients. *In vitro*, overexpression of AhR in BEAS-2B and H1355 cells significantly increased the *IL-6* mRNA level in cells as well as the IL-6 protein level in the culture medium (Figure 2a). Consistent reduction of AhR expression was obtained in two stable AhR RNA interference clones, si1414-6 and si1414-7, and *IL-6* mRNA, and protein levels were also significantly lower in si1414-6 and si1414-7 compared with wild-type and vector control cells (Figure 2b). Similarly, transient interference targeting AhR significantly reduced *IL-6* mRNA levels in H1355 cells (data not shown). These results suggest that AhR expression positive modulates IL-6 expression in human lung cells.

AhR upregulates IL-6 expression via modulation of NFκB activity

As IL-6 is regulated by NFκB (Aggarwal, 2004), we further investigated whether AhR modulated IL-6 expression via increased NFκB activity. Indeed, we demonstrated that overexpression of AhR increased NFκB reporter activity in H1355 cells, and interference with AhR expression significantly reduced NFκB reporter activity in H1355 cells (Figure 3a). Furthermore, the NFκB inhibitor Bay117085 and interference with RelA expression greatly abolished the increase in *IL-6* mRNA levels caused by AhR overexpression

Table 2 Correlation of AhR with CYP1A1, nuclear RelA and IL-6 in NSCLC

	All cases	*Number of CYP1A1 expressers (%)*	P-*value*[a]	*Number of nuclear RelA*[b] *expressers (%)*	P-*value*[a]	*Number of IL-6 expressers (%)*	P-*value*[a]
Total	200	125 (63)		74 (37)		146 (73)	
Cytosolic AhR			0.029		0.008		0.001
Low	105	58 (55)		29 (28)		66 (63)	
High	95	67 (71)		45 (47)		80 (84)	
Nuclear AhR			0.639		<0.001		<0.001
Negative	136	83 (61)		36 (26)		89 (65)	
Positive	64	42 (66)		38 (59)		57 (89)	
Ever smoker	110	75 (68)		38 (36)		84 (76)	
Cytosolic AhR			0.040		0.310		0.361
Low	66	40 (60)		20 (30)		48 (73)	
High	44	36 (80)		18 (41)		36 (82)	
Nuclear AhR			0.282		0.003		0.008
Negative	73	47 (64)		17 (23)		50 (68)	
Positive	37	28 (76)		20 (54)		34 (92)	
Never smoker	86	47 (55)		36 (42)		58 (67)	
Cytosolic AhR			0.082		0.008		<0.001
Low	37	16 (43)		9 (24)		16 (43)	
High	49	31 (63)		27 (55)		42 (86)	
Nuclear AhR			0.480		0.001		0.044
Negative	61	35 (57)		18 (30)		37 (61)	
Positive	25	12 (48)		18 (72)		21 (84)	

Abbreviations: AhR, aryl hydrocarbon receptor; IL-6, interleukin; NSCLC, non-small cell lung carcinoma.
[a]Pearson's χ^2-test was used to compare categorical variables with frequencies; statistically significant at *P*-value <0.05.
[b]Three cases did not have RelA immunohistochemistry.

(Figure 3b). Some studies have shown that the ligand activation of the AhR signaling pathway is usually accompanied by NFκB activation (Chen *et al*., 2005; Chang *et al*., 2007b). The question remains as to whether AhR overexpression activates the classical AhR signaling pathway (resulting in increased CYP1A1 expression via DRE), which then cross talks with the NFκB pathway in human lung cells (Figure 1). Here, we found that AhR overexpression failed to increase either DRE reporter activity or CYP1A1 mRNA levels (Figure 3c). These results suggest that AhR expression upregulates IL-6 expression via a modulation of NFκB activity without the involvement of the DRE-mediated responses.

AhR overexpression increases nuclear translocation of RelA without association with p50

AhR ligand-induced NFκB activation is usually associated with the nuclear translocation of RelA and p50 (Camacho *et al*., 2005). In our following experiments, we investigated whether AhR overexpression also stimulated the nuclear translocation of RelA and p50 as previous reported in ligand-activated AhR (Figure 1). Utilizing an immunofluorescence assay, we found that both TCDD treatment and AhR overexpression resulted in the nuclear translocation of both RelA and AhR (Figure 4). Surprisingly, only TCDD treatment, but not AhR overexpression, caused the nuclear translocation of p50 (Figure 4). Similar results were observed using western immunoblotting (Figure 5a). This raises the question as to whether RelA and AhR associated with each other during nuclear translocation. Using the immunoprecipitation assay, we showed that AhR was associated with HSP90, RelA, p50, and IκBα in the cytosol (Figure 5b). After TCDD treatment, AhR was associated with Arnt in the nucleus (Figure 5b), and RelA was associated with p50 in the nucleus (Figures 4 and 5c). However, in the absence of ligand, AhR overexpression resulted in an association of AhR with RelA, but not of AhR with Arnt, in the nucleus (Figures 5b and c). The absence of a nuclear association of AhR with Arnt after AhR overexpression is consistent with the absence of DRE-regulated responses (Figure 3c). Thus, it appears that ligand-activated AhR and overexpressed AhR modulate NFκB activation in different mechanisms. Overexpressed AhR is associated with RelA and modulates RelA nuclear translocation (Figure 7).

The AhR/RelA complex is bound to the κB element of the IL-6 promoter

It has been demonstrated that RelA/p50 complex binds the κB element of the IL-6 promoter and upregulates IL-6 expression (Vanden Berghe *et al*., 2006). The question is whether the AhR/RelA complex performs a similar function to the RelA/p50 complex. In H1355 cells,

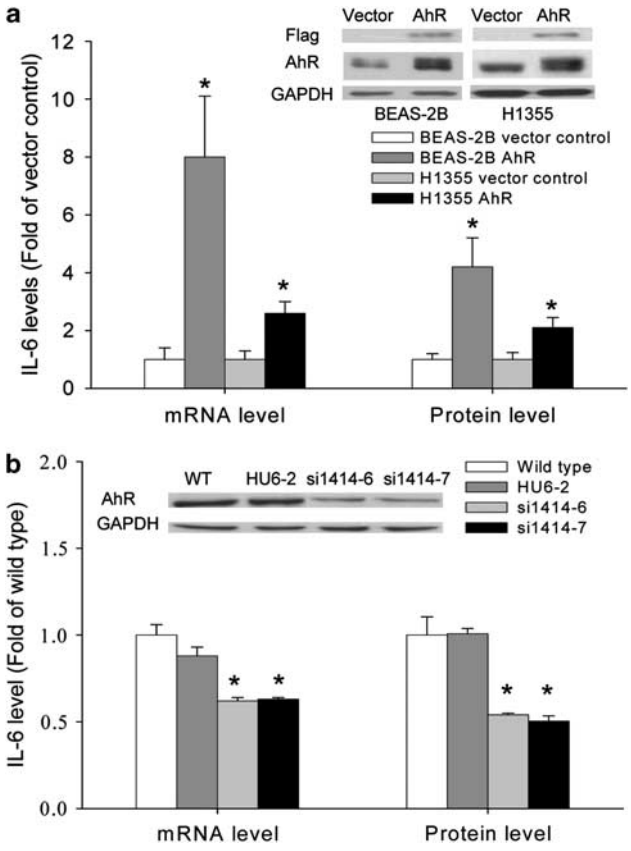

Figure 2 Effects of AhR expression on IL-6 mRNA and protein levels. (**a**) AhR expression vector was transiently transfected into BEAS-2B and H1355 cells for 48 h. The mRNA and protein levels of IL-6 were, respectively, quantified with real-time reverse transcriptase (RT)–PCR and enzyme-linked immunosorbent assay (ELISA). (**b**) The IL-6 mRNA and protein levels were quantified by real-time RT–PCR and ELISA in the clones that had undergone stable AhR interference using H1355 cells. $*P<0.05$, compared with vector control.

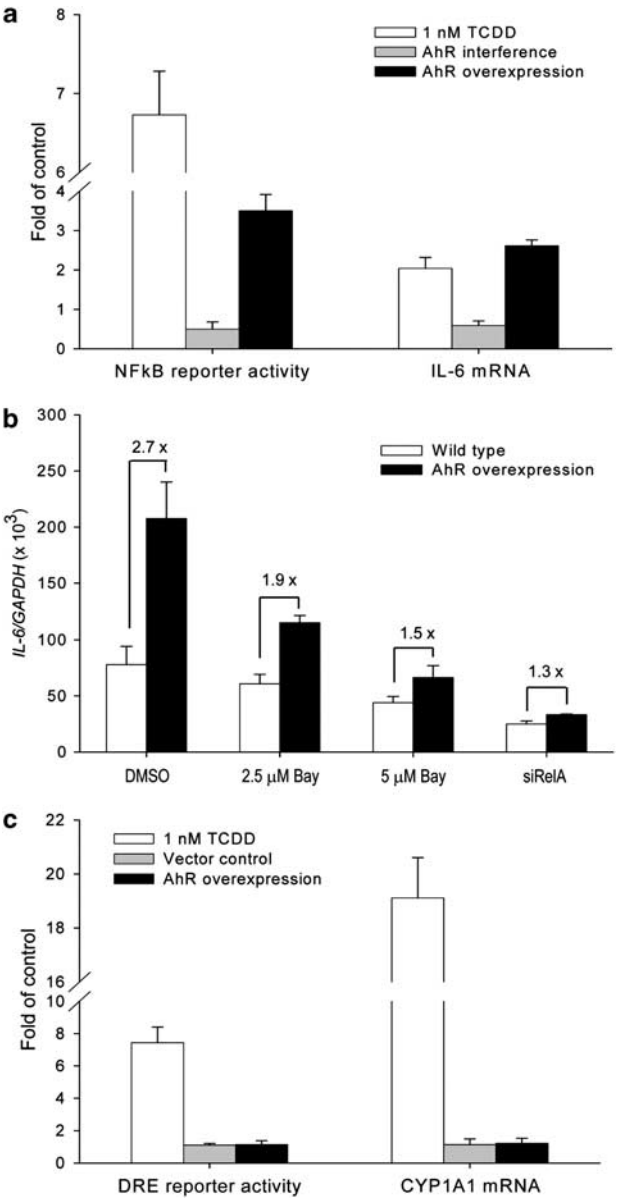

Figure 3 Effects of AhR expression on NFκB and DRE reporter activity. (**a**) AhR expression vector or inducible- short-hairpin AhR vector was co-transfected with *NFκB* reporter gene vector in H1355 cells for 48 h. NFκB-binding activity was determined by luciferase assay. IL-6 mRNA levels were quantified by real-time reverse transcriptase (RT)–PCR. (**b**) H1355 cells were transiently transfected with AhR expression vector or empty vector, and then the cells were either treated with 2.5 or 5 μM Bay117085 or co-transfected with siRelA for 24 h. The level of IL-6 mRNA levels was detected by real-time RT–PCR. (**c**) H1355 cells were co-transfected with AhR expression vector and the DRE reporter for 48 h. AhR-binding activity was detected by luciferase assay. CYP1A1 mRNA levels were quantified with real-time RT–PCR.

TCDD treatment increased the association of RelA and p50 on the κB element of IL-6 promoter (Figure 6), and thus it appears that TCDD enhances the formation of the RelA/p50 complex, which then binds to the κB element and increases IL-6 expression. On the other hand, AhR overexpression in H1355 cells increased the association of RelA with AhR without involving p50 and the complex then also binds to the κB element; AhR interference in si1414-6 cells reduced these associations. These results suggested that AhR positively modulates the association of the AhR/RelA complex with the κB element (Figure 7). Consistent with the increased NFκB activity caused by AhR overexpression, the association of AhR/RelA complex on the κB element would seem to increase NFκB activity, resulting in upregulation of IL-6 expression.

Discussion

Identifying unique molecular mechanisms among never smokers with lung cancer is becoming one of the focuses of lung cancer research. Elevated IL-6 expression is common to many cancers. In our present study, we demonstrated that AhR, without exposure to a ligand, associates with RelA, which then positively modulates NFκB activity and then upregulates IL-6 expression in human lung cells. The association was also significantly

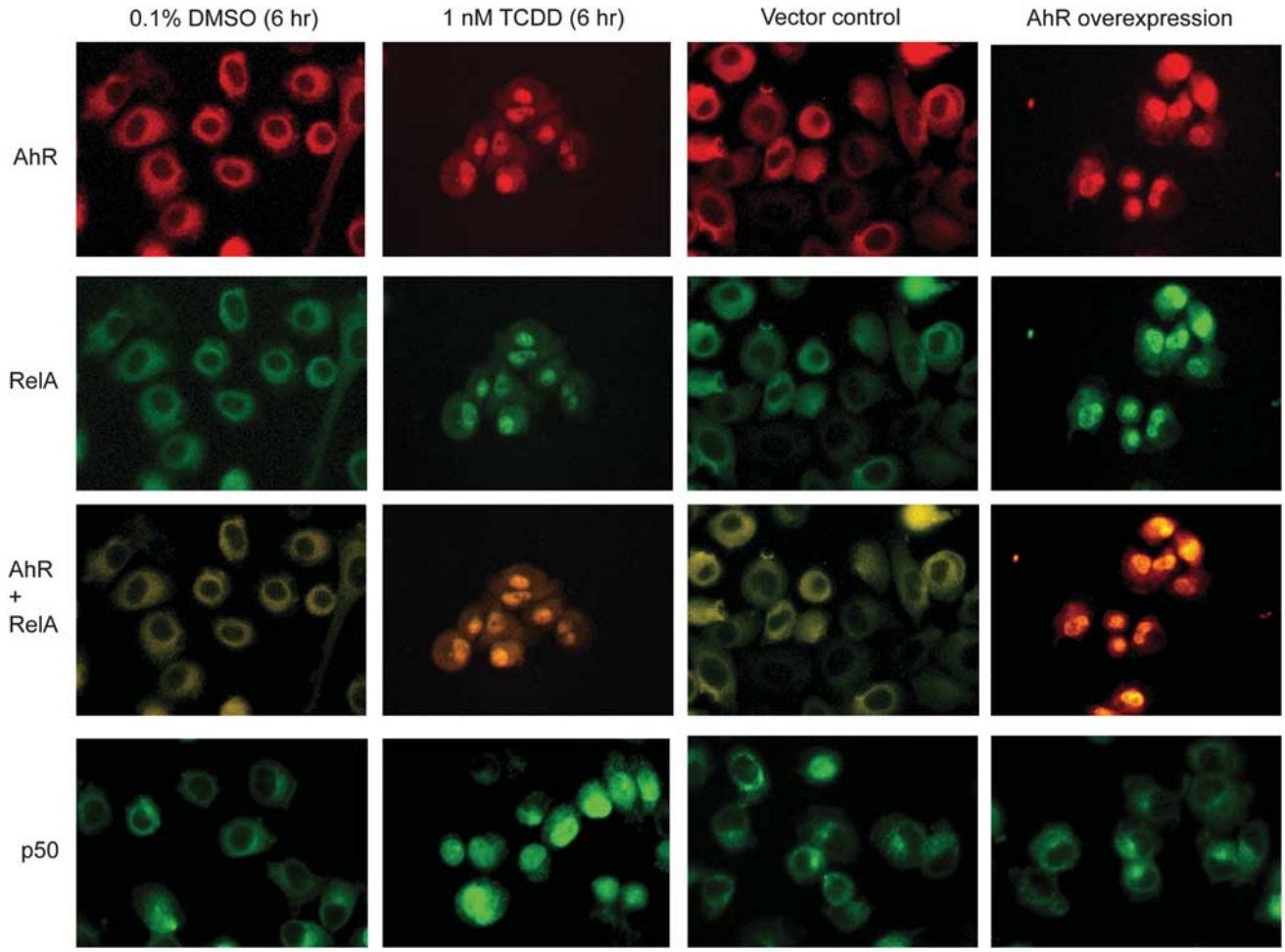

Figure 4 Effects of AhR overexpression on the cellular localization of AhR, RelA and p50. H1355 cells were treated with 1 nM TCDD for 6 h or transiently transfected with AhR expression vector for 48 h. The cells were then fixed with formaldehyde, and the location of AhR, RelA and p50 determined by immunofluorescence staining.

revealed in non-smoking lung cancer patients. Thus, we identified a new mechanism for lung tumorigenesis in non-smokers. Therefore, AhR becomes a potential molecular target for the prevention or treatment of lung cancer in never smokers.

In NSCLC patients, 32% of them express AhR in the nuclei of tumor cells. However, surprisingly, nuclear expression of AhR was not correlated with CYP1A1 expression in NSCLC patients, regardless of smoking status. Notwithstanding this, nuclear expression of AhR might have other functions, such as increasing *IL-6* expression as it does in our present study. This suggests that the nuclear expression of AhR is not a consequence of exogenous ligand-activated nuclear translocation of AhR. The presence of endogenous AhR ligands has been proposed by some studies (Oesch-Bartlomowicz *et al*., 2005; Mukai and Tischkau, 2007); however, the binding sites for endogenous and exogenous molecules on the AhR protein might be different. Another possibility is that AhR fails to be exported out of the nucleus correctly. The exact mechanism involved in the nuclear localization of AhR remains unsolved.

Although both liganded and unliganded AhR are able to upregulate IL-6 expression via increasing NFκB activity, the detailed mechanisms are different (Figure 7). TCDD activated (liganded) AhR increases the binding of the RelA/p50 complex to the IL-6 promoter. It is believed that TCDD increases NFκB activity by generating reactive oxygen species (Dhar *et al*., 2002; Lin *et al*., 2007). On the other hand, unliganded AhR associates with RelA without involving p50 and then binds to the NFκB site of the IL-6 promoter. A similar association between AhR and RelA proteins on c-myc promoter has been reported in breast cancer cells (Kim *et al*., 2000). The exact domains mediating the interaction of AhR with RelA as well as those involved in binding to other proteins associated the AhR/RelA complex remain unclear and further investigation is needed.

Our results show that AhR overexpression increased NFκB activity and enhanced the association of the AhR/RelA complex with NFκB site on the IL-6 promoter (Figure 7). Epigenetic regulation, such as phosphorylation or acetylation, has an important role in NFκB activity and functionality. p300/CBP is a

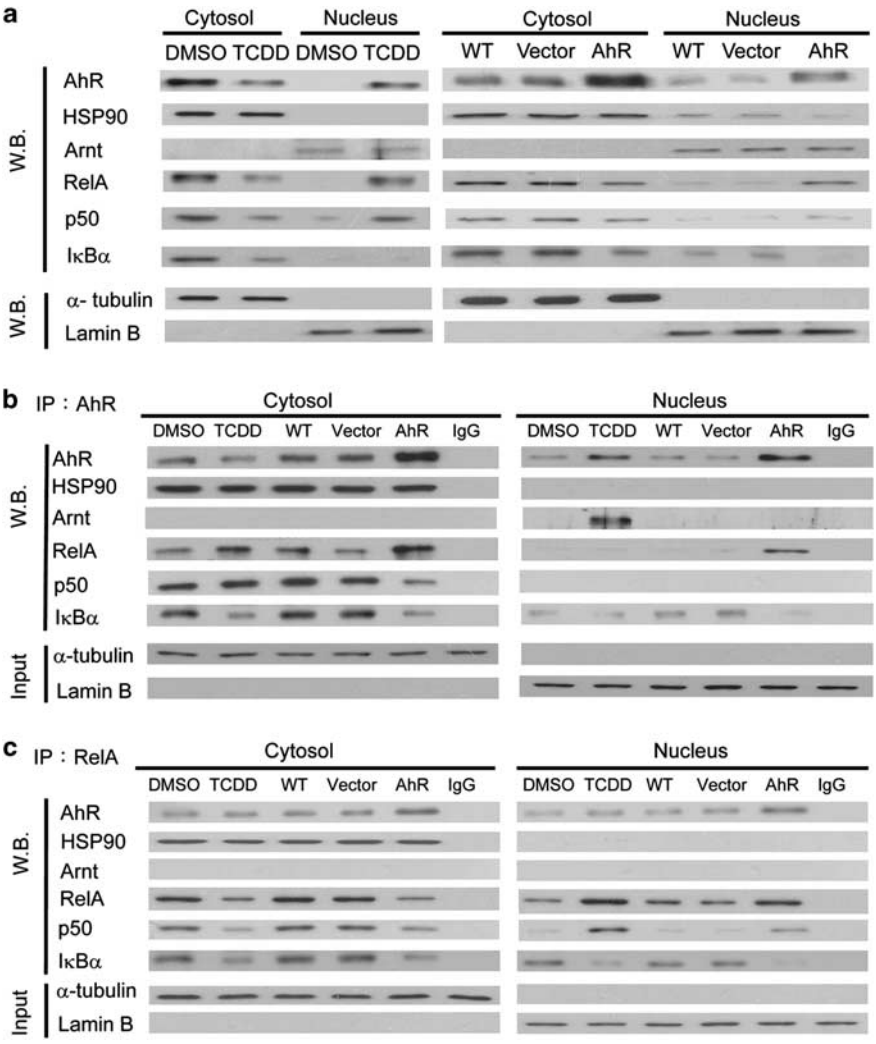

Figure 5 Effects of TCDD-induced AhR and overexpressed AhR on protein location and association. (**a**) H1355 cells were treated with 1 nM TCDD for 6 h or transiently transfected with an AhR expression vector for 48 h. Cytosolic and nuclear proteins were extracted for Western immunoblotting. (**b**, **c**) Protein extracts from **a** were immunoprecipitated with AhR or RelA antibody and detected by western immunoblotting.

coactivator for AhR and NFκB and has histone acetyltransferase activity (Tian, 2009). p300/CBP is also able to increase acetylation of RelA, which prolongs NFκB–DNA binding (Chen *et al*., 2001). On the other hand, histone deacetylase has been reported to reduce RelA acetylation and suppressed NFκB-regulated gene expression (Chen *et al*., 2001). We also observed that AhR overexpression reduced histone deacetylase activity (data not shown). Nonetheless, some studies in other cells have reported that the AhR/RelA interaction negatively regulates gene expression by increasing acetylation status (Ke *et al*., 2001; Tian, 2009). Thus, it seems likely that the positive effect of AhR/RelA on NFκB activity might be cell-type specific.

Our results also show that AhR overexpression reduced the level of IκBα protein in the cytosol and nucleus of H1355 cells. Upon NFκB activation, cytosolic IκBα is phosphorylated, ubiquitinized and then degraded by the ubiquitin–proteasome system. After IκBα degradation, the cytosol IκBα is rapidly replenished by newly synthesized IκBα, which is transcriptionally regulated by activated nuclear NFκB. Large amounts of newly synthesized IκBα may exceed the capacity of the cytosol NFκB proteins and thus permit free IκBα to accumulate in the nucleus (Arenzana-Seisdedos *et al*., 1997). In the nucleus, IκBα will associate with RelA; the complex is then exported out of the nucleus and thus terminates transcriptional activation. Ohtake *et al*. (2007) reported that AhR is an atypical E3 ubiquitin ligase that is required for ubiquitination. Thus, overexpression of AhR in cells might increase the E3 ubiquitin ligase activity of AhR, which, in turn, might reduce IκBα protein levels. However, more evidence is required to reach the conclusion that IκBα protein is a target for the E3 ubiquitin ligase activity of AhR.

Regardless of the mechanisms involved, both liganded and unliganded AhR upregulates IL-6 expression in lung cancer cells. Disrupting AhR signaling or expression therefore might reduce the expression of IL-6, which is known to have multiple roles in tumorigenesis.

Some flavonoids present in plants have already been demonstrated to block ligand-activated AhR signaling and therefore these substances have potential therapeutic usefulness in cancer prevention (Fukuda *et al*., 2007; Izawa *et al*., 2008; Lu *et al*., 2008). Such flavonoids or other targeted drugs may be useful for the prevention or treatment of lung cancer patients where there is AhR involvement.

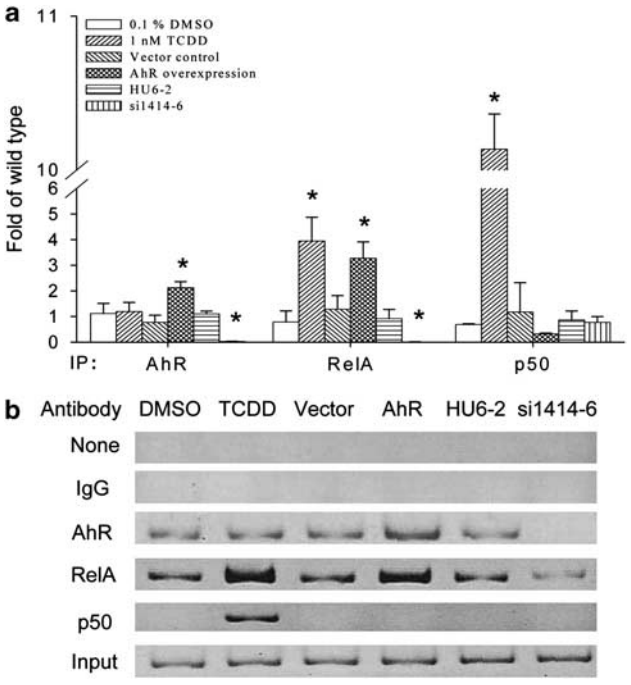

Figure 6 Effects of AhR expression on IL-6 promoter κB element binding activity. (**a**) H1355 cells were treated with 1 nM TCDD for 6 h or transiently transfected with AhR expression vector for 48 h. The cells were then lysed and assayed by chromatin immunoprecipitation/real-time PCR. (**b**) Products from **a** were visualized on 8% polyacrylamide gel electrophoresis by ethidium bromide staining. $*P<0.05$, compared with wild type.

Materials and methods

Study subjects and human tissue microarray constructions

Retrospectively, 200 paraffin tissue blocks, obtained from 200 NSCLC patients (stages I–IV) treated at the Chung Shan Medical University Hospital in Taichung, Taiwan, were collected to construct two tissue microarrays. 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 squamous-cell carcinoma 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, Dr Han Chang, reviewed all the studied cases and selected the area of the tumor for sampling. This study was approved by the Institutional Review Board of the Chung Shan Medical University Hospital, Taichung, Taiwan.

Clinical features

Clinical features, namely, gender, age, cancer stage and smoking status at diagnosis, were verified from the hospital medical records or by telephone interview. Smoking status was assessed as either ever smokers or never smokers. Never smokers were defined as those who had never smoked previously. Individuals who were currently smoking, or had ever smoked, were counted as smokers.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Lin *et al*., 2003). The primary antibodies consisted of anti-AhR (Biomol, Plymouth Meeting, PA, USA), anti-NFκB p65 subunit (clone 12H11, Millipore, Temecula, CA, USA), anti-IL-6 (clone H183, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CYP1A1 (clone H-70, Santa Cruz Biotechnology).

Assessment of AhR, RelA (p65), IL-6 and CYP1A1 immunohistochemistry

The immunoreactivity for AhR, RelA, IL-6 and CYP1A1 were evaluated via immunostaining of the lung tumor and bronchi-

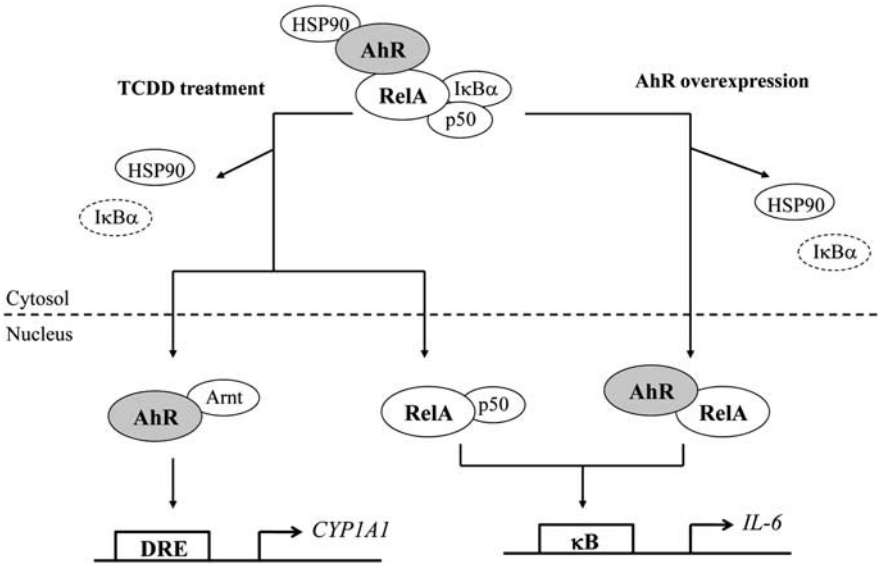

Figure 7 A model outlining AhR–RelA association and the regulation of IL-6 expression by AhR overexpression.

olar cells in the tissue sections. Assessment of AhR (Biomol) immunohistochemistry was done as previously described (Chang *et al*., 2007a). For RelA (Millipore), IL-6 (H-183, Santa Cruz Biotechnology) and CYP1A1 (H-70, Santa Cruz Biotechnology) evaluation, the immunoreactivity of the tumor or bronchiolar cells was compared with those in the adjacent stoma that showed negative immunoreactivity. When the RelA, IL-6 and CYP1A1 immunostaining intensity of the lung tumor or bronchiolar cells ($>10\%$ of cells in the tissues) was stronger than the adjacent stroma, this sample was considered to have positive expression. The percentage of highly expressing cells was then calculated from at least 300 examined cells.

Plasmids and RNA interference
To establish the AhR overexpression plasmid, human AhR complementary DNA was inserted into pcDNA3 together with two FLAG sequences 99 bp upstream of the start site. For AhR RNA interference, the short-hairpin AhR template (5′-GCCAC TCACCTCTTCAGAA-3′) was cloned into an inducible vector (pSingle-tTS-shRNA) and then transfected into H1355 for selection of stable clones as described previously (Wang *et al*., 2009a). RelA expression was transiently interfered with SignalSilence NF-kB p65 siRNA II (Cell Signaling, Danvers, MA, USA).

Cell culture and transient transfection
Human bronchial epithelial cell line BEAS-2B cells were cultured in LHC-9 (Gibco, Grand island, NY, USA). The human lung AD cell line H1355 cells were cultured in 5% fetal calf serum/RPMI1640 (Gibco) supplemented with penicillin/streptomycin. For transient transfection, 8 μg of expression vector (for 6 cm dish) was used to transfect cells using Lipofectamine 2000 (Invitrogen, Grand island, NY, USA). The transfection efficiency was about 80%.

Quantitative real-time reverse transcriptase PCR
Total RNA was extracted using the TRI reagent (MRC, Cincinnati, OH, USA). A total amount of 3 μg of total RNA was subjected to a reverse transcription step using the ABI High-Capacity cDNA Archive Kit (ABI, Foster City, CA, USA). Real-time PCR quantification was then performed using SYBR GREEN Master Mix (ABI) and TaqMan universal PCR Master Mix (Roche, Branchburg, NJ, USA). Glyceraldehyde-3-phosphate dehydrogenase was used for normalization. The primers and probes for AhR, CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase were from the Assay-on-Demand Gene Expression Assay Mix (ABI). The primers for IL-6 were 5′-CCGGGAACGAAAGAGAA GCT-3′ and 5′-CGCTTGTGGAGAAGGAGTTCA-3′.

Enzyme-linked immunosorbent assay
H1355 cells, AhR RNA interference stable clones or AhR transiently transfected H1355 cells were seeded into six-well dishes. The culture medium was then collected at 48 h. IL-6 concentration was determined using the Human IL-6 ELISA kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Reporter gene assay
AhR expression vector was co-transfected with pNF-κB-Luc (Stratagene, La Jolla, CA, USA) or 4 × DRE-TATA-Luc (give from Dr Tsui-Chun Tsou, Division of Environmental Health and Occupational Medicine, National Health Research Institutes, Zhunan, Taiwan). Inducible short-hairpin AhR vector was co-transfected with pNF-κB-Luc (Stratagene). At 48 h after transfection, a luciferase assay was performed as described previously (Chang *et al*., 2007b).

Immunofluorescence
The H1355 cells with or without AhR overexpression were fixed with 10% formaldehyde on a cover glass for 6 h. After permeabilizing with phosphate-buffered saline with Tween 20 (1 × phosphate-buffered saline with 0.2% Tween 20) for 1 h, the cells were incubated with AhR (Biomol), RelA (Millipore) or p50 (Cell Signaling) antibody for 24 h at room temperature and then incubated with FITC (Molecular Probes, Eugene, OG, USA) or TRITC (Zymed, Grand island, NY, USA) for 1 h. After washing, the cells were incubated with 4′,6-diamidino-2-phenylindole for 5 min. Finally the fluorescence was imaged using fluorescence microscopy (Leica DMRXA, Wetzlar, Germany).

Western immunoblotting
Western blotting was performed as previously published (Chang *et al*., 2007a) with anti-AhR (Biomol), anti-Flag (Sigma, Louis, MO, USA), anti-HSP90 (Assay Designs, Ann Arbor, MI, USA), anti-Arnt (H-172, Santa Cruz Biotechnology), anti-RelA (C-20, Santa Cruz Biotechnology), anti-p50 (Cell Signaling), anti-IκBα (C-21, Santa Cruz Biotechnology), anti-α-tubulin (Genetex, Hsinchu City, Taiwan) and anti-LaminB (C-20, Santa Cruz Biotechnology) antibodies. The protein bands were detected by chemiluminescence.

Chromatin immunoprecipitation assay
Cell lysates were collected from H1355 cells, AhR-overexpressed H1355 cells and AhR RNA interference stable clones. Each lysate was incubated with 1 μg of anti-AhR (Biomol), anti-RelA (C-20, Santa Cruz Biotechnology) or anti-p50 (Cell Signaling) antibodies and PureProteome Protein A Magnetic Bead (Millipore) for chromatin immunoprecipitation assay. Chromatin immunoprecipitation analysis was performed using a procedure with the following modifications (Sharma and Fondell, 2002). The chromatin immunoprecipitation and input DNA were used for the real-time PCR analysis. The primers of κB element on IL-6 promoter were 5′-AGACATGCCAAAG TGCTGAG-3′ and 5′-ATGTGGGATTTTCCCATGAGTC-3′. Real-time PCR products were also run on an 8% polyacrylamide gel electrophoresis and analyzed by ethidium bromide staining.

Statistical analysis
Pearson's χ^2-test was performed to examine the association between the biomarkers AhR, RelA, CYP1A1 and IL-6 and the clinical features. Multivariate logistic regression testing was performed to determine the association between expressions of biomarkers. Differences between groups of *in vitro* experiments were compared using the Student's *t*-test. Differences in data were considered statistically significant at $P<0.05$.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by grants from the National Health Research Institutes (99A1-EOPP02-014) and the National Science Council (NSC 97-2320-B-040-016 and NSC 97-2314-B-400-010) of Taiwan.

References

Aggarwal BB. (2004). Nuclear factor-kappaB: the enemy within. *Cancer Cell* **6**: 203–208.

Ando M, Wakai K, Seki N, Tamakoshi A, Suzuki K, Ito Y *et al*. (2003). Attributable and absolute risk of lung cancer death by smoking status: findings from the Japan Collaborative Cohort Study. *Int J Cancer* **105**: 249–254.

Arenzana-Seisdedos F, Turpin P, Rodriguez M, Thomas D, Hay RT, Virelizier JL *et al*. (1997). Nuclear localization of I kappa B alpha promotes active transport of NF-kappa B from the nucleus to the cytoplasm. *J Cell Sci* **110**(Part 3): 369–378.

Barouki R, Coumoul X, Fernandez-Salguero PM. (2007). The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. *FEBS Lett* **581**: 3608–3615.

Bryant A, Cerfolio RJ. (2007). Differences in epidemiology, histology, and survival between cigarette smokers and never-smokers who develop non-small cell lung cancer. *Chest* **132**: 185–192.

Camacho IA, Singh N, Hegde VL, Nagarkatti M, Nagarkatti PS. (2005). Treatment of mice with 2,3,7,8-tetrachlorodibenzo-p-dioxin leads to aryl hydrocarbon receptor-dependent nuclear translocation of NF-kappaB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J Immunol* **175**: 90–103.

Carolan BJ, Harvey BG, De BP, Vanni H, Crystal RG. (2008). Decreased expression of intelectin 1 in the human airway epithelium of smokers compared to nonsmokers. *J Immunol* **181**: 5760–5767.

Chang JT, Chang H, Chen PH, Lin SL, Lin P. (2007a). Requirement of aryl hydrocarbon receptor overexpression for CYP1B1 up-regulation and cell growth in human lung adenocarcinomas. *Clin Cancer Res* **13**: 38–45.

Chang LW, Chang YC, Ho CC, Tsai MH, Lin P. (2007b). Increase of carcinogenic risk via enhancement of cyclooxygenase-2 expression and hydroxyestradiol accumulation in human lung cells as a result of interaction between BaP and 17-beta estradiol. *Carcinogenesis* **28**: 1606–1612.

Chen CL, Hsu LI, Chiou HY, Hsueh YM, Chen SY, Wu MM *et al*. (2004a). Ingested arsenic, cigarette smoking, and lung cancer risk: a follow-up study in arseniasis-endemic areas in Taiwan. *JAMA* **292**: 2984–2990.

Chen J, Yan Y, Li J, Ma Q, Stoner GD, Ye J *et al*. (2005). Differential requirement of signal pathways for benzo[a]pyrene (B[a]P)-induced nitric oxide synthase (iNOS) in rat esophageal epithelial cells. *Carcinogenesis* **26**: 1035–1043.

Chen L, Fischle W, Verdin E, Greene WC. (2001). Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science* **293**: 1653–1657.

Chen YC, Chen JH, Richard K, Chen PY, Christiani DC. (2004b). Lung adenocarcinoma and human papillomavirus infection. *Cancer* **101**: 1428–1436.

Dalwadi H, Krysan K, Heuze-Vourc'h N, Dohadwala M, Elashoff D, Sharma S *et al*. (2005). Cyclooxygenase-2-dependent activation of signal transducer and activator of transcription 3 by interleukin-6 in non-small cell lung cancer. *Clin Cancer Res* **11**: 7674–7682.

Denison MS, Nagy SR. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* **43**: 309–334.

Devesa SS, Bray F, Vizcaino AP, Parkin DM. (2005). International lung cancer trends by histologic type: male:female differences diminishing and adenocarcinoma rates rising. *Int J Cancer* **117**: 294–299.

Dhar A, Young MR, Colburn NH. (2002). The role of AP-1, NF-kappaB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. *Mol Cell Biochem* **234–235**: 185–193.

Dietrich C, Kaina B. (2010). The aryl hydrocarbon receptor (AhR) in the regulation of cell-cell contact and tumor growth. *Carcinogenesis* **31**: 1319–1328.

Dijsselbloem N, Vanden Berghe W, De Naeyer A, Haegeman G. (2004). Soy isoflavone phyto-pharmaceuticals in interleukin-6 affections. Multi-purpose nutraceuticals at the crossroad of hormone replacement, anti-cancer and anti-inflammatory therapy. *Biochem Pharmacol* **68**: 1171–1185.

DiNatale BC, Schroeder JC, Francey LJ, Kusnadi A, Perdew GH. (2010). Mechanistic insights into the events that lead to synergistic induction of interleukin 6 transcription upon activation of the aryl hydrocarbon receptor and inflammatory signaling. *J Biol Chem* **285**: 24388–24397.

Fukuda I, Mukai R, Kawase M, Yoshida K, Ashida H. (2007). Interaction between the aryl hydrocarbon receptor and its antagonists, flavonoids. *Biochem Biophys Res Commun* **359**: 822–827.

Gao SG, Dong XY, Wang LJ, He J. (2007). The study on the chromosome aneuploidy in human lung cancer]. *Zhonghua Yi Xue Za Zhi* **87**: 1701–1703.

Hayashibara T, Yamada Y, Mori N, Harasawa H, Sugahara K, Miyanishi T *et al*. (2003). Possible involvement of aryl hydrocarbon receptor (AhR) in adult T-cell leukemia (ATL) leukemogenesis: constitutive activation of AhR in ATL. *Biochem Biophys Res Commun* **300**: 128–134.

Ishida M, Mikami S, Kikuchi E, Kosaka T, Miyajima A, Nakagawa K *et al*. (2010). Activation of the aryl hydrocarbon receptor pathway enhances cancer cell invasion by upregulating the MMP expression and is associated with poor prognosis in upper urinary tract urothelial cancer. *Carcinogenesis* **31**: 287–295.

Izawa H, Kohara M, Aizawa K, Suganuma H, Inakuma T, Watanabe G *et al*. (2008). Alleviative effects of quercetin and onion on male reproductive toxicity induced by diesel exhaust particles. *Biosci Biotechnol Biochem* **72**: 1235–1241.

Jensen BA, Leeman RJ, Schlezinger JJ, Sherr DH. (2003). Aryl hydrocarbon receptor (AhR) agonists suppress interleukin-6 expression by bone marrow stromal cells: an immunotoxicology study. *Environ Health* **2**: 16.

Kasai A, Hiramatsu N, Hayakawa K, Yao J, Maeda S, Kitamura M. (2006). High levels of dioxin-like potential in cigarette smoke evidenced by *in vitro* and *in vivo* biosensing. *Cancer Res* **66**: 7143–7150.

Ke S, Rabson AB, Germino JF, Gallo MA, Tian Y. (2001). Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor-alpha and lipopolysaccharide. *J Biol Chem* **276**: 39638–39644.

Kim DW, Gazourian L, Quadri SA, Romieu-Mourez R, Sherr DH, Sonenshein GE. (2000). The RelA NF-kappaB subunit and the aryl hydrocarbon receptor (AhR) cooperate to transactivate the c-myc promoter in mammary cells. *Oncogene* **19**: 5498–5506.

Liaw YP, Huang YC, Lien GW. (2005). Patterns of lung cancer mortality in 23 countries: application of the age-period-cohort model. *BMC Public Health* **5**: 22.

Lin P, Chang H, Tsai WT, Wu MH, Liao YS, Chen JT *et al*. (2003). Overexpression of aryl hydrocarbon receptor in human lung carcinomas. *Toxicol Pathol* **31**: 22–30.

Lin PH, Lin CH, Huang CC, Chuang MC, Lin P. (2007). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces oxidative stress, DNA strand breaks, and poly(ADP-ribose) polymerase-1 activation in human breast carcinoma cell lines. *Toxicol Lett* **172**: 146–158.

Lu F, Zahid M, Wang C, Saeed M, Cavalieri EL, Rogan EG. (2008). Resveratrol prevents estrogen-DNA adduct formation and neoplastic transformation in MCF-10F cells. *Cancer Prev Res (Phila)* **1**: 135–145.

Matsumoto Y, Ide F, Kishi R, Akutagawa T, Sakai S, Nakamura M *et al*. (2007). Aryl hydrocarbon receptor plays a significant role in mediating airborne particulate-induced carcinogenesis in mice. *Environ Sci Technol* **41**: 3775–3780.

Mukai M, Tischkau SA. (2007). Effects of tryptophan photoproducts in the circadian timing system: searching for a physiological role for aryl hydrocarbon receptor. *Toxicol Sci* **95**: 172–181.

Oesch-Bartlomowicz B, Huelster A, Wiss O, Antoniou-Lipfert P, Dietrich C, Arand M *et al*. (2005). Aryl hydrocarbon receptor activation by cAMP vs dioxin: divergent signaling pathways. *Proc Natl Acad Sci USA* **102**: 9218–9223.

Ohtake F, Baba A, Takada I, Okada M, Iwasaki K, Miki H *et al*. (2007). Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature* **446**: 562–566.

Peng TL, Chen J, Mao W, Song X, Chen MH. (2009). Aryl hydrocarbon receptor pathway activation enhances gastric cancer cell invasiveness likely through a c-Jun-dependent induction of matrix metalloproteinase-9. *BMC Cell Biol* **10**: 27.

Pollenz RS. (2002). The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation. *Chem Biol Interact* **141**: 41–61.

Puga A, Barnes SJ, Chang C, Zhu H, Nephew KP, Khan SA *et al*. (2000). Activation of transcription factors activator protein-1 and nuclear factor-kappaB by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochem Pharmacol* **59**: 997–1005.

Scagliotti GV, Longo M, Novello S. (2009). Nonsmall cell lung cancer in never smokers. *Curr Opin Oncol* **21**: 99–104.

Sharma D, Fondell JD. (2002). Ordered recruitment of histone acetyltransferases and the TRAP/Mediator complex to thyroid hormone-responsive promoters *in vivo*. *Proc Natl Acad Sci USA* **99**: 7934–7939.

Shimba S, Komiyama K, Moro I, Tezuka M. (2002). Overexpression of the aryl hydrocarbon receptor (AhR) accelerates the cell proliferation of A549 cells. *J Biochem* **132**: 795–802.

Smith PC, Hobisch A, Lin DL, Culig Z, Keller ET. (2001). Interleukin-6 and prostate cancer progression. *Cytokine Growth Factor Rev* **12**: 33–40.

Su JM, Lin P, Wang CK, Chang H. (2009). Overexpression of cytochrome P450 1B1 in advanced non-small cell lung cancer: a potential therapeutic target. *Anticancer Res* **29**: 509–515.

Subramanian J, Velcheti V, Gao F, Govindan R. (2007). Presentation and stage-specific outcomes of lifelong never-smokers with non-small cell lung cancer (NSCLC). *J Thorac Oncol* **2**: 827–830.

Tan XL, Wang T, Xiong S, Kumar SV, Han W, Spivack SD. (2009). Smoking-related gene expression in laser capture-microdissected human lung. *Clin Cancer Res* **15**: 7562–7570.

Thun MJ, Hannan LM, Adams-Campbell LL, Boffetta P, Buring JE, Feskanich D *et al*. (2008). Lung cancer occurrence in never-smokers: an analysis of 13 cohorts and 22 cancer registry studies. *PLoS Med* **5**: e185.

Tian Y. (2009). Ah receptor and NF-kappaB interplay on the stage of epigenome. *Biochem Pharmacol* **77**: 670–680.

Trikha M, Corringham R, Klein B, Rossi JF. (2003). Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. *Clin Cancer Res* **9**: 4653–4665.

Tse LA, Yu IT, Au JS, Yu KS, Kwok KP, Qiu H *et al*. (2009). Environmental tobacco smoke and lung cancer among Chinese nonsmoking males: might adenocarcinoma be the culprit? *Am J Epidemiol* **169**: 533–541.

Vanden Berghe W, Dijsselbloem N, Vermeulen L, Ndlovu N, Boone E, Haegeman G. (2006). Attenuation of mitogen- and stress-activated protein kinase-1-driven nuclear factor-kappaB gene expression by soy isoflavones does not require estrogenic activity. *Cancer Res* **66**: 4852–4862.

Wang CK, Chang H, Chen PH, Chang JT, Kuo YC, Ko JL *et al*. (2009a). Aryl hydrocarbon receptor activation and overexpression upregulated fibroblast growth factor-9 in human lung adenocarcinomas. *Int J Cancer* **125**: 807–815.

Wang XR, Yu IT, Chiu YL, Qiu H, Fu Z, Goggins W *et al*. (2009b). Previous pulmonary disease and family cancer history increase the risk of lung cancer among Hong Kong women. *Cancer Causes Control* **20**: 757–763.

Whitlock Jr JP. (1999). Induction of cytochrome P4501A1. *Annu Rev Pharmacol Toxicol* **39**: 103–125.

Yu IT, Chiu YL, Au JS, Wong TW, Tang JL. (2006). Dose-response relationship between cooking fumes exposures and lung cancer among Chinese nonsmoking women. *Cancer Res* **66**: 4961–4967.

Zerbini LF, Wang Y, Cho JY, Libermann TA. (2003). Constitutive activation of nuclear factor kappaB p50/p65 and Fra-1 and JunD is essential for deregulated interleukin 6 expression in prostate cancer. *Cancer Res* **63**: 2206–2215.