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ORIGINAL ARTICLE

Subcellular localization of apurinic endonuclease 1 promotes lung tumor aggressiveness via NF-κB activation

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Apurinic endonuclease 1 (Ape1) is not only involved in base excision repair, but also activates some transcriptional factors through its redox activity. However, which subcellular localization of Ape1 is involved in the activation of transcriptional factor remains unclear. We first observed that Cox-2 expression was associated with cytoplasmic Ape1 expression in lung tumors and cancer cell lines. We thus hypothesize that nuclear factor (NF)-κB is activated by cytoplasmic Ape1 to cause Cox-2 expression. Herein, we generated cytoplasmic and nuclear Ape1 in Ape1-knockdown lung cancer cells by exogenous expression of Ape1 containing various deletions and/or mutations of the nuclear localization sequence. It was observed that cytoplasmic Ape1, but not nuclear Ape1, induced Cox-2 expression through NF-κB activation. NF-κB activation by cytoplasmic Ape1 was diminished by the Ape1 redox activity inhibitor resveratrol. Cells expressing cytoplasmic Ape1 exhibited tumor progression and metastasis *in vitro* and *in vivo* as xenografts, but cells expressing nuclear Ape1 did not. Patients with tumors containing elevated cytoplasmic Ape1 had a poor prognosis and a 3.722-fold risk of tumor recurrence and/or metastasis. Cytoplasmic Ape1 could therefore enhance lung tumor malignancy through NF-κB activation, suggesting that combination of cisplatin and specific redox inhibitor could improve chemotherapeutic response in patients with tumors containing elevated cytoplasmic Ape1.
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Introduction

Apurinic endonuclease 1 (Ape1), also known as redox factor-1 (Ref-1), is thought to be a multifunctional protein involved in reduction–oxidation (redox) regulation and base excision DNA repair. Ape1 was originally identified as a DNA repair enzyme with apurinic and apyrimidinic endonuclease activity in the base excision repair pathway (Bhakat *et al*., 2009; Tell *et al*., 2009). The redox state of cysteine residues in the DNA-binding domain of several transcription factors, such as Erg-1 (Fantini *et al*., 2008), HIF-1α (Huang *et al*., 1996), p53 (Gaiddon *et al*., 1999) and nuclear factor (NF)-κB p50 subunit (Nishi *et al*., 2002; Ando *et al*., 2008), has been shown to affect DNA binding. NF-κB is a transcription factor important for the expression of numerous genes contributing to inflammation, and innate and adaptive immune responses (Aggarwal, 2004; Karin, 2006). NF-κB activation also has a critical role in human tumorigenesis (Delhase *et al*., 1999; Chen *et al*., 2005; Van Waes, 2007; Stathopoulos *et al*., 2007, 2008), particularly in inflammatory-induced cancer (Aggarwal, 2004; Tang *et al*., 2006; Maeda and Omata, 2008). NF-κB primarily occurs as a heterodimer comprising the p50/p65 subunits; this form is inactive and is retained in the cytoplasm by binding to inhibitory proteins such as IκBα, IκBβ and IκBε. Phosphorylation of IκB by IκB kinase causes ubiquitination and proteasomal degradation of IκB (Wu and Ghosh, 2003; Wu *et al*., 2006), and subsequent translocation of p50/p65 to the nucleus, where it acts as a transcriptional regulator of several genes, such as *Cox-2* (Aggarwal, 2004). It has been shown that reduction of Cys-62 in the p50 subunit by the Ape1 chaperone is essential for activation of the DNA-binding capacity of NF-κB (Nishi *et al*., 2002).

Ape1, normally localized in the nucleus, is a regulator of the cellular response to oxidative stress and protects cells from apoptosis (Kakolyris *et al*., 1998; Tell *et al*., 2009). Several human cancers, including lung (Kakolyris *et al*., 1999; Puglisi *et al*., 2001), prostate (Kelly, 2000), cervical (Xu *et al.,* 1997), ovarian (Moore *et al*., 2000; Freitas *et al*., 2003), breast (Puglisi *et al*., 2002) and hepatocellular (Di Maso *et al*., 2007) carcinomas, have been reported to have poorer prognosis in patients'

tumor, with cytoplasmic and/or nuclear localization of Ape1. However, the causal role of cytoplasmic localization of Ape1 on lung tumor malignancy is yet to be elucidated (Tell *et al*., 2009). Therefore, we established stable Ape1-knockdown clones of lung cancer cells by transfection of a small hairpin RNA targeting Ape1. The knockdown clones were then transfected with cDNA expression vectors containing the full-length Ape1 (FL) gene or the *Ape1* gene containing various deletion and/or point mutations of the N-terminal nuclear localization sequence that result in either nuclear or cytoplasmic Ape1 localization. This system was used to elucidate (1) whether cytoplasmic Ape1 is responsible for Cox-2 induction through NF-κB activation, (2) the role of cytoplasmic Ape1 in activation of the NF-κB pathway, (3) whether tumor malignancy is enhanced by cytoplasmic Ape1 through NF-κB activation *in vitro* and *in vivo*, and (4) whether cytoplasmic Ape1 is associated with an increased risk of tumor recurrence, metastasis or poor clinical outcome among post-operative lung cancer patients.

Results

Increased p65 nuclear translocation is associated with Cox-2 induction in lung cancer cells containing cytoplasmic Ape1

Eight lung cancer cell lines and two normal lung cell lines were used to examine the association between Ape1 and Cox-2 expression. Western blot analysis showed that Ape1 protein was overexpressed in the lung cancer cells, but not in the normal cells (Figure 1a), but different levels of Cox-2 expression were observed in the lung cancer cells. Cytoplasmic Ape1 expression in H441, H157and CH27 cells was higher than in A549, H1975, Calu-1, H520 and CL-3 cells. Efficient separation of nuclear and cytoplasmic fractions was verified by analyzing each of the fractions for the presence of α-tubulin (cytoplasmic protein) and Sp1 (nuclear protein). Interestingly, Cox-2 expression was elevated in H157, CH27 and H441 cells, which contain cytoplasmic Ape1 and nuclear p65. However, Cox-2 expression was also elevated in A549 and H1975 cells, which contain nuclear Ape1 and nuclear p65. To verify whether cytoplasmic Ape1 could contribute to Cox-2 expression, Ape1 expression of H441, H157, H520 and A549 cells was knocked down by shApe1. Data showed that Cox-2 expression was markedly decreased in H157 and H441 cells containing cytoplasmic Ape1, but not in H520 and A549 cells after Ape1 knockdown (Supplementary Figure S1a–e). These results suggest that Ape1 critically contributes to Cox-2 expression in those lung cancer cells associated with cytoplasmic distribution of Ape1, but not all cells, in association with increased p65 nuclear translocation.

Cytoplasmic Ape1-activated NF-κB results in Cox-2 induction

To explore whether cytoplasmic Ape1 induces Cox-2 expression through NF-κB activation, Ape1-knockdown H157 stable clones were established by transfection with a small hairpin RNA targeting Ape1. Ape1 and Cox-2 expression levels were suppressed in both stable clones, particularly in clone 7 (Supplementary Figure S1f). Amino-acid residues 1–36 in the N-terminus of Ape1 have been shown to contain nuclear localization sequences and E12A/D13A mutation was further shown to be a nuclear export signal of Ape1 subcellular localization (Jackson *et al*., 2005). Therefore, we generated Ape1 cDNA expression vectors encoding full-length Ape1 (FL) or encoding Ape1 with deletion of residues 1–7 and mutation of residues 12 and 13 (ND7), deletion of residues 1–20 (ND20), deletion of residues 1–30 (ND30) or deletion of residues 1–41 (ND41) (Figure 1b). These Ape1 vectors were then transiently transfected to Ape1-knockdown H157 cells (shApe1 #7 background), and Calu-1 and CL-3 cells with low cytoplasmic Ape1. To express these Ape1 vectors in the Ape1-knockdown H157 cells, these vectors were subjected to site-directed mutagenesis within the sequence of shApe1-targeting codons 180–186 of the *Ape1* gene (Figure 1c). Western blot analysis indicated elevated cytoplasmic Ape1 levels in ND20-, ND30- and ND41-transfected cells, but not in FL-, ND7- and vector-transfected cells. Inversely, elevated nuclear Ape1 was observed in FL- and ND7-transfected cells, but not in ND20-, ND30-, ND41- and vector-transfected cells (Figure 1d; Supplementary Figures S2 and S3a). In addition, enhanced Cox-2 and nuclear p65 expression and NF-κB DNA-binding capacity were observed in ND20- and ND30-transfected cells containing cytoplasmic Ape1, but not in FL-, ND7-, ND41- and vector-transfected cells containing nuclear Ape1 (Figures 1d and e; Supplementary Figure S3), suggesting that cytoplasmic Ape1 is associated with NF-κB activation through increased p65 nuclear translocation.

We hypothesized that Cox-2 induction through increased p65 nuclear translocation is mediated through Ape1-induced IκBα/IκBβ degradation. Consistent with this, enhanced and more rapid degradation of IκBα and IκBβ was both observed in Ape1-knockdown cells transiently transfected with ND20 or ND30 vector than in those transiently transfected with ND7 or ND41 vector or control vector (Figure 2a). In ND20- and ND30-transfected cells, IκBα/IκBβ degradation and Cox-2 expression were enhanced in unison over time (Figure 2a). Similar results were obtained using Ape1-knockdown cells stably transfected with the ND20 or ND30 vector. Cox-2 overexpression in ND-20 and ND-30 stable clones was consistent with increased IκBα/IκBβ degradation and p65 nuclear localization, but a similar association was not observed in Ape1-knockdown clones stably transfected with FL, vector control or shNC plasmid (Figures 2b and c; Supplementary Figure S4). EMSA analysis indicated that the DNA-binding capacity of NF-κB in whole-cell lysates from ND20 and ND30 stable clones was higher than that in FL, shNC and vector control stable clones (Figure 2d). F5M fluorescence assay further showed that the reductive degree of p50 in ND20 and ND30 stable

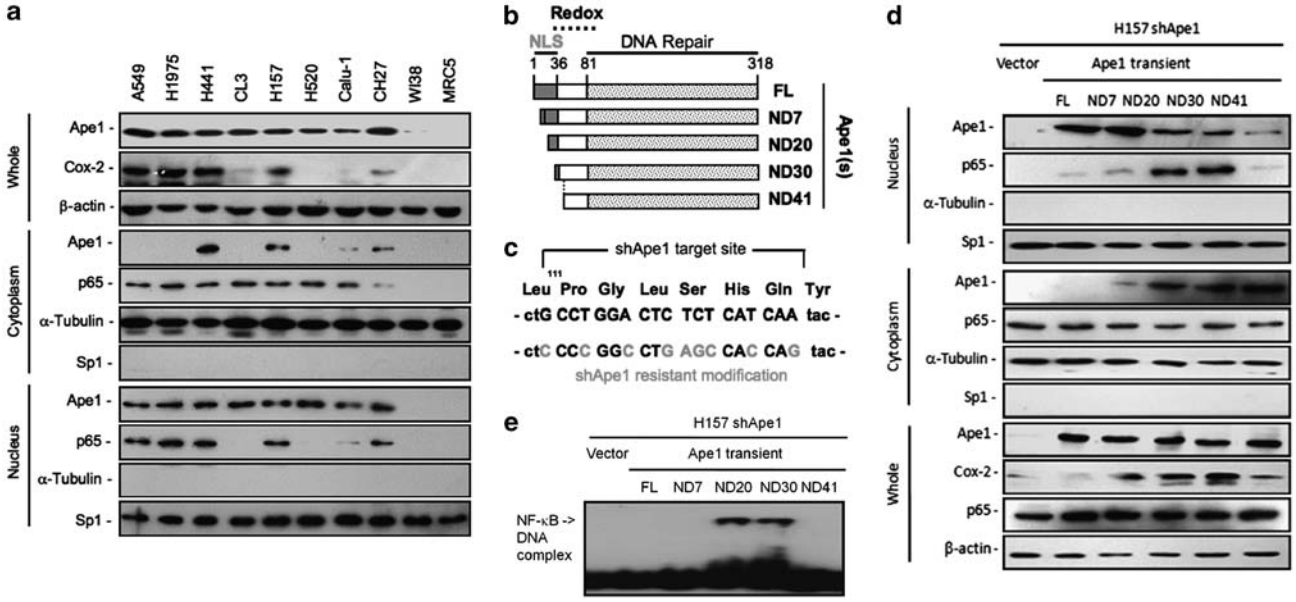

Figure 1 Correlation of Cox-2 expression in lung cancer cells with the subcellular localization of Ape1. (**a**) Western blot analysis was conducted to evaluate Ape1, Cox-2 and p65 expression in cytoplasmic and nuclear fractions, and in whole-cell lysates prepared from the indicated cells. Ape1 expression in WI38 and MRC5 normal lung fibroblast cells was compared with that in eight lung cancer cell lines. β-Actin, α-tubulin and Sp1 were analyzed as loading controls for whole-cell lysates, cytoplasmic and nuclear fractions, respectively. (**b**) Schematic diagram of Ape1 cDNA expression vectors containing deletion and/or mutation of the N-terminal NLS of Ape1 (**c**) shApe1 target sequence in the Ape1 cDNA expression vectors was mutated by site-directed mutagenesis to create shApe1-resistant construct for the recombinant Ape1 expressions. (**d**) Western blot analysis of Cox-2, Ape1and p65 expression in cytoplasmic and nuclear fractions and whole-cell lysates prepared from a shApe1 H157 stable cell line in 48 h after transient transfection with FL, ND7, ND20, ND30 or ND41 vector. β-Actin, α-tubulin and Sp1 were analyzed as loading controls for whole-cell lysates, cytoplasmic and nuclear fractions, respectively. (**e**) The DNA-binding capacity of NF-κB of whole lysates from the stable shApe1 cells was determined by EMSA analysis after transient transfection with ND7, ND20, ND30 or ND41 vector for 48 h.

clones was higher than in FL, shNC and vector control stable clones (Figure 2e). Chromatin immunoprecipitation (ChIP) assays also showed that p65 and p50 were bound to the NF-κB binding region in the Cox-2 promoter in the ND20 and ND30 stable clones, but not in the FL, shNC and vector control stable clones (Figure 2f). In addition, MG132, a specific inhibitor of the 26S proteasome complex, was used to show that IκBα/IκBβ was predominantly degraded by the 26S proteasome in stable clones of ND20 and ND30 (Supplementary Figure S5). Collectively, these data indicate that cytoplasmic Ape1, not nuclear Ape1, is responsible for Cox-2 induction through NF-κB activation.

The redox activity of cytoplasmic Ape1 is responsible for Cox-2 induction via NF-κB activation

To explore whether the redox activity of cytoplasmic Ape1 is required for NF-κB activation, the ND20 and ND30 stable clones were treated with the Ape1 redox activity inhibitor resveratrol (Yang *et al*., 2005, 2008; Athar *et al*., 2007). Cox-2 expression and blockade of IκBα/IκBβ degradation in these cells gradually decreased in the presence of resveratrol from 4 to 16 h, but Cox-2 expression was slightly restored at 24 h (Figure 2g). It is possible that the redox inhibition of resveratrol was completely exhausted by 24 h, even though blockade of IκBα/IκBβ degradation by resveratrol persisted at 24 h. Importantly, Cox-2 reduction and blockade of IκBα/IκBβ degradation was observed in parallel from 4 to 24 h in the presence of the NF-κB inhibitor BAY11-7082 (Supplementary Figure S6a). However, Cox-2 induction by cytoplasmic Ape1 was not reduced by CRT0044876, an inhibitor of Ape1 endonuclease activity (Supplementary Figure S6b), suggesting that Cox-2 induction by cytoplasmic Ape1 was not related to the DNA repair activity of Ape1. These results reveal that the redox activity of cytoplasmic Ape1 could be responsible for Cox-2 expression by directly elevating the DNA-binding capacity of NF-κB and indirectly enhancing NF-κB transactivation.

S-nitrosation of Ape1 confers cytoplasmic translocation of Ape1 and NF-κB activation

Nitric oxide (NO) has been reported to control nuclear export of Ape1 through S-nitrosation of cysteine residues 93 and 310 in human HepG2 hepatoma and HEK293T embryonic kidney cells (Qu *et al*., 2007). To verify whether the subcellular localization of Ape1 is regulated by nitrosation in lung cancer cells, the lung cancer cell lines H157, containing high cytoplasmic Ape1 levels, or H520, containing low cytoplasmic Ape1 levels (Figure 1), were treated with the NO scavenger carboxy-PTIO or the NO donor S-nitrosoglutathione (GSNO), respectively. Cytoplasmic Ape1 in H520 cells was increased by GSNO with a concomitant increase in Cox-2 expression (Figure 3a). In addition, cytoplasmic

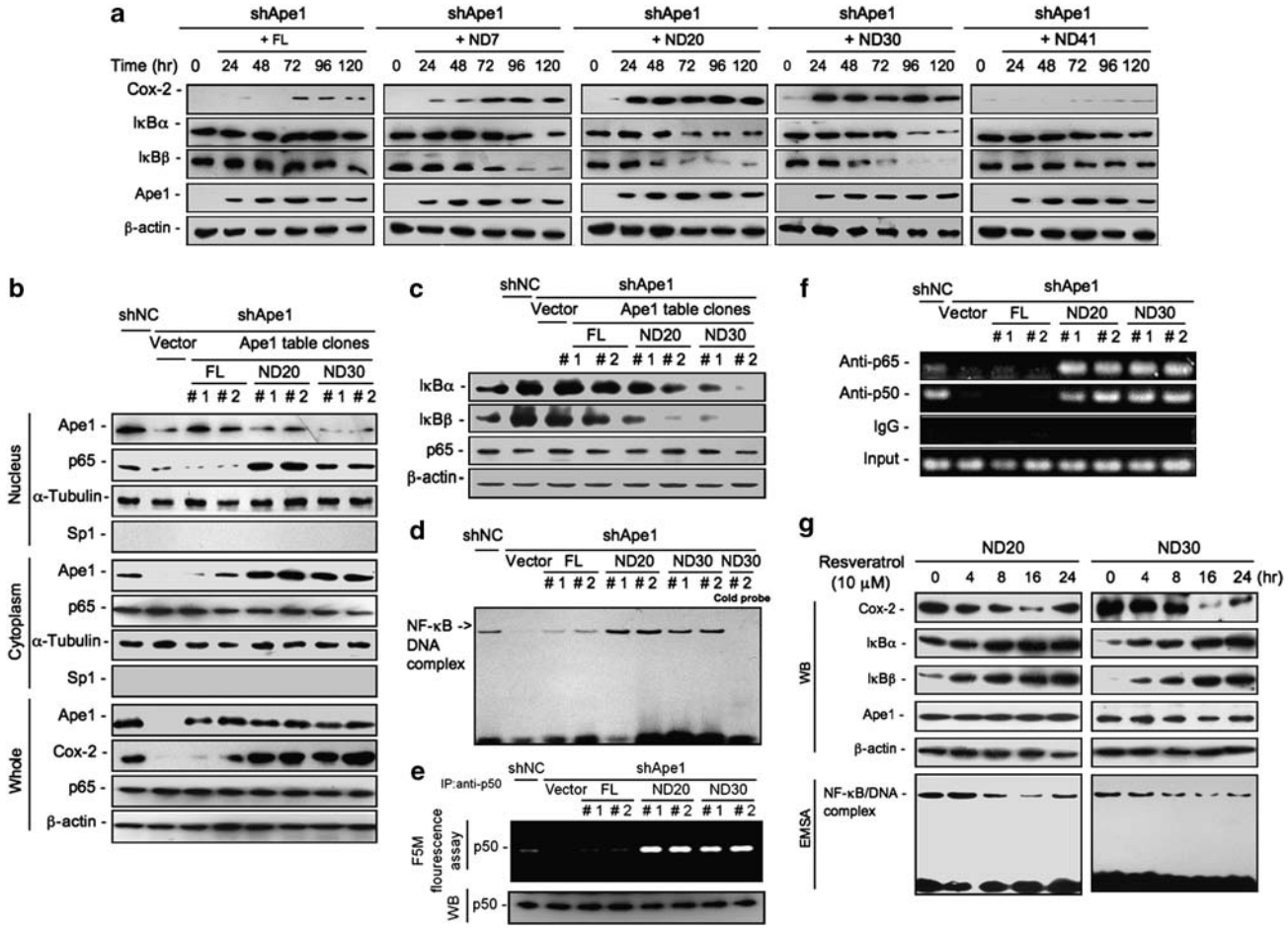

Figure 2 Cox-2 induction by cytoplasmic Ape1 is mediated by increased IκBα and IκBβ degradation, p65 nuclear localization, and enhanced NF-κB DNA binding in Ape1-knockdown H157 cells transiently transfected with the ND20. (**a**) Western blot analysis of Cox-2, Ape1, IκBα and IκBβ expression in the Ape1-knockdown H157 cell line 48 h after transient transfection with FL, ND7, ND20, ND30or ND41 vector. (**b**) Western blot analysis of Cox-2, Ape1 and p65 nuclear localization in Ape1-knockdown H157 cells stably transfected with FL, ND20or ND30 vector (FL, ND20, or ND30 stable clones). β-Actin, α-tubulin and Sp1were analyzed as loading controls for whole-cell lysates, cytoplasmic fractions and nuclear fractions, respectively. (**c**) Western blot analysis of p65, IκBα and IκBβ in Ape1-knockdown H157 cells stably transfected with FL, ND20or ND30 vector. (**d**) The DNA-binding capability of NF-κB was determined by EMSA analysis of whole lysates from the indicated cell lines and the excess cold probe was used as negative control to verify binding specificity. (**e**) F5M fluorescence assay was used to evaluate the redox state of NF-κB p50 and the procedures are described in Materials and methods. The p50 protein level evaluated by western blot served as the loading control. (**f**) ChIP analysis of NF-κB binding to the Cox-2 promoter *in vivo* was performed using p65 and p50 antibody as indicated. (**g**) Western blot analysis showing time-dependent inhibition of Cox-2 expression and concomitant elevation of IκBα and IκBβ expression in ND20 and ND30 stable clones by treatment of 10 μM for 0–24 h; the DNA-binding capacity of NF-κB in whole-cell lysates from ND20 and ND30 stable clones was evaluated by EMSA and was significantly decreased by resveratrol in a time-dependent manner.

Ape1 expression in H157 cells was decreased by carboxy-PTIO concordant with decreased Cox-2 expression (Figure 3b). Ape1 NO-cysteine levels were also decreased or increased by GSNO or carboxy-PTIO, respectively (Figures 3a and b). However, Cox-2 expression in the ND30 stable clone was not influenced by carboxyl-PTIO (Figure 3c), whereas cytoplasmic Ape1 and Cox-2 expression levels in Ape1-knockdown cells transiently transfected with the FL vector were significantly increased by GSNO treatment (Figure 3d). When Ape1-knockdown cells were transiently transfected with FL vector containing C93A and/or C310A, the expression levels of cytoplasmic Ape1 and Cox-2 were ablated despite the addition of GSNO (Figure 3d). These results not only support the previous study showing that S-nitrosation of Ape1 cysteines 93 and 310 is critical for Ape1 nuclear export (Qu *et al*., 2007), but also indicate that Ape1 S-nitrosation contributes to Cox-2 induction in lung cancer cells.

Cytoplasmic Ape1 promotes the tumor activity of lung cancer cells in vitro

To clarify whether cytoplasmic Ape1 promotes tumor activity, soft agar colony formation and matrigel invasion assays were conducted to compare the oncogenic activity and invasiveness of lung cancer cells containing cytoplasmic or nuclear Ape1. Soft agar assays showed that colony formation in the ND20 and ND30 stable clones was significantly higher than in the

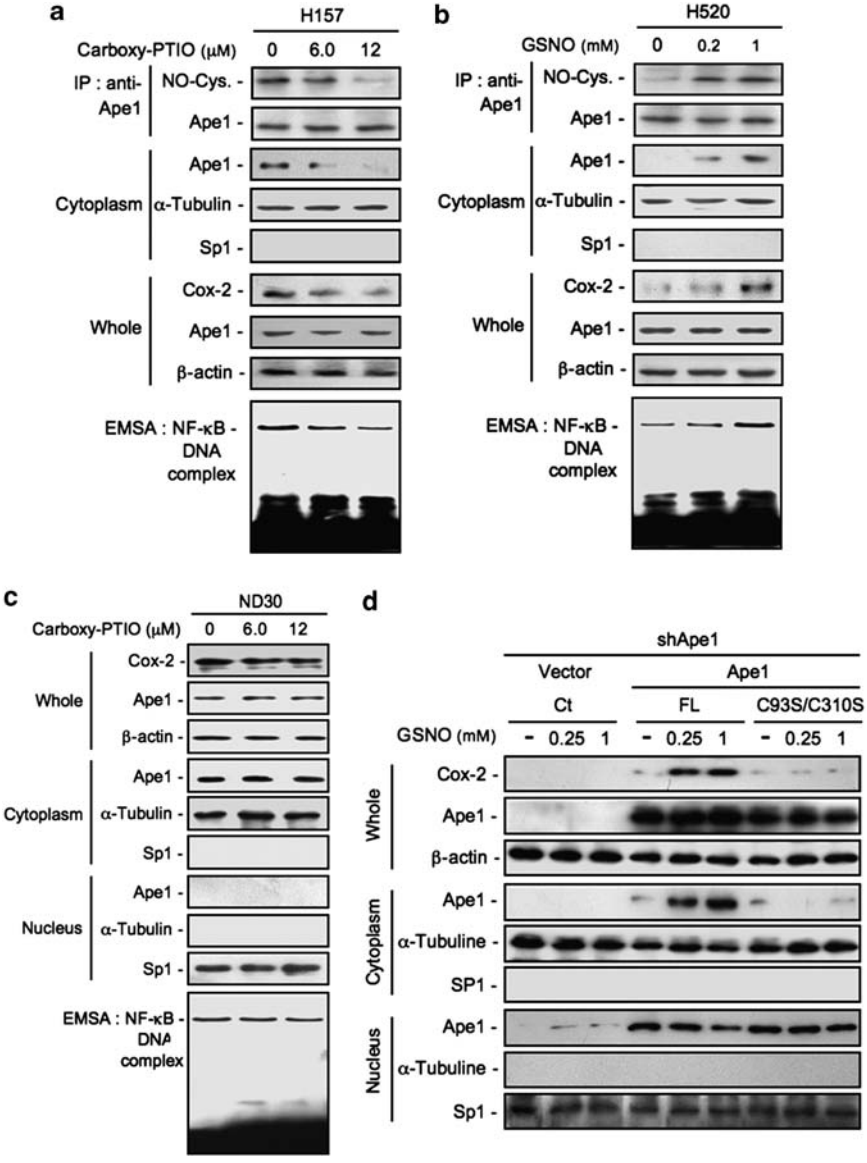

Figure 3 Nitrosation of Ape1 leads to Ape1 nuclear export and Cox-2 induction. The whole-cell lysates and cytoplasmic fractions of (**a**) H157 cells (containing relatively high levels of cytoplasmic Ape1) treated with various concentrations of carboxyl-PTIO, (**b**) H520 cells (containing relatively low levels of cytoplasmic Ape1) treated with various concentrations of GSNO and (**c**) the ND30 stable clone (containing cytoplasmic Ape1) treated with various concentrations of carboxyl-PTIO for 24 h were prepared and immunoprecipitated with anti-Ape1 followed by western blot analysis using anti-nitrocysteine (upper panel). Cox-2, total Ape1 and cytoplasmic Ape1 levels were also evaluated by western blot and β-actin was analyzed as a loading control (middle panels). Whole-cell lysates were also subjected to EMSA analysis to evaluate theDNA-binding capacity of NF-κB (lower panel). (**d**) Western blot analysis of whole-cell lysates, cytoplasmic fractions and nuclear fractions prepared from Ape1-knockdown H157 cells transiently transfected with control, FL or mutant Ape1 (C93S/C310S) vector and subsequently treated with 10 μM GSNO for 24 h was performed using anti-Ape1 and anti-Cox-2. β-Actin, α-tubulin and Sp1 were analyzed as loading controls for whole-cell lysates, cytoplasmic fractions and nuclear fractions, respectively.

FL, shNCor vector control stable clones (Figure 4a). The Boyden-chamber assay indicated that the invasiveness of the ND20 and ND30 stable clones was about 2–4-fold higher than the FL, shNC or vector control stable clones (Figure 4b). Importantly, the substantial invasiveness of the ND30 stable clones (~90% invasive) was suppressed by resveratrol compared with the solvent control; similar suppression was observed using the NF-κB inhibitors sulfathalazine and BAY11-7082, but a lower suppression (~50% invasive) was observed in the presence of the Cox-2 inhibitor NS398 (Figure 4c). These results clearly show that activation of NF-κB by cytoplasmic Ape1 promotes the tumor activity of lung cancer cells *in vitro*.

Cytoplasmic Ape1 promotes tumor growth and lung tumor metastasis in nude mice in vivo

We next examined whether cytoplasmic Ape1 promotes tumor growth and metastasis in an animal model. The

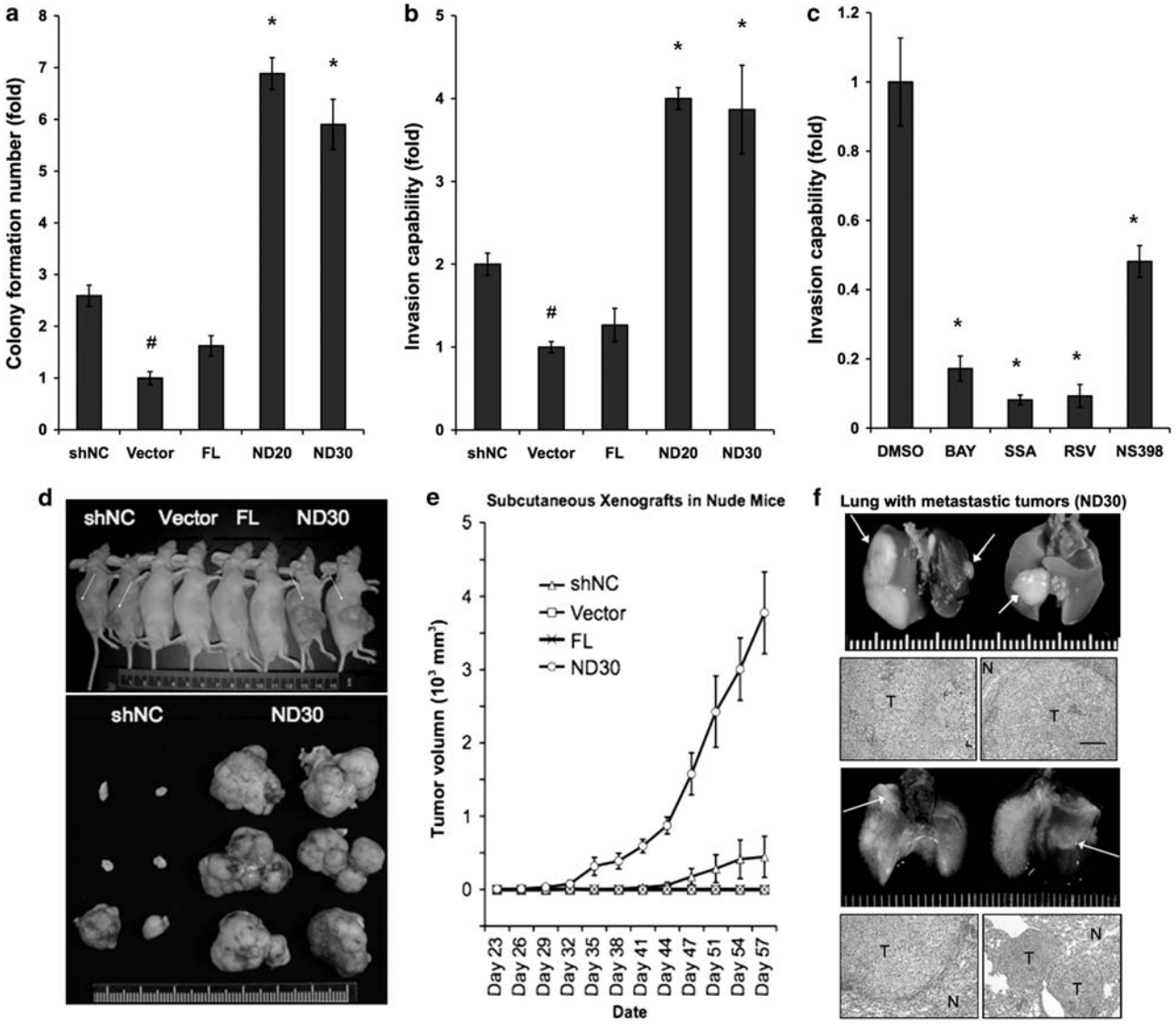

Figure 4 Cytoplasmic Ape1 expressed in ND20 and ND30 stable clones enhances tumor malignancy *in vitro* and *in vivo* compared with FL, shNC and vector control clones lacking cytoplasmic Ape1. (**a**) Anchorage-independent colony formation of ND20 and ND30 stable clones grown in soft agar plates for 11 days was compared with that of FL, shNC and vector control stable clones. $^{\#}P<0.01$ compared with shNC clone. $^{*}P<0.01$ compared with the FL stable clone by Student's *t*-test. (**b**) Invasion capability of ND20 and ND30 stable clones was evaluated by Boyden-chamber assay (24 h) and compared with that of FL, shNCand vector control stable clones. Data represent means ± s.e.m. from three independent experiments. $^{\#}P<0.01$ compared with shNC clone. $^{*}P<0.01$ compared with FL stable clone by Student's *t*-test. (**c**) ND30 stable clones were treated with BAY11-7082 (10 μM), sulfathalazine (SSA) (100 μM), resveratrol (RSV) (10 μM) or NS398 (100 μM) for 1 h, and invasion activity was evaluated by Boyden-chamber assay (24 h). Data represent means ± s.e.m. from three independent experiments. $^{*}P<0.01$ compared with FL clone (Student's *t*-test). (**d**) Tumor burden (as the arrow indicated) in nude mice subcutaneously injected with the ND30 stable clone was compared with those injected with FL, shNC or vector control stable clones 57 days after injection (seven mice in each group). (**e**) Tumor volume of tumors from nude mice subcutaneously injected with ND30, FL, shNC and vector control stable clones was evaluated at 3-day intervals from day 23 to day 57 after injection. Mean ± s.e.m. (1000 mm^3) values were calculated from the tumor burdens of seven nude mice of each group. (**f**) Lung tumor nodules were identified in four of theseven nude mice injected in the tail vein with the ND30 stable clone on day 120 after injection, but no lung tumor nodules were observed in nude mice injected with shNC, vector or FL stable clones. Lung tumor nodule histology was confirmed by H & E staining (scale bar = 2 mm). Abbreviations: N, normal lung tissue; T, lung tumor tissue.

ND30, FL, shNCor vector control stable clones were subcutaneously injected into seven nude mice each (1×10^7 cells/injection). No tumor burden was observed among mice injected with FL and vector control stable clones by day 57 after injection. In contrast, tumor formation was observed in mice injected with ND30 cells by day 23 and the tumor volume increased substantially between days 23 and 57; tumors in shNC-injected mice were observed on day 38 and the volume only slightly increased by day 57 (Figures 4d and e). To verify whether cytoplasmic Ape1 enhances lung tumor metastasis, 10^6 cells from ND30, FL, shNCor vector control stable clones were injected into the tail vein of nude mice (seven mice per clone). All mice were

killed on day 120 after injection. No lung tumor nodules were found in the mice injected with FL or shNC clones, but four of the seven mice injected with ND30 clones had remarkable lung tumor burden; tumors in two of these four mice occupied most of the lung lobes and several tumor nodules were observed in the lungs of the other two mice (Figure 4f). Tumor histology was confirmed by hematoxylin and eosin staining (Figure 4f). The three mice injected with the ND30 clone that had no lung tumor nodules harbored tumors in the vicinity of the tails, indicating unsuccessful tail vein injections. Nevertheless, these results clearly suggest that cytoplasmic Ape1 promotes tumor growth and lung tumor metastasis in nude mice *in vivo*.

Activation of NF-κB enhances the innate immune/inflammatory response and regulates the expression of many genes involved in tumor malignancy (Aggarwal, 2004; Karin, 2006; Maeda and Omata, 2008). Consistent with this, we determined that Cox-2 induction by cytoplasmic Ape1-mediated NF-κB activation enhances lung tumor malignancy. To confirm that NF-κB activation by cytoplasmic Ape1 promotes tumor malignancy, cDNA microarray analysis was performed to compare the gene expression profiles from ND30 stable clones containing cytoplasmic Ape1 with those from FL stable clones containing nuclear Ape1 (data not shown). Expression of NF-κB-regulated genes, such as the inflammation-related genes *IL-1β*, *Cox-2*, *IL-8* and *IL-6*, the anti-apoptosis and cell survival-related gene *Bcl-2*, the invasion-related genes *ICAM1* and *MMP-9*, the angiogenesis-related gene *CXCL1*, and the drug resistance-related gene *Mdr-1*, were twofold higher in ND30 stable clones than in FL stable clones. This supports the hypothesis that NF-κB-regulated gene expression activated by cytoplasmic Ape1 enhances lung tumor malignancy.

Cytoplasmic Ape1 is an indicator of poor prognosis likely because of increased tumor recurrence and/or metastasis in surgically resected lung cancer patients

To verify whether the promotion of tumor growth and lung metastasis by cytoplasmic Ape1 observed in cells and in animal models is also observed in human lung tumors, Ape1 and Cox-2 expression in 100 lung tumors surgically resected from lung cancer patients was evaluated using immunohistochemistry. Of the 100 tumors, 51 (51%) had elevated Ape1 immunostaining; Ape1 protein in tumor cells was detected in the nucleus, in the cytoplasm or in both, with a prevalence of 19, 11 and 38%, respectively (Table 1; Supplementary Figure S7). Interestingly, elevated Cox-2 expression was more common among tumors with cytoplasmic or cytoplasmic and nuclear Ape1 than among those with nuclear Ape1 (Table 1; $P=0.015$). Univariate and multivariate analyses were conducted to verify whether cytoplasmic Ape1 was associated with the clinical outcome of the patients. Patients with tumors containing high cytoplasmic Ape1 levels had a 2.2-fold higher hazard ratio of poor prognosis than those with low cytoplasmic Ape1 levels (Table 2). For 74 of the 100 patients, follow-up information regarding tumor recurrence and/or metastasis was available; 22 patients were diagnosed with recurrent tumors and/or distant metastatic tumors after surgery. Among these twenty-two patients, eight had local tumor recurrence and fourteen had tumor metastasis, the latter of which included five patients with bone metastasis, three with lung to lung metastasis, one with liver metastasis, one with liver and bone metastasis, one with local recurrence and multiple metastasis, one with pleural effusion and multiple mediastinal lymph node metastasis, one with pericardial effusion, and one with pleural effusion. Surprisingly, patients with tumors containing high cytoplasmic Ape1 levels had a 3.722-fold hazard ratio for tumor recurrence and/or metastasis than those with low cytoplasmic Ape1 levels (95% CI; 1.290–10.742, $P=0.015$) (Table 3). Kaplan–Meier analysis indicated that the survival of the 22 patients with tumor recurrence and/or metastasis was lower than the 52 patients without tumor recurrence and/or metastasis ($P=0.004$, Supplementary Figure S8). These results clearly indicate that cytoplasmic Ape1 may increase the risk of tumor recurrence and/or metastasis in post-operative lung cancer patients. Moreover, cytoplasmic Ape1 may be an independent prognostic indicator for lung cancer patients following surgical therapy.

Table 1 Association of Cox-2 expression with Ape1 expression in lung tumors of the cytoplasm, nucleus and cytoplasm/nucleus subcellular localizations

Ape1 localization	*Cox-2 protein*			P-*value*
	Patient no.	*Low* n *(%)*	*High* n *(%)*	
	100	49 (49)	51 (51)	—
Nucleus/cytoplasm				
Low/Low	32	21 (66)	11 (34)	0.015
High/Low	19	12 (63)	7 (37)	—
Low/High	11	3 (27)	8 (73)	—
High/High	38	13 (34)	25 (66)	—
Cytoplasm				
Low	51	33 (65)	18 (35)	0.001
High	49	16 (33)	33 (67)	—

$>50\%$ or $\leqq 50\%$ of tumor cells in lung tumors with Ape1 protein expression were defined to have 'high' or 'low' expression of Ape1. *P*-values were obtained from χ^2 or Fisher's exact test.

Discussion

Reduction of the disulfide bond of NF-κB by the redox activity of Ape1 has been shown to increase the DNA-binding activity of NF-κB (Nishi *et al*., 2002; Ando, *et al*., 2008). However, it is unclear whether nuclear or cytoplasmic Ape1 elicits this effect. In the present study, we identified that the DNA-binding capacity of NF-κB and reductive degree of p50 were consistent with p65 nuclear localization and Cox-2 induction in ND20 or ND30 stable clones containing cytoplasmic Ape1, but not in FL or ND7 stable clones containing nuclear Ape1

Table 2 Univariate and multivariate Cox regression analysis of the influence of cytoplasmic Ape1 on the clinical outcome of lung cancer patients after surgical therapy

Parameter	*Patient no.*	*Univariate*			*Multivariate*		
		HR	*(95% CI)*	P-*value*	HR	*(95% CI)*	P-*value*
Age, years							
<65	50	1	—	—	1	—	—
>65	50	1.323	(0.791–2.212)	0.286	1.453	(0.849–2.487)	0.173
Gender							
Female	36	1	—	—	1	—	—
Male	64	0.924	(0.539–1.585)	0.775	1.333	(0.618–2.878)	0.464
Smoking status							
Nonsmoking	52	1	—	—	1	—	—
Smoking	48	0.878	(0.524–1.470)	0.621	0.891	(0.417–1.905)	0.766
Tumor histology							
AD	48	1	—	—	1	—	—
SQ	52	0.593	(0.353–0.998)	0.049	0.587	(0.317–1.087)	0.090
Tumor stage							
I, II	51	1	—	—	1	—	—
III	49	2.398	(1.405–4.093)	0.001	2.639	(1.528–4.558)	0.001
Cytoplasmic Ape1 level							
Low	51	1	—	—	1	—	—
High	49	2.224	(1.303–3.794)	0.003	2.243	(1.305–3.855)	0.003

Abbreviations: AD, adenocarcinoma; CI, confidence interval; HR, hazard ratio; SQ, squamous cell carcinoma.
Tumors were assigned as having 'high' cytoplasmic Ape1 levels if 50% of tumor cells in tumor paraffin sections of lung tumors contained cytoplasmic Ape1. Tumors were assigned as having 'low' cytoplasmic Ape1 levels if <50% of tumor cells in tumor paraffin sections of lung tumors contained cytoplasmic Ape1.

Table 3 Multivariate analysis of the association between cytoplasmic Ape1 levels on tumor recurrence and/or metastasis of 74 lung cancer patients after surgical therapy

Cytoplasmic Ape1 level (n)	*OR*	*(95% CI)*	P-*value*
Low (40)	1	—	—
High (34)	3.722	(1.290–10.742)	0.015

Abbreviations: Ape1, apurinic endonuclease 1; CI, confidence interval; OR, odds ratio.
Information regarding tumor recurrence and/or metastasis after surgical therapy was available for 74 of 100 lung cancer patients.
Tumors were assigned as having 'high' cytoplasmic Ape1 levels if 50% of tumor cells in tumor paraffin sections of lung tumors contained cytoplasmic Ape1. Tumors were assigned as having 'low' cytoplasmic Ape1 levels if <50% of the tumor cells in tumor paraffin sections of lung tumors contained cytoplasmic Ape1.

(Figure 2). In addition, although similar cytoplasmic Ape1 levels were observed in Ape1-knockdown H157 cells transiently transfected with the ND20, ND30 or ND41 (a mutant lacking a portion of the redox activity domain) vector, p65 nuclear localization and Cox-2 expression were not observed in the cells transfected with ND41 vector (Figure 1). In the ND20 and ND30 stable clones, the DNA-binding capacity of NF-κB and Cox-2 expression were also significantly suppressed by resveratrol (Figure 2g). These results clearly indicate that the redox activity of cytoplasmic Ape1 is critical for Cox-2 induction through NF-κB activation. However, in the cells in which Cox-2 induction is not correlated with cytoplasmic Ape1 levels, such as A549 and H1975, Cox-2 expression was not reduced by adding an equal concentration of resveratrol (Supplementary Figure S6c), and this indicates that resveratrol may specifically inhibit cytoplasmic Ape1-mediated NF-κB activation and Cox-2 induction.

The role of NF-κB in chronic inflammation-driven tumor promotion has been shown in different experimental models (Aggarwal, 2004; Karin, 2006; Maeda and Omata, 2008). NF-κB activity is regulated by its association with the inhibitory IκB proteins, among which IκBα and IκBβ are the most abundant (Wu and Ghosh, 2003). Although both IκB isoforms are phosphorylated by the same IκB kinase complex, their subsequent ubiquitination and degradation are quite different. Rapid and transient signal-induced IκBα proteasome degradation in various cells is required for nuclear import and DNA binding by NF-κB p50/p65 for acute response (Wu and Ghosh, 2003; Maeda and Omata, 2008). However, nuclear NF-κB can drive IκBα expression, generating a negative feedback loopand NF-κB activation in cells can be suppressed in a compensatory manner by IκBβ expression. Moreover, IκBβ has been shown to inhibit anchorage-independent growth and lung tumor metastasis (Thompson *et al*., 1995). Likewise, enhanced IL-1β expression in tumor cells is associated with dramatically reduced IκBβ expression and subsequent persistent activation of NF-κB. It is therefore conceivable that activation of NF-κB consists of two overlapping phases, a transient

phase mediated by IκBα and a persistent phase mediated by IκBβ (Jiang *et al*., 2001). In the present study, degradation of IκBα and IκBβ was observed in the ND20 and ND30 stable cell lines containing cytoplasmic Ape1. Furthermore, when these cells were transfected with IL-1β shRNA, increased IκBβ and decreased Cox-2 expression was observed (Supplementary Figure S9), suggesting that cytoplasmic Ape1 induces IL-1β, resulting in enhanced IκBβ degradation and persistent NF-κB activation.

Elevated Ape1 expression is commonly observed in lung cancer cells and lung tumor tissues. Previous reports have indicated that Ape1 overexpression is induced by oxygen reactive species to remove oxidative DNA damage and to inhibit apoptosis (Angkeow *et al*., 2002; Pines *et al*., 2005). Alteration of p53 by gene mutation or other mechanisms may promote the accumulation of oxygen reactive species through decreased expression of genes encoding antioxidants such as magnesium superoxide dismutase and glutathione peroxidase (Sablina *et al*., 2005). In addition, NF-κB activation has been associated with p53 alteration (Liu *et al*., 2006; Weisz *et al*., 2007; Dey *et al*., 2008; Maeda and Omata, 2008). A recent report showed that p53 binds to the Ape1 promoter and abrogates the transcription activity of Ape1 (Zaky *et al*., 2008). In addition, p53/Mdm2-dependent ubiquitination of Ape1 confers cytoplasmic localization (Busso *et al*., 2009). Therefore, it is expected that p53 mutation would result in Ape1 overexpression through accumulation of oxygen reactive species. Consistent with this, in our preliminary study, cytoplasmic Ape1 was significantly higher in those tumors containing mutated p53 than in those containing wild-type p53 (data not shown). In addition, expression of the human papillomavirus 16/18 E6 oncoprotein has been shown in lung tumors from Taiwanese female cancer patients and was associated with p53 inactivation (Cheng *et al*., 2004). Therefore, we expect that human papillomavirus E6-positive lung tumors may show increased cytoplasmic Ape1 levels through E6-mediated p53 degradation. Indeed, we observed that the prevalence of elevated cytoplasmic Ape1 in human papillomavirus E6-positive tumors was higher than in human papillomavirus E6-negative tumors (data not shown). Therefore, we propose that tumor progression and malignancy in p53-mutated or E6-positive tumors could result, in part, from cytoplasmic Ape1-mediated NF-κB activation.

Elevated levels of Ape1 have been linked to resistance to chemotherapy and poor prognosis (Bapat *et al*., 2008), and inhibition of Ape1 DNA repair activity and/or its redox activity may be a promising avenue to develop novel cancer therapeutics and chemoprevention. Therefore, we speculate that inhibition of Ape1 redox activity may enhance cisplatin sensitivity to kill lung cancer with cytoplasmic Ape1. Our preliminary data support the hypothesis showing that ND30 cells containing cytoplasmic Ape1 had lower cisplatin sensitivity than that of FL cells containing nuclear Ape1 (Supplementary Figure S10a). Interestingly, the cisplatin sensitivity of ND30 cells was markedly increased when the cells were pre-treated with resveratrol (Supplementary Figure S10b).

In summary, we show that NF-κB activation by the redox activity of cytoplasmic Ape1, but not nuclear Ape1, is likely responsible for the oncogenic and invasive properties of lung cancer cells. Data from nude mice models and lung cancer patients showed that cytoplasmic Ape1 is associated with tumor growth and metastasis, and may be indicative of a poor prognosis for lung cancer patients. Therefore, we strongly suggest that the Ape1 redox activity inhibitors such as resveratrol and E3330 (Shimizu *et al*., 2000), combined with cisplatin, may improve chemotherapeutic efficacy for lung cancer patients with tumors containing elevated cytoplasmic Ape1.

Materials and methods

Plasmid DNA

Ape1 cDNA was cloned into pcDNA3.1 Zeo(+) (Invitrogen, Carlsbad, CA, USA) by PCR amplification with newly created *Xho*I and *Eco*RI sites attached on the Ape1 5 ends of forward and reverse primers, and H157 cDNA as template, respectively. Several N-terminal deletions and missense mutations of Ape1 coding sequence were then created by PCR amplification of the Ape1-pcDNA3.1 Zeo(+) template using the oligos described in the Supplementary Information. Site-directed mutagenesis was performed to generate the shApe1-resistant and C93S/C310S mutant Ape1 constructs using the complementary oligos described in the Supplementary Information. All plasmid clones were verified by DNA sequencing.

Mouse models

Eight-week-old female BALB/c nude mice ($n=60$; National Laboratory Center, Taipei, Taiwan) were acclimated for 1–2 weeks. The mice were housed in pathogen-free conditions and fed a diet of animal chow and water throughout the study. Mice were randomized in four groups ($n=7$ each) and injected subcutaneously with 1×10^7 cells from shNC, vector control, FL or ND30 stable clones in phosphate-buffered saline (PBS) or injected through the tail vein with 10^6 cells in PBS. For the mice with subcutaneous xenografts, tumors were measured with a calipers every 3 days, starting on day 23 after injection when tumors became palpable and visible. Tumor volumes were calculated using the equation width $\times$ length$^2\times0.5$. The mice were anesthetized and then killed by overdose with anesthesia on day 59. Subcutaneous tumors were surgically excised and photographed, and a portion of each tumor was placed in 10% formalin for paraffin embedding and a portion was frozen at $-80\,^{\circ}C$ for subsequent analysis. For the mice with tail vein injection, mice were anesthetized and then killed by an overdose with anesthesia on day 120 after injection. All the organs were surgically excised and photographed, and a portion of each tumor was placed in 10% formalin for paraffin embedding and a portion was frozen at $80\,^{\circ}C$ for subsequent analysis.

Lung tumor specimen

One hundred lung tumors, including adenocarcinomas and squamous cell carcinomas, were collected from non-small-cell lung cancer patients admitted in series to the Taichung Veteran's General Hospital, Taiwan between 1993 and 2003. Written informed consent for the use of tumor specimens from

lung cancer patients, as approved by the Institutional Review Board at Taichung Veteran's General Hospital, was obtained from each patient before surgery. Information regarding patient smoking history was collected using a standard written questionnaire. Only those patients who reported never to have smoked were categorized as non-smokers. Tumor types and stages were determined by qualified pathologists according to the 1981 World Health Organization classification. Overall survival was calculated from the day of surgery to the date of death until December 2006. A total of 74 of the 100 cases used in this study were successfully followed up in this experiment. Post-operative follow-up was scheduled at 1 month, 2 months and every 3 months thereafter during the first 2 years after surgery and every 6 months thereafter, or more frequently if needed. Chest X-ray, chest computed tomography scan, carcinoembryonic antigen analysis and other serum analyses were obtained at every follow-up visit. The end of the follow-up period was defined as May 2004. The mean follow-up period for patients was 36.1 months (range, 3.3–68.9 months).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)