ABL Regulation by AXL Promotes Cisplatin Resistance in Esophageal Cancer

Jun Hong, DunFa Peng, Zheng Chen, Vikas Sehdev, and Abbes Belkhiri

Abstract

Esophageal adenocarcinoma (EAC) is characterized by resistance to chemotherapy and poor outcome. Although cisplatin (CDDP) has been used as a first-line therapy in patients with EAC, resistance remains a major clinical problem. The AXL receptor tyrosine kinase, originally isolated as a transforming gene from leukemia, is overexpressed in several solid tumors. Herein, we assessed AXL protein expression in human EACs and examined its role in CDDP resistance in human EAC cells. AXL overexpression was detected in more than 50% of tumors examined. Elevating AXL in nonoverexpressing cells doubled the CDDP IC50 and increased cell survival three-fold, while attenuating AXL in overexpressing cells reduced survival two-fold. The effects of AXL modulation on cell survival were associated with changes in cellular and molecular markers of apoptosis. Mechanistic investigations revealed that AXL blocked CDDP-induced activation of endogenous p73β (TP73), reducing its protein half-life, and inhibited CDDP-induced levels of p-c-ABL(Y412) and p-p73β(Y99). These changes were associated with a disruption of c-ABL/p73β protein interactions due to association with c-ABL in the cytoplasm, thereby blocking nuclear accumulation of c-ABL and phosphorylation of p73β in response to DNA damage. Together, our results establish that AXL promotes CDDP resistance in esophageal adenocarcinoma and argue that therapeutic targeting of AXL may sensitize these cancers to DNA-damaging drugs. Cancer Res; 73(1): 1–10. ©2012 AACR.

Introduction

The incidence of esophageal adenocarcinoma (EAC) has sharply increased by 6-fold in the United States in the last few decades (1, 2). One of the major risk factors for developing EAC is gastroesophageal reflux disease (GERD), which may lead to Barrett’s esophagus, a known premalignant lesion that transforms into invasive cancer by progressing through intermediate stages of low- and high-grade dysplasia (reviewed in ref. 3). Unfortunately, the majority of patients with EAC are mostly diagnosed at an advanced stage of the disease, which may reflect their poor prognosis (4). Generally, the treatment of advanced EAC involves neoadjuvant chemotherapy and radiation followed by esophagectomy (5).

The cancer chemotherapeutic agent cisplatin (CDDP, cis-diamminedichloroplatinum) exerts its cytotoxic effect by inducing DNA damage and causing apoptosis through activation of the tumor-suppressor p53 protein (6). Although deficient p53 expression renders cancer cells less responsive to cisplatin-based chemotherapy, drug resistance is not complete. In fact, the p73 protein, a member of the p53 family, can also induce apoptosis in response to genotoxic stress (7) and cisplatin (6). A previous study showed that cisplatin increased p73 protein levels through activation of the nonreceptor tyrosine kinase c-ABL. Yuan and colleagues (8) showed that c-ABL binds and phosphorylates the p73 protein on a tyrosine residue (Y99) in response to DNA damage, and disruption of the c-ABL/p73 interaction blocked apoptosis. Cisplatin, alone or in combination with other drugs, has been used in the treatment of patients with advanced esophageal cancer, but cisplatin resistance remains a serious clinical challenge (9, 10). In addition to aberrant p53 expression, negative regulation of p73 could potentially play a significant role in promoting cisplatin resistance. This is of major importance given the fact that the majority of EACs are deficient or mutant in p53 (11, 12).

The AXL receptor tyrosine kinase is a member of the TAM subfamily that also includes Tyro3 and Mer, and was originally isolated as a transforming gene from human leukemia cells (13, 14). Elevated AXL expression and interaction with its ligand Gas6 (growth arrest-specific 6) have been associated with cell survival, proliferation, and migration in solid tumors (15–17). These effects are mediated through activation of the MAPK/ERK and PI3K/AKT pathways (reviewed by Linger and colleagues, ref. 18). A recent study identified AXL activation as a novel mechanism of acquired resistance to EGFR inhibitors in non–small cell lung cancer (19, 20). Overexpression of AXL has been reported in various neoplasms, including melanomas (21), lung (22), and breast cancers (23). A previous study indicated that AXL was increasingly upregulated during a multistep...
esophageal carcinogenesis and as an adverse prognostic marker in EAC (24).

The primary aim of this report was to study the role of AXL in mediating CDDP resistance in EAC and identify the molecular mechanism that regulates this effect. We have uncovered that the AXL protein is frequently overexpressed in human EAC primary tumors and cell lines. We also show that AXL suppresses c-ABL/p73 signaling in response to CDDP, thereby blocking apoptosis and promoting drug resistance in EAC. These findings provide evidence that the AXL–c-ABL–p73 axis might be exploited as a therapeutic target to sensitize tumors to DNA-damaging drugs in EAC.

Materials and Methods

Cell lines and reagents

The human esophageal adenocarcinoma cancer cell lines OE33, OE19, SK-GT-4, and FLO-1 were kindly provided by Dr. David Beer (University of Michigan, Ann Arbor, MI). The cells (OE33, OE19, SK-GT-4, FLO-1, and JH-EsoAd1) were kindly provided by Dr. Wael El-Rifai (Vanderbilt University Medical Center). The JH-OE33, OE19, SK-GT-4, and FLO-1 were kindly provided by Dr. Hiroshi Nakagawa (University of Pennsylvania, Philadelphia, PA). HEEC cells were cultured in EpiCM-2 medium (ScienCell) supplemented with 5% FBS. EPC2 cells were grown in keratinocyte serum-free medium supplemented with 40 mg/mL bovine pituitary extract and 1 mg/mL EGF (Invitrogen). The cell lines (HEK-293, EPC2, and HEEC) have not been authenticated. All the cells used in this study were not passaged longer than 6 months after receipt, and were confirmed free of mycoplasma as determined by MycoSEQ Mycoplasma Real-Time PCR Detection Kit (Applied Biosystems). Cisplatin (APP Pharmaceutical) stock solution (3.3 mmol/L) prepared in sterile water was provided by the TVC Outpatient Pharmacy, Vanderbilt University Medical Center, Nashville, TN. Cycloheximide (CHX) was purchased from Sigma-Aldrich. AXL, PUMA, c-ABL, p-c-ABL (Y412), cleaved caspase-3 and -9, cleaved PARP, and β-actin antibodies were obtained from Cell Signaling Technology. The p73 antibody was obtained from Bethyl Laboratories. The Lamin B antibody was purchased from Santa Cruz Biotechnology.

AXL expression and plasmids

The constructs of pcDNA4/AXL-myc-His and pcDNA4 (a gift from Dr. Rosa Marina Melillo, University of Naples, Italy; ref. 25) were used to generate stable expression cells. Briefly, OE33 cells were transfected using Lipofectamine 2000 (Invitrogen). Stably transfected OE33 cells expressing AXL or vector control (empty pcDNA4) were selected with 100 μg/mL zeocin (Invitrogen) according to standard protocols (26).

The AXL coding sequence from pcDNA3.1/AXL plasmid was subcloned into the adenoviral shuttle vector (pACCMV). The recombinant adenovirus-expressing AXL was generated by cotransferring HEK-293 cells with the shuttle and backbone adenoviral (pJM17) plasmids using the Calcium Phosphate Transfection kit (Applied Biological Materials). The pcDNA3/FLAG-p73β and pcDNA3/GFP-c-Ab1 IV (mouse type IV) plasmids were kindly provided by Dr. Alex Zaika (Vanderbilt University Medical Center). The pcDNA3-lacZ plasmid was a gift from Dr. Michael K. Cooper (Vanderbilt University).

Short hairpin RNA

Lentivirus particles expressing control short hairpin (shRNA) or a cocktail of 5 different clones of AXL shRNA were produced and validated by Sigma-Aldrich. FLO-1 cells that express high levels of endogenous AXL were transduced with lentivirus particles and selected with 1 μg/mL puromycin for 10 days.

Immunoblot analysis

Cells were lysed in RIPA buffer (50 mmol/L Tris-HCl buffer, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with 1× Halt protease inhibitor cocktail and 1× Halt phosphatase inhibitor cocktail (Pierce). Proteins were separated and Western blot analysis was carried out as described previously (27).

Immunoprecipitation

Cells were lysed in RIPA buffer supplemented with 1% Halt protease and phosphatase inhibitors (Pierce). The protein concentration was determined by the Bio-Rad Protein Assay. Immunoprecipitations of equal total protein amounts (200 μg) were conducted at room temperature for 1 hour by using a primary antibody previously bound to 50 μL Dynabeads Protein G (Invitrogen). The beads were washed 3 times with ice-cold PBS. The beads in each tube were heated to 100°C for 5 minutes in 30 μL of 2× sample buffer. The proteins were then eluted by magnet and subjected to immunoblot analysis.

Immunohistochemistry

Tissue microarrays (TMA) containing 27 deidentified archival cases of esophageal adenocarcinomas, including 7 esophageal normal epithelial tissue samples, were kindly provided by Dr. Wael El-Rifai (Vanderbilt University Medical Center). Thereafter, 5 μm of TMA sections were used for immunohistochemical (IHC) staining of AXL receptor tyrosine kinase with polyclonal goat AXL antibody (1:200; AF154; R&D Systems). The intensity and frequency of staining were graded as described previously (28).
Cell viability assay
Cells (5 × 10³ per well) were seeded in triplicate onto a 96-well plate. The next day, cells were treated with vehicle or various concentrations of CDDP for 48 hours. Cell viability was determined using the CellTiterr-Glo Luminescent Cell Viability Assay kit (Promega) according to the supplier’s instructions.

Clonogenic survival assay
Cells were plated in triplicate at low density (2 × 10³ per well) in 6-well plates. The next day, cells were treated with vehicle or CDDP for 48 hours. Culture media were replaced and cells were grown for 2 weeks. Cell colonies were then fixed with 2% paraformaldehyde and stained with 0.05% crystal violet. Cell colonies were semiquantitatively analyzed by densitometry using ImageJ software (NIH Image).

Apoptosis analysis
Cells (10⁵ per well) were plated in triplicate in 6-well plates and treated with vehicle or CDDP for 48 hours. Cells were then harvested and stained with Annexin-V fluorescein isothiocyanate (FITC) and propidium iodide (PI; R&D Systems). The samples were subjected to fluorescence-activated cell sorting (FACS) analysis by a flow cytometer (Becton Dickinson). Apoptotic cell death was determined by counting the cells that stained positive for Annexin-V FITC and negative for PI.

CHX-based p73β protein stability assay
OE33 cells were transiently cotransfected with pcDNA3/p73β-Flag in combination with pcDNA4/AXL-myc-His or pcDNA4 vector control using Lipofectamine 2000. The next day, cells were treated with 80 µg/mL of CHX and harvested at different time points. Proteins were analyzed by Western blotting to evaluate p73β protein stability. Intensities of protein bands were semiquantitatively analyzed by densitometry using ImageJ software (NIH Image). The intensities of p73β protein bands for each time point were normalized to their corresponding β-actin. The protein degradation curves were generated by plotting relative band intensities as a function of the time period of CHX treatment. Linear regression was applied and the protein half-life (t₁/₂) of p73β, which is expressed as the time for degradation of 50% of the protein, was calculated from the fitted line equation (29).

Luciferase assay
To investigate the transcriptional activity of p73, we used the PG13-Luc vector that contains 13-tandem repeats of the p53 consensus DNA binding site. In cells harboring nonfunctional p53, activated endogenous p73 protein binds to the p53 binding site; therefore, transcription is induced and the reporter gene is expressed (30). OE33 cells stably expressing AXL or pcDNA4 empty vector and OE19 cells infected with recombinant adenovirus expressing AXL or vector control were seeded in triplicate in 24-well plates (25 × 10³ per well). The next day, cells were transiently cotransfected with 200 ng of the PG13-Luc and 100 ng of pcDNA3-lacZ plasmid, under the control of the cytomegalovirus promoter, using Fugene 6 (Promega) according to the manufacturer’s instructions. The next day, cells were treated with CDDP (10 µmol/L) for 48 hours. The luciferase activity was measured using the luciferase reporter assay kit (Promega) according to the manufacturer’s instructions. The β-galactosidase activity was determined by incubation of cell lysates with the enzyme substrate ONP-β-D-galactopyranoside, and measuring light absorbance at 410 nm. Firefly luciferase activities were normalized to β-galactosidase levels.

Statistical analysis
The results were expressed as the mean with ± SD. The statistical significance of the studies was determined by either the parametric unpaired Student t test or 2-way ANOVA followed by the Bonferroni post hoc test. Differences with 0.05 are considered significant. The difference in AXL protein expression between normal and EAC tissue samples was assessed by the Fisher exact test.

Results
Frequent overexpression of AXL in human esophageal adenocarcinoma
The data from Western blot analysis indicated increased levels of AXL and p-AXL(Y779) proteins in 3/5 EAC cell lines and that 2/2 normal esophageal squamous cell lines were negative for AXL expression (Fig. 1A). We evaluated AXL protein expression in tissue microarrays containing 27 EAC and 27 normal esophageal tissue samples. Representative AXL IHC staining of normal esophageal squamous epithelial tissue sample (NS; left), showing a weak membrane staining and strong cytoplasmic and membrane staining. A strong cytoplasmic and membrane staining.
Table 1. AXL receptor tyrosine kinase is frequently overexpressed in esophageal adenocarcinomas

<table>
<thead>
<tr>
<th>IHC Index Score</th>
<th>#P value</th>
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<tr>
<td>NS 2</td>
<td>0.01</td>
</tr>
<tr>
<td>EAC 8 (29.6%)</td>
<td>0.0015</td>
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Abbreviations: IHC, immunohistochemistry; NS, normal esophageal squamous epithelial tissue; EAC, esophageal adenocarcinoma tissue

The data on the cell viability assay indicated that reconstitution of AXL expression in OE33 cells significantly increased cell survival relative to control cells in response to 48-hour treatment with various concentrations of CDDP (P < 0.05; Fig. 2A). In fact, the CDDP IC50 was 45.8 μmol/L in AXL-expressing cells as opposed to 23.8 μmol/L in control cells (Fig. 2A). To further confirm the role of AXL in regulating cell survival, we subjected FLO-1 cells stably expressing AXL shRNA or control shRNA to the CellTiter cell viability assay after treatment with various concentrations of CDDP for 48 hours. The results clearly indicated that knockdown of endogenous AXL significantly sensitized cells to CDDP (P < 0.001; Fig. 2B). In fact, the CDDP IC50 was 102.2 μmol/L in FLO-1/control shRNA cells, and 34.1 μmol/L in FLO-1/AXL shRNA cells (Fig. 2B).

To confirm the short-term survival assay data, we subjected OE33 cells stably expressing AXL or empty vector, and FLO-1 cells stably expressing AXL shRNA or control shRNA to long-term clonogenic survival assay. The data indicated that the reconstitution of AXL expression in OE33 cells enhanced cell survival by 3-fold relative to control (P < 0.01) in response to CDDP (Fig. 2C). Conversely, knockdown of endogenous AXL in FLO-1 cells decreased cell survival by approximately 2-fold relative to control (P = 0.01) in response to CDDP (Fig. 2D).

AXL suppresses DNA damage-induced apoptosis and activation of caspases

The data on Annexin V/PI staining and FACS analysis indicated that the reconstitution of AXL expression in OE33 cells inhibited early apoptosis events by 25% relative to control in response to CDDP (P = 0.014; Fig. 3A). In line with this result, Western blot analysis indicated significantly higher protein levels of cleaved forms of caspase-9 and -3, and PARP in control cells than AXL-expressing cells after treatment with CDDP (Fig. 3B). In contrast, knockdown of endogenous AXL in FLO-1 cells increased early apoptosis by 73.3% relative to control (P < 0.01) in response to CDDP (Fig. 3C). Accordingly, Western blot analysis results showed that knockdown of endogenous AXL significantly increased protein levels of cleaved forms of caspase-9 and -3 and PARP relative to control in response to CDDP (Fig. 3D).

AXL blocks DNA-damage-induced activation and nuclear accumulation of p73 and decreases p73 protein stability

To examine if AXL expression has an effect on DNA-damage-induced activation of p73, we used OE33 and OE19 cell models. Data from Western blot analysis indicated that the level of endogenous p73β protein was significantly higher in control cells than AXL-expressing cells in response to CDDP (Fig. 4A and 4B). Accordingly, protein expression of p73 downstream transcriptional targets PUMA and HDM2 was significantly induced by CDDP in control cells but not in AXL-expressing cells (Fig. 4A and B). Consistent with these data, qRT-PCR analysis showed that relative mRNA expression of PUMA and HDM2 were 3.5- and 2-fold higher in control cells than AXL-expressing cells, respectively, in response to CDDP (Supplementary Fig. S1). To confirm the role of AXL in regulating p73 transcriptional activity, we conducted the luciferase reporter assay using pG13-luc plasmid. In OE33 cells, luciferase activity was 54.7% higher in control cells than AXL-expressing cells (P = 0.005) after treatment with CDDP (Fig. 4C). Similarly, in OE19 cells, the luciferase activity was 81.1% higher in control cells than AXL-expressing cells (P = 0.01) in response to CDDP (Fig. 4D). Western blot analysis of cytosolic and nuclear protein fractions unequivocally confirmed CDDP-induced accumulation of p73β protein in the nucleus in OE33 control cells (Fig. 4E). Conversely, CDDP treatment had no effect on p73β level or localization in AXL-expressing OE33 cells (Fig. 4E).

On the basis of our finding that AXL suppressed CDDP-induced activation of p73β, we hypothesized that AXL could potentially regulate p73β protein stability. To test this hypothesis, we assessed the protein stability of exogenous p73β transiently expressed in OE33/AXL or OE33/pcDNA4 stable cells by Western blot analysis after treatment with CHX. Indeed, the data on protein degradation indicated that AXL significantly reduced the protein t½ of p73β from 11.4 to 6.5 hours relative to control (Fig. 4F).

AXL attenuates DNA-damage-induced phosphorylation of c-ABL and disrupts c-ABL/p73 protein association

Because the nonreceptor tyrosine kinase c-ABL is the primary regulator of p73 in response to DNA damage, we postulated that AXL could negatively regulate c-ABL, thereby inhibiting...
Figure 2. AXL promotes survival of esophageal adenocarcinoma cells. A, cell viability of OE33 cells stably expressing AXL or empty vector in response to CDDP was assessed by CellTiter-Glo Luminescent Cell Viability Assay. Western blot analysis of AXL in OE33/AXL and OE33/pcDNA4 stable cells is shown (top). Cell survival of AXL-expressing cells was significantly higher than control cells in response to CDDP (bottom). B, cell viability of FLO-1 cells stably expressing AXL shRNA or control shRNA in response to CDDP was determined as in A. Immunoblot of AXL is shown (top). Knockdown of AXL in FLO-1 cells significantly decreased cell viability in response to CDDP (bottom). C, OE33 cells stably expressing AXL or pcDNA4 were subjected to clonogenic survival assay after treatment with vehicle or CDDP (2.5 μmol/L) for 48 hours. Quantitative data (right) showed significantly higher cell survival in AXL-expressing cells than control cells (P < 0.01). D, FLO-1 cells stably expressing AXL shRNA or control shRNA were treated with CDDP (5 μmol/L) for 48 hours and subjected to clonogenic survival assay. Quantitative data (right) indicated that knockdown of endogenous AXL significantly decreased cell survival relative to control in response to CDDP (P = 0.01). Results are representative of at least 3 experiments and shown as the mean ± SD. *, P < 0.05; **, P < 0.01.

p73 activation. Data from Western blot analysis indicated that CDDP treatment significantly increased p-c-ABL(Y412) and p-p73β(Y99) in OE33/pcDNA4 cells. Conversely, AXL expression substantially attenuated CDDP-induced phosphorylation of c-ABL and p73β proteins in OE33/AXL cells (Fig. 5A). Interestingly, the basal levels of c-ABL and p73β proteins were higher in AXL-expressing cells than control cells (Fig. 5A). We next examined if AXL interferes with c-ABL binding to p73β in OE33 cells. Immunoprecipitation and data from Western blot indicated that endogenous c-ABL bound to exogenous p73β protein as expected. However, the reconstitution of AXL expression significantly disrupted the c-ABL/p73β protein complex (Fig. 5B). To confirm that AXL blocked CDDP-induced apoptosis through regulation of c-ABL, we verified whether AXL could directly suppress c-ABL-induced cell death. The data from the cell viability assay showed that transient expression of c-ABL in OE33 cells induced approximately 40% less cell survival relative to control cells (P = 0.02; Fig. 5C). On the other hand, expression of AXL in combination with c-ABL completely restored survival to the level of control cells (Fig. 5C). Data from Western blot analysis confirmed protein expression of c-ABL alone or in combination with AXL in OE33 cells (Fig. 5D).

AXL associates with c-ABL protein and prevents DNA-damage-induced accumulation of c-ABL in the nucleus

On the basis of our results showing that AXL interfered with c-ABL binding to p73 and blocked phosphorylation of c-ABL, we hypothesized that AXL could interact with c-ABL forming a protein complex that prevents accumulation of c-ABL in the nucleus and its interaction with p73 in response to DNA damage. To test this hypothesis, we conducted immunoprecipitation with the AXL antibody followed by Western blot analysis of AXL and c-ABL proteins in OE33 and HEK-293 cells. The data showed protein association of exogenous AXL with endogenous c-ABL in OE33 cells (Fig. 6A), or with exogenous c-ABL in HEK-293 cells (Fig. 6B). We next treated OE33 cells...
stably expressing AXL or pcDNA4 with vehicle or CDDP (10 \( \mu \)mol/L) for 48 hours, and subjected the cytosolic and nuclear protein fractions to Western blot analysis of c-ABL and AXL proteins. The results clearly indicated that cytosolic c-ABL significantly decreased in response to CDDP in control cells, but AXL expression counteracted this effect (Fig. 6C). Overall, the protein level of cytosolic c-ABL was significantly higher in AXL-expressing cells than control cells. Conversely, the nuclear c-ABL protein expression level was higher in control cells than AXL-expressing cells (Fig. 6C). In addition, the data showed that AXL protein expression was limited to the cytosolic fraction (Fig. 6C). Taken together, these results strongly suggest that AXL sequesters c-ABL in the cytosol and prevents targeting of c-ABL to the nucleus in response to DNA damage.

Discussion

Although the DNA-damaging agent CDDP alone or in combination with other drugs has been used as a first-line therapy in patients with advanced esophageal cancer, resistance to CDDP, unfortunately, remains a major clinical problem (9, 10). Identification of the mechanisms that control resistance to CDDP is essential to predicting response to therapy, and to developing new drugs that can overcome resistance. On the basis of the fact that AXL was implicated in promoting cell survival and proliferation through activation of downstream growth and survival pathways in various cancers (15, 18), we evaluated AXL protein expression and examined its role in CDDP resistance in EAC.

In this study, we showed frequent overexpression of the AXL protein in EAC primary tumors (51.8%) and that AXL was exclusively expressed in EAC cell lines, but not in normal esophageal squamous cell lines. Our results clearly indicated that reconstitution of AXL expression enhanced survival and attenuated apoptosis after treatment with CDDP. Conversely, knockdown of endogenous AXL sensitized cells to CDDP. Our findings strongly indicate that AXL promotes resistance to CDDP in EAC cells.
CDDP induces DNA damage causing apoptosis through activation of the tumor suppressor p53 protein (6). However, CDDP can also induce apoptosis in p53-deficient cells through activation of the p73 protein, a member of the p53 family (6). This is particularly of major importance, given the majority of EACs are deficient or mutant in p53 (11). To identify the mechanism by which AXL mediates resistance to CDDP, we hypothesized that AXL could block activation of p73 in response to CDDP in EAC cells. Indeed, our data showed that the reconstitution of AXL expression significantly attenuated CDDP-induced transactivation of endogenous p73β as confirmed by decreased levels of p73β, PUMA, and HDM2 proteins, and PG13-luc luciferase activity. Interestingly, AXL expression increased the basal endogenous p73β protein level, albeit blocking CDDP-induced activation of p73β. This suggests that the reconstitution of AXL oncoprotein expression in our p53-deficient EAC cells could decrease the rate of growth or induce some apoptosis through activation of p73. Consistent with this observation, our results indicated significantly less colony formation (Fig. 2C) and more apoptosis (Fig. 3A) in vehicle-treated AXL-expressing cells than control cells. A previous report showed that endogenous p73α and p73β proteins were upregulated in p53-deficient cancer cells in response to oncogenes (30). Additional studies will be required to fully determine the mechanism by which AXL regulates endogenous p73 basal expression independent of stress signals. As the protein stability of p73 was shown to be enhanced by CDDP (6), we
postulated that AXL could destabilize p73 protein, thereby blocking its activation in response to DNA damage. Indeed, we showed that AXL expression significantly decreased the somewhat stable exogenous p73β protein t1/2 from 11.4 to 6.5 hours.

Several studies indicated that the functional nonreceptor tyrosine kinase, c-ABL, is required for CDDP-induced upregulation of p73 protein stability, because activated c-ABL binds through its SH3 domain to the carboxy-terminal homo-oligomerization domain of p73 and phosphorylates it on a tyrosine residue at position 99 in response to DNA damage (6, 8, 31). We confirmed that CDDP blocked CDDP-induced phosphorylation of c-ABL and p73β proteins, and showed that AXL expression disrupted c-ABL/p73β protein association. Accordingly, our data showed that AXL significantly attenuated c-ABL-induced cell death, strongly suggesting that AXL regulation of p73 is mediated by c-ABL. The c-ABL localization is controlled by 3 nuclear localization signal (NLS) and 1 nuclear export signal (NES) that are responsible for shuttling of c-ABL between the cytoplasm and nucleus (32). It has been shown that nuclear c-ABL relays proapoptotic signals from ATM to p53 and p73 in response to DNA damage (8, 33). Another study indicated that c-ABL increases p73 protein levels in the nucleus in a kinase-dependent manner. Phosphorylation of p73 (Y99) promotes tight interaction with the SH2 domain of c-ABL that may enhance p73 protein stability (34). On the basis of these studies and our findings, we postulated that AXL interacts with cytosolic c-ABL, thereby blocking nuclear accumulation of c-ABL in the apoptotic response to DNA damage. Our results clearly showed protein association between AXL and either exogenous or endogenous c-ABL, although we did not confirm direct binding of AXL to c-ABL.

Furthermore, we showed that AXL blocked nuclear accumulation of c-ABL protein in response to DNA damage, thus disrupting the apoptotic signaling cascade. In a similar study, Raina and colleagues (35) indicated that the MUC1 transmembrane glycoprotein sequesters c-ABL in the cytoplasm and thereby attenuates apoptosis in response to DNA damage mediated by anticancer agents. Interestingly, our data showed that AXL dramatically increased the basal protein expression level of c-ABL in the cytosol independent of DNA damage response. Further studies will be necessary to elucidate the mechanism by which AXL regulates cytosolic c-ABL expression, and investigate its functional implication.

In conclusion, our results indicate that frequent overexpression of AXL in EAC underlies a CDDP resistance phenotype. We show that AXL expression inhibits c-ABL/p73 signaling in response to DNA damage, thus blocking apoptosis and mediating drug resistance in EAC. Therefore, our findings provide evidence that the AXL–c-ABL–p73 axis could be exploited as a therapeutic target to sensitize tumors to DNA-damaging drugs in EAC.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Hong, V. Sehdev, A. Belkhiri
Development of methodology: J. Hong, V. Sehdev, A. Belkhiri
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Hong, D-F. Peng, Z. Chen, V. Sehdev
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Hong, D-F. Peng, Z. Chen
Writing, review, and/or revision of the manuscript: J. Hong, V. Sehdev, A. Belkhiri

References

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Hong
Study supervision: A. Belkhiri

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