Genetics and Molecular Research

Roles of ERα and ERβ in estrogen-induced DDP chemoresistance in non-small cell lung cancer

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ABSTRACT. The role of estrogen in inducing chemoresistance is not yet fully understood. The objective of this study was to observe the relationship between estrogen levels and cellular response to chemotherapeutic drugs in non-small cell lung cancer (NSCLC) and to reveal the potential mechanisms involved. Cell viability was analyzed after pre-treating NSCLC cells with different levels of estrogen (E2), followed by treatment with an anti-tumor drug for 48 h. The roles of various estrogen receptors (ERs) were examined *in vitro* by blocking the activity of each ER individually. The ER pathway was further confirmed in NSCLC tissues. It was found that 10-1000 nM E2 resulted

in a decreased cellular response to DDP in H1650 cells compared to the use of cisplatin alone ($P < 0.05$). However, this result was not demonstrated in H1299 cells, which lack p53. Both ERα and ERβ were associated with E2-induced cisplatin chemoresistance, though they had opposite functions. p53 expression did not correlate with the expression of ERα or ERβ individually. However, a statistically significant correlation between p53 expression and ERα to ERβ mRNA ratio was observed ($P < 0.001$, $R = -0.676$). These findings suggest that E2-induced DDP chemoresistance depends on the balance between ERα and ERβ expression and the p53 pathway.

Key words: Estrogen; Estrogen receptors; p53; NSCLC; Chemoresistance

INTRODUCTION

Lung cancer has been a common disease worldwide among the male population for many years. In addition to its prevalence in men, this disease has become the most common cause of cancer death in women in the United States, 2013, accounting for 71,660 deaths; 26% of the total cancer-attributable deaths (Siegel et al., 2015). Increasing evidence has shown that compared to men, women have higher adenocarcinoma prevalence (Jemal et al., 2007), decreased DNA repair capacity (DRC) (Wei et al., 2000), and an increased frequency of K-ras and EGFR mutations (Ahrendt et al., 2001; Ben Aissa and March, 2012). Despite these odds, it has been shown that women have a superior response to therapy than men. Moreover, women are more likely to receive molecular targeted therapy compared to men (Graham et al., 2013). Some studies have shown that being female acts as a possible protective factor for non-small cell lung cancer (NSCLC) survival (Nakamura et al., 2011; Scaglia et al., 2013), while one study (Minami et al., 2000) suggested that this effect is only confined to older, post-menopausal women. Women over the age of 60 showed a distinct survival advantage relative to men and younger women.

The differences between lung cancer characteristics in men and women may partly be attributed to personality traits, attitudes, behaviors, and social role. However, the gender-related difference in circulating estrogen level may be a key factor that influences the response to therapy. Estrogens are steroid compounds that function as the primary female sex hormones. Although estrogens are present in both men and women, levels are significantly higher in women of reproductive age. There are three estrogen hormones that occur naturally in women, among which 17 β-estradiol (E2), produced by the ovaries, is the most potent and has the highest affinity for estrogen receptors (Ruggiero and Likis, 2002; Ascenzi et al., 2006).

Estrogens exert their biological effect through two classical estrogen receptor (ER) subtypes, ERα and ERβ, as well as the novel estrogen receptor G-protein coupled receptor 30 (GPR30) (Prossnitz et al., 2008). Previous studies demonstrated the roles of ERs in lung cancer. For example, it was observed that lung cancer expresses both ERα and ERβ, and blocking both receptors led to the arrest of tumor growth, suggesting that these receptors promote cell proliferation (Kawai et al., 2005a). However, the roles of ERα and ERβ in tumor survival are complicated. ERα-positive and ERβ-negative status is associated with poor prognosis in lung cancer patients. Moreover, high ERβ expression levels may be positively correlated with overall survival and have prognostic value for NSCLC progression (Kawai et

al., 2005b; Liu et al., 2013). Another study found that ERα is elevated in tumors but is not predictive of survival (Stabile et al., 2011). Additionally, studies on the relationship between ER expression and chemotherapy sensitivity are scarce.

This study hypothesized that estrogen levels and ERs are associated with chemotherapeutic response in NSCLC patients. The NSCLC cell lines NCI-H1650 and NCI-H1299 were chosen for our analysis. Cells were pre-treated with E2 at varying concentrations, followed by treatment with cisplatin (DDP) or gemcitabine at their respective IC_{50} concentrations. Results were verified by examining the expression of ERα, ERβ, and p53 in patient tissues.

MATERIAL AND METHODS

Ethics statement

This study was approved by the local ethical committee of the Xi'an Jiao tong University Institutional Review Board for clinical research. All subjects were fully informed of the nature of the study, and all gave their written consent.

Cell lines

NCI-H1299 (p53-null) and NCI- H1650 (wide type p53) NSCLC cell lines were purchased from ATCC (Manassas, VA, USA). Cells were maintained in RPMI 1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS) (ATCC) and 100 U/mL penicillin-streptomycin (GIBCO, USA). All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. For all experiments involving ER ligands, cells were washed with sterile PBS and cultured in serum-free medium for 24 h prior to the addition of estrogens.

Patients

Lung tumor tissues and the corresponding tumor-free lung tissues from 24 patients with NSCLC were collected during primary debulking surgery at a general hospital in China from January 2011 to December 2013. The patients selected met the following criteria: a) presented with primary NSCLC; b) previously untreated; c) offered complete patient health history; and d) surgery as the first treatment. All tissues were obtained according to the tumor removal protocol for lung cancer patients from the Xi'an Jiao tong University. Tissues were immediately frozen in liquid nitrogen and stored at -80°C.

Compounds

The selective ERα antagonist (MPP dihydrochloride), ERβ receptor antagonist (PHTPP), and GPR30 receptor antagonist (G15) used in this study were purchased from Tocris Bioscience (Ellisville, MO, USA). E2, DDP, and gemcitabine were purchased from Sigma (St. Louis, MO, USA). The ligands were prepared as 0.1 M stocks in DMSO or 100% ethanol and stored at -20°C. Prior to use, ligands were further diluted into cell culture medium for treatment groups.

Treatment protocol and cell viability analysis

Cells were starved in serum-free medium for 24 h before E2 treatment. ER activities were blocked by pretreating with the appropriate selective ER antagonist for 1 h before the addition of E2. Treatment with chemotherapeutic drugs was conducted 1 h after E2 exposure. The effect of chemotherapy agents on cell growth was determined by MTT assay. MTT assay kits were purchased from ATCC, and assays were performed according to the manufacturer's protocol. Percent cell viability was calculated using a standard curve and was normalized to untreated control cells.

Quantitative real-time PCR

RNA was isolated from tissues using an RNA isolation kit (QIAGEN, Valencia, CA, USA). RNA (1 μg) was reverse transcribed into cDNA with an Omniscript RT kit (QIAGEN, Germany) according to the manufacturer's protocol. Gene expression was quantified using a real-time quantitative RT-PCR machine (BIO RAD iQ5 Optical Module), and primers (IDT, USA) for ERs and other genes were designed to detect two exons (Table 1). The 18S gene was also measured as an endogenous housekeeping control gene. Reaction mixtures contained 1X SYBR Green PCR Supermix (Bio-rad, USA), 0.1 μL each primer, and 1 μL cDNA template in a total volume of 25 μL. The final primer concentration is 100 nM, and the final concentration of cDNA is 50 ng in each reaction mixture. Thermal cycling conditions included activation at 95°C for 10 min, followed by 40 cycles each of denaturation at 95°C (15 s) and annealing at 60°C (1 min).

Table 1. Sequences of the primers used in PCR analysis.

RNAs	Primer sequences
Human estrogen receptor a mRNA	5'-GGAGACGGACCAAAGCCACT-3'
	5'-TTCCCAACAGAAGACAGAAGATG-3'
Human estrogen receptor β mRNA	5'-CACGTCAGGCATGCGAGTAAC-3'
	5'-ACCCCGTGATGGAGGACTTG-3'
Human GPR30 mRNA	5'-ACGAGACTGTGAAATCCGCAACCA-3'
	5'-ATCAGGCTGGAGGTGCACTTGGAA-3'
Human p53 mRNA	5'-TGACTGTACCACCATCCACTA-3'
	5'-AAACACGCACCTCAAAGC-3'
18s rRNA	5'-GGGAGGTAGTGACGAAAAATAACAAT-3'
	5'-CCCTCCAATGGATCCTCGTTAAAGGA-3'

Statistical analysis

All values were expressed as the means ± standard deviation (SD). Statistically significant differences were calculated by one way ANOVA or independent t-test analysis. Gene expression correlations were evaluated by a chi-square test. Two-tailed $P < 0.05$ was considered to be statistically significant.

RESULTS

Cell type-specific relationship between E2 concentration and DDP chemoresistance

To determine if E2 modulates the response of NSCLC cells to chemotherapy, the

relative viabilities of H1650 (wide type p53) and H1299 (p53-null) cells were analyzed after exposure to different concentrations of E2 in combination with chemotherapeutic drugs (DDP or gemcitabine) at their respective IC_{50} concentrations (Table 2) for 48 h. Compared to untreated controls, E2 alone did not affect proliferation of H1299 or H1650 cells between 0.1 and 1,000 nM. The response of H1650 cells to DDP was altered upon exposure to E2. Pretreatment with E2 at higher concentrations, between 10 to 1,000 nM, followed by DDP at its IC_{50}, resulted in greater cell viability compared to treatment with DDP alone ($P < 0.05$) (Figure 1A). Compared to gemcitabine treatment alone, no difference in cell viability was observed when gemcitabine was combined with E2. In H1299 cells, which lack p53, neither DDP nor gemcitabine affected cell viability when combined with E2, compared to when using an antitumor drug alone (Figure 1B). These results suggest that higher concentrations of E2 induce DDP-based chemoresistance in a cell specific manner.

Table 2. Relative cell viability caused by anti-tumor drugs (%, means ± SD).

Cells	Treatment	E2 concentration (nM)					
		0	0.1	1	10	100	1000
H1650	E2	100 ± 0.13	89.65 ± 0.07	97.09 ± 0.24	87.4 3± 0.08	87.85 ± 0.10	100 ± 0.20
	E2 + DDP	49.73 ± 0.04	46.41 ± 0.08	53.93 ± 0.00	72.17 ± 0.06*	91.31 ± 0.17*	100 ± 0.03*
	E2 + Gemcitabine	46.76 ± 0.03	44.80 ± 0.02	53.51 ± 0.06	51.41 ± 0.04	50.26 ± 0.02	58.55 ± 0.05
H1299	E2	100 ± 0.37	103 ± 0.12	104 ± 0.33	91.85 ± 0.20	92.41 ± 0.11	102 ± 0.22
	E2+DDP	46.51 ± 0.07	68.26 ± 0.05	53.67 ± 0.02	56.97 ± 0.08	59.65 ± 0.05	63.80 ± 0.02
	E2 + Gemcitabine	42.08 ± 0.02	56.48 ± 0.11	43.73 ± 0.02	58.31 ± 0.05	47.33 ± 0.05	44.95 ± 0.45

Anti-tumor drug treatment at their IC_{50} concentrations. H1650 cells: DDP, 5 μM; Gemcitabine, 0.05 μM; H1299 cells: DDP, 36 μM; Gemcitabine, 0.5 μM. *$P < 0.05$, compared with E2 Concentration at 0 nM group.

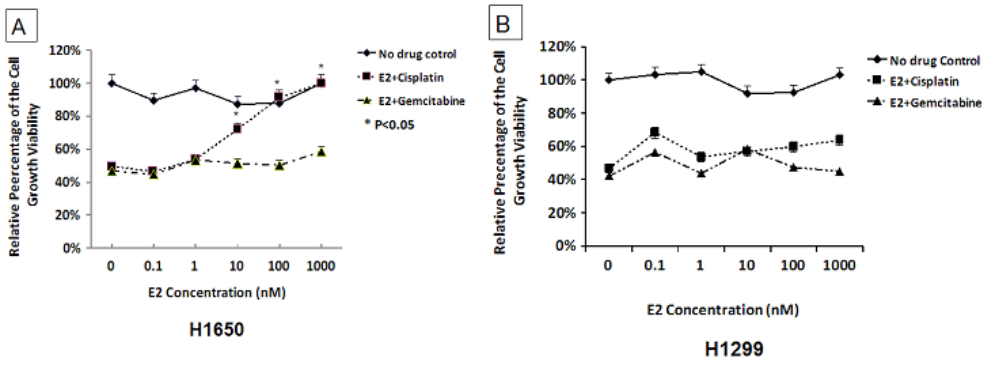

Figure 1. Viability of H1650 (**A**) cells and H1299 (**B**) cells treated with the indicated compounds for 48 h.

Relationship between ERα/ERβ expression and E2-induced DDP chemoresistance in NSCLC cells

To further evaluate whether ERs were involved in DDP chemoresistance, E2 and selective ER antagonists were utilized. Cells treated with both 10 nM E2 and DDP showed significantly higher cell viability than cells treated with DDP alone ($P < 0.05$). Blocking the

activity of all three receptors by combining their selective antagonists (MPP, PHTPP, and G15) before E2 treatment abolished this effect. Blocking ERα and GPR30 with 1 μM of MPP and G15, respectively, for 1 h before E2 treatment resulted in decreased cell viability compared to cells treated with E2 and DDP without ER antagonism ($P < 0.05$), which was even lower when DDP was used alone ($P < 0.05$). In contrast, inhibiting ERβ and GPR30 with 1 μM their respective antagonists did not significantly change viability when compared to the E2 and DDP combination treatment group (Figure 2). Moreover, blocking both ERα and ERβ with MPP and PHTPP, respectively, before E2 treatment resulted in no difference in cell viability compared with cells where all three receptors were blocked. This result suggests that ERα and ERβ are associated with DDP chemoresistance induced by E2, and ERα and ERβ have opposing effects in terms of regulating E2-induced DDP chemoresistance. GPR30 had no effect in this process. The presence of ERα and the absence of ERβ activity were found to be associated with E2-induced DDP chemoresistance.

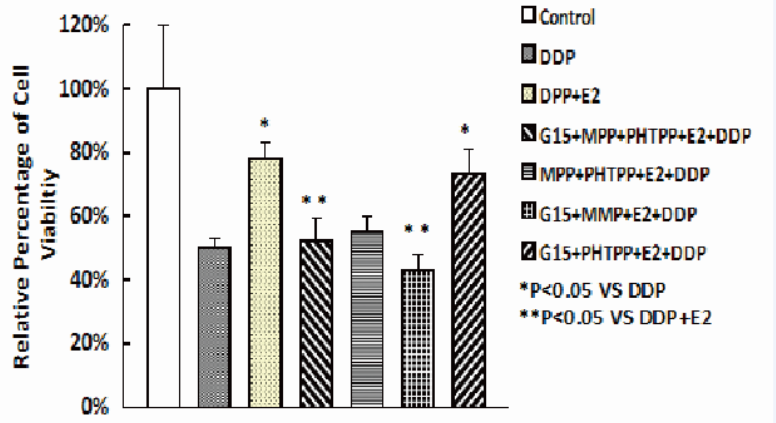

Figure 2. Role of ERs in E2-based DDP-chemoresistance. Cell viability is shown upon treatment with the indicated compounds.

Dependence of E2-induced DDP-based chemoresistance on p53 and the relationship with ERα/ERβ ratio

The expression of ERα and ERβ mRNA in NSCLC and their corresponding tumor-free lung tissues was analyzed. NSCLC tissues were compared to normal tissues from the same patient. Tumor tissues overexpress both ERα and ERβ mRNA compared to normal tissues (Figure 3). The expression of p53 was examined in all tissues to further explore the relationship between ERs and p53. The results showed that p53 mRNA expression levels in tumor tissue had no relationship with the expression of either ERα or ERβ. However, a statistically significant negative correlation between p53 expression and the ratio of ERα to ERβ mRNA was observed ($P < 0.001$, Pearson's $R = -0.676$) (Table 3). When the ratio of ERα to ERβ mRNA expression was greater than 1, the expression of p53 in tumor tissues was lower than the tumor-free lung tissues; when the ratio of ERα to ERβ mRNA expression was less than 1, the expression of p53 was elevated in tumor tissues compared to normal tissues. This result suggests that although the expression of p53 may not be correlated with ERα or ERβ individually, its expression is correlated with the balance between ERα and ERβ.

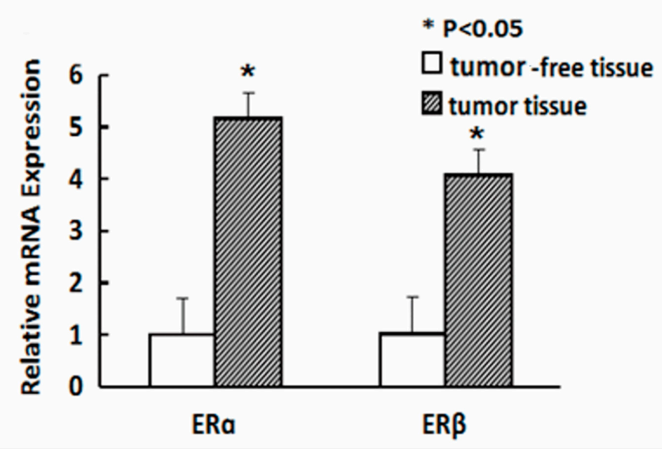

Figure 3. Comparison of ER mRNA levels between tumor-free tissues and NSCLC tumor tissues.

Table 3. Correlation between p53 expression and the ERα:ERβ ratio.

p53 expression	Ratio of ERα to ERβ expression		P value	Pearson's R**
	Above 1	Below 1		
High	N = 3	N = 11		
Low	N = 9	N = 1	0.000	-0.676

**Correlation is statistically significant at 0.01 level.

DISCUSSION

E2, produced by the ovaries, is the most abundant estrogen hormone and is the most potent due to its high affinity for ERs. In pre-menopausal women, circulating E2 levels fluctuate from 40 to 200-400 pg/mL (0.2 to 0.73-1.47 nM) during the menstrual cycle. After menopause, E2 levels drop to less than 20 pg/mL (0.07 nM) (Ascenzi et al., 2006). Estrogen plays an important role in the regulation of normal physiological processes as well as in tumor development. Female hormones may induce the proliferation and growth of cells or render poor survival and prognosis of patients with breast and gynecologic cancers (Anonymous, 2001; Fernandez et al., 2003; Mourits and De Bock, 2006; Willey and Cocilovo, 2007). On the other hand, E2 inhibits the growth of tumor cells in digestive cancers, which was confirmed by an epidemiological study that showed that the risk of developing digestive cancers is generally decreased in patients receiving estrogen-replacement therapy (ERT) (al-Azzawi and Wahab, 2002; Slattery et al., 2003). By recognizing gender-related differences in lung cancer development, estrogen has been investigated as a factor in lung carcinogenesis.

Recently, however, the effect of E2 in women has become highly controversial. Adami et al. (1989) showed an increased risk (RR, 1.26) of lung cancer in women receiving HRT. Taioloi and Wynder (1994) also showed that the use of HRT and its interaction with smoking leads to an increased risk of lung cancer in women, and early menopause was associated with a decreased risk. However, other data that showed an association between HRT and reduced risk of lung cancer in current smokers (OR, 0.59; 95%CI, 0.38 to 0.92) indicated that HRT may actually exert a protective effect (Mourits and De Bock, 2006). Moore et al. (2003)

analyzed the influence of menopausal status lung cancer outcomes. The study showed that premenopausal women tended to have more advanced disease than postmenopausal women, though premenopausal women still had fewer lung cancer-related deaths than older men. It appears that higher circulating levels of estrogen couple with lung cancer susceptibility and poor prognosis.

Past studies and the results from the current work suggest that normal, physiological plasma E2 concentrations do not affect patient responses to DDP. However, patients with higher E2 concentrations than normal, which may be found in patients with early age at menarche, late age at menopause (Bernstein et al., 1991; Moore et al., 1991; Clavel-Chapelon and E3N group, 2002), or who use exogenous hormones, may have an altered response to chemotherapy.

In this study, high (10-1000 nM) levels of E2 induced DDP chemoresistance in in H1650 cells. This phenomenon was not observed at lower E2 concentrations (0.1-1 nM). The study then focused on the role of ERs in the cellular response to chemotherapy. Two ER subtypes affected DDP response: ERα and ERβ. Through selective ER antagonism, we found that E2-induced DDP chemoresistance was associated with both ERα and ERβ. GPR30 was not involved in this process, as no difference in cell viability was observed whether it was active or blocked. The presence of ERα and the absence of ERβ promoted E2-induced chemoresistance, while the presence of ERβ and the absence of ERα made cells more sensitive to DDP. These results are partly consistent with the report that the presence of ERα expression and the absence of ERβ were associated with poor cancer patient survival (Kawai et al., 2005b).

The other main question addressed in this study was whether ER expression patterns influence E2-induced chemoresistance through the p53 pathway. Although a significant interaction was found between E2 concentration and response to antitumor drugs in H1650 cells with wildtype p53, E2 treatment did not affect the response to chemotherapy in H1299 cells that lack p53. This suggests that p53 can mediate cellular responses to DDP-based chemotherapy; which is supported by studies that found that active p53 in DDP-treated cells may contribute to the cellular response (Fojta et al., 2003). Our data indicate that E2-induced DDP chemoresistance acts in an ER-dependent manner and is determined by the expression pattern of both ERα and ERβ. Further work was conducted to reveal the relationship between ERs and p53 expression in NSCLC patient tissues. In agreement with the *in vitro* findings, mRNA levels of both ERα and ERβ were elevated in tumor tissue compared to tumor-free tissues. Neither ERα nor ERβ independently demonstrated a relationship with p53 mRNA concentration. However, the ratio of ERα to ERβ was found to be significantly correlated with p53 mRNA concentration. ERα predominance in in NSCLC tissues often correlated with a low level of p53 mRNA, while ERβ predominance often correlated with a high level of p53 mRNA. This result was consistent with the data produced *in vitro*, and it can also be explained by the proposed ER-mediated pathway (Figure 4).

Patient response to DDP-based chemotherapy was dependent on serum estrogen level as well as the balance between expression of ER subtypes. Thus, the cellular response was determined by the balance between chemoresistance mediated by ERα expression and chemosensitivity mediated by ERβ expression. p53 was especially intriguing, as it was correlated with the ratio of ERα and ERβ, apoptosis, and DNA repair pathways.

One major limitation of the present study was the absence of evidence showing the causal relationship between ER expression levels and p53 expression levels. Therefore, it

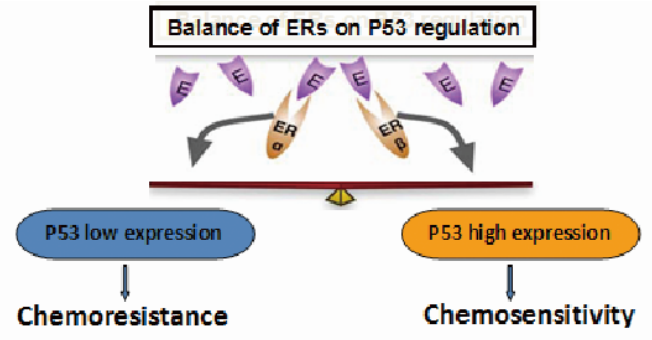

Figure 4. Proposed ER-mediated pathway of p53 regulation in DDP chemotherapy.

cannot be concluded that overexpression of p53 leads to a higher ratio of ERα/ERβ or vice versa. In addition, a more detailed demonstration of the relationship between ER levels and p53 in chemoresistance was not carried out. This analysis was not performed because of the relatively small sample size. A more detailed study on the correlation between p53 and ER expression during chemoresistance should be performed.

In conclusion, we showed that elevated levels of circulating estrogens are associated with a decreased response to DDP in a p53-dependent manner. Furthermore, the presence of ERα and the absence of ERβ resulted in DDP chemoresistance, and this phenomenon was regulated by p53, indicating that ERs act as mediators of chemoresistance. In the future, it may be possible to prescreen lung cancer patients for serum E2 level and ER expression to select those that may benefit from anti-estrogen or ERα-blocking agent therapy in addition to traditional chemotherapeutic agents.

Conflicts of interest

The authors declare no conflict of interest.

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