The Role of Estrogen Receptors in Proliferation of Non-Small Cell Lung Cancer

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Abstract

To explore the expression pattern of ERs in NSCLC tissues and assess their relationship with tumor histopathological variable. In our study, Ers expression was examined using Real-time PCR with specimens of 28 NSCLC patients. It was showed that both ERα and ERβ were over expression in NSCLC tissues, and also the mRNA concentration of both ERα and ERβ were significantly higher in primary tumor T2 stage than in T1 stage and higher in squamous carcinoma than in adenocarcinoma. However, the activation of ERα and ERβ were completely different. To further explore the role of Ers in development and progression of NSCLC, we used Ers selective siRNA or antagonist in vitro experiments. The results showed that ERβ but not ERα can mediate E2 induced cell growth, since siRNA targeting ERβ but not ERα gene can induce cell cycle arrest at G1 phase by down regulation of cyclinD1 expression, and also cell cycle regulators p21WAF1 and p53 were involved in this signaling pathway.

Keywords: Estrogen receptors; Non-small cell lung cancer; siRNA; P53; Proliferation

Introduction

Estrogens and its receptors are key signaling molecules that regulate various physiological processes, such as cell growth, development, and differentiation [1,2]. Ers are also involved in the development of many types of malignant tumors, including breast and gynecologic cancers [3-6], endocrine gland cancers [7-15], digestive cancers [16-18] and lung cancer [19-22]. Estrogens exert their biological effect through two classical estrogen receptors(Ers) subtypes, ERα and ERβ, and novel estrogen receptor G protein coupled receptor 30 (GPR30) [23].

Lung cancer, especially Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality for both men and women worldwide. Estrogen signaling pathways may play an important role in development of lung cancer. The cellular response to estrogen is mainly mediated by ERα and ERβ. There was increasing evidence to show that NSCLC express both ERα and ERβ in nucleus as well as extra-nuclear sites [19]. It was observed that ERβ but not ERα is the dominant form in normal lung cancer cell line and tissues, and loss of ERβ leading to abnormal lung structure and systemic hypoxia [24,25]. Moreover, when expression of either ERα or ERβ was suppressed by small interfering RNA (siRNA), the proliferation of NSCLC cells were significantly reduced [20], and also, blockage of Ers by Ers antagonist ICI 182780 reduced [20], and also, blockage of Ers by Ers antagonist ICI 182780 in vitro experiments. The results showed that ERβ but not ERα can mediate E2 induced cell growth, since siRNA targeting ERβ but not ERα gene can induce cell cycle arrest at G1 phase by down regulation of cyclinD1 expression, and also cell cycle regulators p21WAF1 and p53 were involved in this signaling pathway.

Materials and Methods

Patients

Lung cancer tissues and normal tissues from 28 patients with NSCLC were collected during primary debulking surgery from January 2009 to January 2011. The patients were selected according to the following criteria: (a) primary none small cell lung cancer; (b) previously untreated; (c) complete patient characteristics information; and (d) surgery as the first treatment.

The patient information was recorded as follows: clinical diagnosis, pathological stage, histologic subtype and histologic grade. The tissues were classified into two groups; one is lung tumor tissues group; and the other is corresponding tumor-free lung tissues from the same patient. All the tissues were obtained according to protocol of lung cancer patients during surgery to remove the tumors. Tissues were frozen immediately in liquid nitrogen and kept at -80°C. Patient characteristics are summarized in Table 1.

Cell culture and transfection

NSCLC cell line, H1650 and A549 (ATCC, USA), were cultured in

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RPIMI medium 1640 (ATCC, USA) supplemented with 10% fetal calf serum (ATCC, USA) at 37°C under 5% humidified CO₂ and 100 μg/ml each of streptomycin and penicillin G (GIBCO, USA). The anti-ERα, anti-ERβ and anti-GPR30 siRNA (Santa cruz, USA) were transfected by Lipofectamine™ RNAiMAX (Invitrogen, USA) according to the manufacturer’s instructions. Briefly, approximately 2 × 10⁶ cells/well were grown overnight in 24-wells plate. When the cells reached 80% confluence, they were transfected with anti-ERα siRNA (30 nM), anti-ERβ siRNA (30 nM) or anti-GPR30 siRNA (30 nM) separately in anti-body free medium using lipofectamine™ RNAiMAX. After incubation for 48 h at 37°C 200 µl RPMI 1640 with 10% FBS was added and then subjected to real-time RT-PCR and western blot analysis.

RNA extraction and reverse transcription

Lung tumor tissue (N=28) and the corresponding tumor-free tissue (N=28) were homogenized and total RNA extraction from tissues was performed using RNAeasy Mini Kit (Qiagen, Valencia, CA), RNA quality was checked with Spectrophotometer (NanoDrop 1000). Almost all samples had high-quality RNA. 1 μg RNA was reverse transcribed into cDNA with Omniscript RT kit (QIAGEN, Germany) according to the manufacturer`s protocol.

Quantitative real-time PCR

Primers (IDT, USA) for estrogen receptors and other genes were designed to locate into two exons, Sequences of the primers used in the real-Time PCR analysis were shown in Table 2. The 18s gene was chosen as an endogenous house-keeping control gene. Quantitative real-time PCR was performed using Cyler IQ Real-time PCR Detection System (BIO RAD iQ5 Optical Module. Reaction mixtures contained 1 × SYBR Green PCR supermix (Bio-rad, USA), 0.1 µl Primer, 1 µl cDNA template in total volume of 25 µl. Thermal cycling conditions included activation at 95°C (10 min) followed by 40 cycles each of denaturation at 95°C (15 sec) and annealing at 60°C (1 min).

Each reaction was performed in triplicate and negative controls were included in each experiment. To compare the expression levels among different samples, the relative mRNA levels were calculated using the comparative delta CT (ΔCt) method.

Western blot analysis

After transfection, cells were lysed in lysis buffer. Equivalent amounts of protein lysates from each sample were separated by electrophoresis through pre-cast 10% Tris-HCl polyacrylamide gel and transferred to PVDF membranes. Nonspecific binding site were block by incubation in 1X PBS. Primary antibodies (Santa cruz, USA) were incubated with the membranes and recognized with secondary antibodies (Santa cruz, USA). ECL-Plus Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA) were used to visualize the complexes.

Cell viability analysis

Cells were seeded in phenol red-free RPMI 1640 medium supplemented with 10% 4X dextran-coated, charcoal-treated FBS (SFS) in 24-well plates or 96-well plates. After overnight attachment, the cell culture medium was removed. The adherent cell was washed twice with sterile PBS, and the cells were then treated with the indicated ligands for a total of 48 h. Selective siRNA or antagonist treatment were 1 h before E2. The effect of treatment on cell growth was determined by MTT assay. The MTT assay kits were purchased from ATCC, and assays were according to the manufacturer’s protocol. The percentage of cell viability was calculated using a standard curve and normalized to non-treated control.

Flow cytometric analysis

Cells were cultured overnight in RPMI-1640 medium and then treated with siRNA of ERα and ERβ for 48 h. The treated cells were fixed and stained with propidium iodide (Sigma USA). At least 1 × 10⁶ stained cells were analyzed using FACSAria (BD Biosciences USA).

Statistical analysis

All data shown as bar graphs are expressed as the mean ± SE. The statistical significance of differences was calculated by one-way ANOVA and two-tailed t test analysis. The correlation between gene expressions was evaluated by chi-square test. Two-tailed P<0.05 was considered to be statistically significant.

Results

Both ERα and ERβ mRNA were over-expression in NSCLC tissues

We examined the expression of ERs in all lung tumor tissues and the corresponding tumor-free lung tissues from same patient. Both the ERα and ERβ mRNA transcript expression levels were over-expression in NSCLC tissues Table 3. However, GPR30 mRNA transcript showed

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**Table 1:** Patient characteristics.

| Characteristic | N=28 |
|---------------|------|
| **Age**       | 63   |
| **Range**     | 43-71|
| **Histologic grade** |      |
| G1 Well differentiated (Low grade) | N=2 |
| G2 Moderately differentiated (intermediated grade) | N=14 |
| G3 Poorly differentiated (High grade) | N=12 |
| **Histologic subtype** |      |
| Adenocarcinoma | N=16 |
| Squamous cell lung carcinoma | N=10 |
| Large Cell Carcinoma | N=1 |
| Adeno-squamous cell carcinoma | N=1 |

**Table 2:** Sequences of the primers used in the real-Time PCR analysis.

| RNAs                      | Primer sequences                        |
|---------------------------|-----------------------------------------|
| Human estrogen receptor α | 5’-GGAGACGCGAACAAAGCCACCT-3’ |
| mRNA                      | 5’-TCCCTCCAATGGATCCTCGTTAAAGGA-3’ |
| Human estrogen receptor β | 5’-CACGTCCAGGGATCCGGAGTAAC-3’ |
| mRNA                      | 5’-ACCCCGTGATGGAGGACTTG-3’ |
| Human GPR30 mRNA          | 5’-ACGAGACTGTGAAATCCCGCAACCA-3’ |
| Human cyclin A2 mRNA      | 5’-CCTGCAAACTGCAAGAATGG-3’ |
| Human cyclin D1 mRNA      | 5’-CCCTCGGTGTCCTCTACTCTCAA-3’ |
| Human P53 mRNA            | 5’-TGAAGTGGACATCCACTCCACCTA-3’ |
| Human P21 mRNA            | 5’-TGGACCTGTACCTGTTCTGTG-3’ |
| 18s rRNA                  | 5’-GGGAGGTAGTGACGAGAAAAATACAAT-3’ |
|                          | 5’-CCCTCAATGGATCCTCGTTAAAGGA-3’ |

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ERβ but not ERα mediates estrogen-dependent growth of NSCLC

To evaluate the role of ERα and ERβ in non-small cell lung cancer, we used receptor selective siRNA to down regulate the ERα, ERβ and GPR30 expression separately in H1650 cell lines, as well as A549 cell lines. and then Real-time RT-PCR and western blotting were performed to determine ERα, ERβ and GPR30 expression levels in transfected NSCLC cell lines (Figure 1). When H1650 cell line were transfected with anti-ERα siRNA, anti-ERβ siRNA or anti-GPR30 siRNA, the mRNA concentration (Figure 1) and proteins level (Figure 2) were down-regulated. The results obtained from A549 cells were same.

To determine whether the expression of ERα or ERβ affect the NSCLC cells proliferation, Cell viability was detected using MTT method. The cell growth in 10 nM E2 treatment group had no significant difference from that in control group. However, blocking of estrogen receptors before E2 treatment significantly decreased the proliferation of cells compared with that in E2-treatment group. Furthermore, the role of ERβ was detected by blocking both ERα and GPR30 expression by selective siRNA but left ERβ expression, and the result shown that the cell growth was increased compared with all ERs blockage group. However, no difference was found in ERβ and GPR30 blockage but ERα expression group compared with all ERs blockage group (Figure 3A). It indicated that up-expression and down-expression of ERβ but not ERα can accelerate and inhibit the cellular proliferation.

To further confirm that ERβ but not ERα is involved in NSCLC cells proliferation, we performed cell proliferation assays with ERα-, ERβ- and GPR30 selective antagonist. Combined with G15 and MPP, but not PHTPP can stimulate the growth of H1650 cells, while combined with PHTPP and G15 shows no difference (Figure 3B)

Inhibition of ERβ induced a G1-phase cell-cycle arrest

Flow cytometric analysis (Figures 4A-4D) showed a significant increase in the number of cells in G0/G1 phase after treatment of H1650 with anti-ERβ siRNA for 48 hr, as compared with that in control cells. It indicated that the induction of a cell cycle arrest at G1 phase was induced by ERβ siRNA in H1650 cells. Further, the role of ERα was further determined by transfection with ERα siRNA, and the result showed no significantly difference in G0/G1 cell population compared with control group.

Table 3: ERα and ERβ mRNA concentration in patients with NSCLC.

| Variables                  | ERα mRNA          | ERβ mRNA          |
|----------------------------|-------------------|-------------------|
| Normal (n=28)              | 1.01 ± 0.03       | 1.02 ± 0.03       |
| Cancer (n=28)              | 5.17 ± 0.07       | 4.08 ± 0.05       |
| P value                    | 0.004*            | 0.002*            |

**Pathological stage**

| Stage                | ERα mRNA | ERβ mRNA |
|----------------------|----------|----------|
| T1, N0, M0 (n=11)    | 1.93 ± 0.23 | 1.62 ± 0.12 |
| T2, N0, M0 (n=17)    | 6.02 ± 0.50  | 5.07 ± 0.52  |
| P value              | 0.009*     | 0.019*     |

**Histologic subtype**

| Subtype          | ERα mRNA | ERβ mRNA |
|------------------|----------|----------|
| Adenocarcinoma (n=16) | 4.09 ± 0.52 | 2.62 ± 0.62 |
| Squamous (n=10)  | 7.64 ± 0.97 | 5.82 ± 0.26 |
| P value          | 0.03*     | 0.05*     |

* Difference is significantly at 0.05 level

Figure 1: mRNA Expression of ERs are reduced by anti-ER siRNA Transfection: As determined by real-time PCR, mRNA expression of ERα, ERβ and GPR30 are reduced by anti-ERs siRNA transfection. * P<0.05 vs control.

Figure 2: Protein expression of ERs were reduced by anti-ER siRNA Transfection: Transfection with siRNA specially reduced ERs protein expression compared with control.

Figure 3A: Inhibition of ERβ induced a G1-phase cell-cycle arrest

Flow cytometric analysis (Figures 4A-4D) and western blotting showed a significant increase in the number of cells in G0/G1 phase after treatment of H1650 with anti-ERβ siRNA for 48 hr, as compared with that in control cells. It indicated that the induction of a cell cycle arrest at G1 phase was induced by ERβ siRNA in H1650 cells. Further, the role of ERα was further determined by transfection with ERα siRNA, and the result showed no significant difference in G0/G1 cell population compared with control group.
Figure 3: ERβ is Associated with Cell Growth induced by Estrogen: A: The inhibitory of ERs by siRNA before E2 treatment decrease the cell growth significantly (*p<0.05 vs control). Pre-treatment with ERα siRNA and GPR30 siRNA transfection followed by 10 nM E2 increase the growth of cells significantly compared with E2+GPR30 siRNA+ERα siRNA+ERβ siRNA (**P<0.05 vs E2+GPR30 siRNA+ERα siRNA+ERβ siRNA). B: Also, treatment cells with ERs selective antagonist (1 µM MPP, PHTPP and G15) can significantly reduce cell growth (*p<0.05 vs control), and inhibitory of ERα and GPR30 by MPP and G15 can increase the cell relative viability (**p<0.05 vs E2 +G15+MPP+PHTPP). So it shows that E2 induced H1650 cell proliferation through ERβ-mediated signaling.

Figure 4: Cell cycle distribution of H1650 Transfected with ERα or ERβ for 48 h: inhibitory of ERβ expression by siRNA induced cell cycle arrest at G1 phase, while the cell cycle distribution of cells transfected with ERα siRNA showed no difference compared with control, * p<0.05 VS control.
To determine whether the anti-ERβ siRNA treated H1650 were arrested at the G0/G1 phase, we examined changes in the expression of panel cell-cycle-specific markers after 48 h. The mRNA concentration as well as the protein level of cyclin-D1 which functions in the transition from G1 to S phase of the cell cycle decreased (Figures 5A and 5B). The mRNA concentration and the protein level of cyclin-A2, which is involved in transition from S to G2, did not change significantly (Figure 5C and 5D). Collectively, these data support the notion that inhibition of ERβ induces cell-cycle arrest at the G0/G1 phase in H1650 cells, which is consistent with flow cytometric analysis. The results obtained from A549 also shown the changes of cyclinD1 and cyclinA2 when the expression of ERβ was blocked (Figure 5B).

**P53 and P21 expression was involved in cell growth inhibition through ERβ-mediated signaling**

Inhibition of ERβ expression by anti-ERβ siRNA markedly increased P53 expression at both mRNA level and protein level in H1650 cells as well as A549 cells. The overexpression was completely induced by anti-ERβ siRNA but not by a scramble siRNA control (Figures 6A-6D). Furthermore, the cyclin-dependent kinase cyclin inhibitors gene, P21 mRNA concentration were also upregulated by inhibition of ERβ siRNA. These finding establish that inhibition of ERβ induced upregulation of P53 and P21 as a direct mediator of the growth-inhibitory response.

**Discussion**

It is well established that ERs play a major role in pathological processes of lung cancer, while the results remain inconsistent. Early studies detected for the presence of the classical ERα only, and then with the development of ERβ antibody, the expression of ERβ was also found in lung cancer [28-30]. Many studied have shown the expression of ERβ in majority of human NSCLC cell lines as well as primary...
The complexes reported. A549 cells transfected with ERβ have increased proliferation in response to estrogen [33]. Our results showed that inhibitory effect of ERβ but not ERα affects NSCLC cells proliferation. MTT method was used in this study for cell viability. The results showed that inhibitory of ERβ but not ERα affects NSCLC cells proliferation. MTT method was used in this study for cell viability. The results showed that inhibitory of ERβ but not ERα affects NSCLC cell line A549 transfected with ERβ has increased proliferation in response to estrogen [33].

The progression of cells proliferation is associated with cell cycle, which is known to be positively regulated by a series of cyclin-dependent kinases (CDKs), as well as cyclins, and is negatively controlled by specific CDK inhibitors (CDKIs) [34-36]. CDKIs are separate into two classes, including this, the CIP/KIP family ((p21Waf1/Cip1, p27Kip1, and p57Kip2) which inhibits all CDKs by directly binding CDK complexes [37]. Wild-type P53 regulates cell transition from G1 to S phase in cell cycle and induce G1 arrest in response to DNA damage [38]. The p21Waf1/Cip1 gene, which is transcriptional target of wild-type P53 [39,40], is activated by wild-type P53 and is downstream effector for P53 function by inducing G1 arrest if cells are exposed to DNA damaging agents [41]. We found that inhibitory of ERβ but not ERα by selective siRNA can lead to upregulation of P53 and P21, which in turn downregulated cyclinD1, which is G1-checkpoint regulator [42], and led to cell-cycle arrest at the G1 phase in H1650 cells. These findings provide evidence for P21-mediated ERβ-induced cell cycle arrest at the G1 phase in H1650 cells.

One major limitation of the present study was small tissue samples. Therefore, it cannot be test the level of ERs protein in both lung cancer tissues as well as tumor free tissues. A further limitation was that a more detailed demonstration of the relationship between the level of GPR30 and lung cancer proliferation was not carried out. This was not completed because of the relatively low expression of GPR30 in lung cancer tissue. A further study with more detail on the correlation between GPR30 and lung cancer should be performed.

Conclusion

In conclusion, we showed that both ERα and ERβ play important role in development of NSCLC. ERβ was involved in proliferation of NSCLC induced by estrogen separately. The discovery of the expression pattern of ERs may open up an opportunity for development of estrogen-based therapy.

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