

RESEARCH COMMUNICATION

An Upstream Estrogen Response Element Linked to Exogenous p53 Tumor Suppressor Gene Expression Differentiates Effects of the Codon 72 Polymorphism

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Abstract

The objective of this study was to assess the effects of an upstream estrogen response element (ERE) on exogenous p53 tumor suppressor gene with a codon 72 polymorphism about which there have been controversial reports in relation to cancer risk. The p53 gene (bases 166–1143 from start codon) with the codon 72 polymorphism, inserted into the pIRES-hrGFP II plasmid with or without upstream ERE, were transfected into HHUA endometrial cancer cells expressing the estrogen receptor. The ERE-linked p53 gene with the proline variant at codon 72 showed lower transfection rates than the gene without ERE or with the arginine variant at codon 72. p21 expression was significantly higher in HHUA cells transfected with the proline variant gene than in those transfected with the arginine variant gene. We consider that the presence of an upstream ERE promotes the transcriptional effects of the exogenous p53 gene with the proline variant, which strengthens the expression of p21, and results in lower transfection rates through cell cycle inhibition.

Keywords: P53 - estrogen response element - codon 72 polymorphism - p21

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Introduction

The proline-rich region (residues 64–93) of the p53 tumor suppressor plays a role in apoptosis and inhibits MDM2-mediated p53 degradation (Baptiste et al., 2002; Lui et al., 2003). Polymorphism at codon 72 of p53 (proline or arginine) correspond, respectively, to the nucleotide sequences CCC or CGC at bases 214–216 from the start codon. Storey et al. suggested that women with the homozygous CGC variant were seven times more susceptible to cervical cancer in comparison to CCC homozygotes (Storey et al., 1998).

Human papillomavirus (HPV) has been the focus of intense study because of its role in causing cervical cancer; the viral E6 protein of HPV binds to and inactivates cellular p53, and the arginine variant of p53 is more susceptible to degradation by the E6 protein (Thomas et al., 1999). Furthermore, a statistical study showed that the arginine variant of p53 was more common in patients with squamous cell cancer of the oropharynx than in controls (Perrone et al., 2007).

Although some reports have controversially shown that patients with the arginine variant have a better prognosis

for lung cancer, we speculate that their prognosis may depend on cell diversity under particular hormonal conditions. *In vitro* studies have shown the intracellular relationship between estrogen receptor (ER) and p53 for transcriptional control of target genes (Menendez et al., 2007). In this paper, we show the effect of the codon 72 polymorphism and the possible effect of an upstream estrogen response element (ERE) (Klinge et al., 2001), -GGTCAXXXTGACC-, linked to the exogenous p53 gene on the induction of p21, which suppresses cyclin-dependent kinase and induces cellular senescence.

Materials and Methods

Human cDNAs were obtained by RT-PCR of total RNA from chorionic tissue, which was acquired from an artificially aborted decidual sac at 11 weeks of gestation under informed consent. RNA was extracted with an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and the cDNA of the p53 gene (bases 166-1143) was amplified with forward (GAAGACCCAGGTCCAGAT) and reverse (TTTATGGCGGGAGGT) primers using PfuUltra high fidelity DNA polymerase (Agilent Technologies, Santa

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Clara, CA, USA). The PCR product was separated by electrophoresis on 1.2% Seakem GTG agarose (Lonza, Rockland, ME, USA) and extracted with Quiaex (Qiagen). A forward primer to link an EcoR1 site, the ERE, and the start codon at the 5' terminal (GAATTC GGT CATAGT GACC AT AT G G A A G A C C C A G G T C C A G A T) and a reverse primer (A G T G T G A T G G A T A T C T G C A G A A T T C T T T A T G G C G G G A G G T) were used for further amplification to produce ERE-linked p53. A forward primer to link an EcoR1 site and the start codon at the 5' terminal (AGGAATTCATGGAAGACCCAGGTCCAGAT) and a reverse primer (CAGAATTCTTTATGGCGGGAGGT) were used for amplification to produce ERE-unlinked p53. These PCR products were purified by agarose gel electrophoresis, ligated into the pGEM-T easy vector (Promega, Madison, WI, USA), and transfected into Escherichia coli DH5 α cells. The amplified plasmids were digested with EcoRI and ligated into pIRES-hrGFP II (Agilent Technologies, Santa Clara, CA, USA). For the polymorphic variant at codon 72 of p53, the corresponding arginine variant (CGC) in the cDNA from chorionic tissue was changed to the proline variant (CCC) with the primers GCCAGAGGCTGCTCCCC and CGTGCAAGTCACAGACTT using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies).

Cell cultures and transfection

HHUA cells, a human endometrial cancer cell line established by Ishiwata et al. that expresses ER β (Ishiwata et al., 1984; Zhi et al., 2007), were supplied by Riken Laboratories (Wako, Japan) and cultured in F-12/minimal essential medium (1:1) (Invitrogen, Carlsbad, CA, USA) containing 10⁻⁹ M estradiol supplemented with 15% charcoal-stripped fetal bovine serum (HyClone Laboratories, Logan, UT, USA) at 37°C in a humidified atmosphere with 5% CO₂. The pIRES-hrGFP II plasmids containing the ERE-unlinked p53 gene (p53R or p53P, corresponding to the arginine or proline variant at codon 72, respectively) or the ERE-linked p53 gene (EREp53R or EREp53P, also corresponding to the arginine or proline variant at codon 72, respectively) (Figure 1)

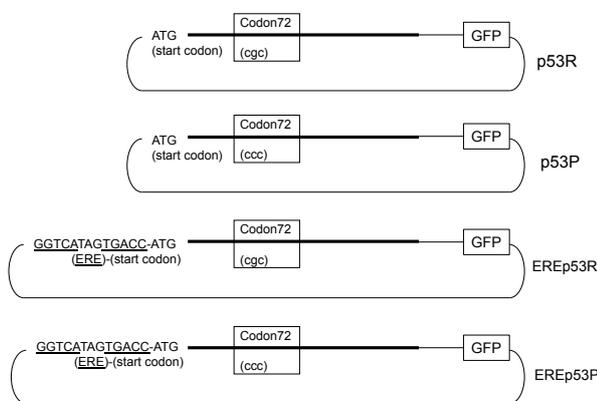


Figure 1. Plasmid Gene Structure of pIRES-hrGFP II Containing the p53 Gene. Codon 72, arginine or proline, corresponds to CGC or CCC in the p53 gene, respectively. ERE is either linked or not linked to the 5'-side of the start codon and inserted into the pIRES-hrGFP II gene

(4 μ g each) were transfected with the polycationic lipid Optifect Reagent (Invitrogen) into proliferating HHUA cells at about 50% confluence in plastic 12-well culture plates containing Opti-MEM Reduced Serum Medium (Invitrogen). Five hours later, the medium was exchanged with fresh F-12/minimal essential medium (1:1) containing 10⁻⁹ M estradiol supplemented with 15% charcoal-stripped fetal bovine serum, and the HHUA cells were then cultured for 48 h or 168 h.

Flow cytometry

The HHUA cells were dispersed by incubation at 37°C with 0.25% trypsin in 0.1 mM EDTA and analyzed by a FACSCalibur flow cytometer equipped with an argon laser (Becton Dickinson, Franklin Lakes, NJ, USA). Cells transfected with p53R, p53P, EREp53R, or EREp53P were sorted for the presence of green fluorescent protein (GFP).

RT-PCR assay

RNA was extracted from the GFP-positive HHUA cells using an RNeasy Mini Kit (Qiagen). For each sorted sample, cDNA was transcribed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) and real-time PCR was carried out using the 7500 Fast Sequence Detection System (Applied Biosystems). The relative levels of p21 mRNA (compared with 18S ribosomal RNA) were analyzed with TaqMan MGB probes and primers for human p21 (Hs00355782_m1) using the multiplex threshold cycle method.

Statistical analysis

The GFP-positive cell rate and relative levels of p21 mRNA were analyzed with nonparametric methods using PASW Statistics 18 software (SPSS, Chicago, IL, USA), and the result of p<0.05 between the two groups by the Mann-Whitney U test was judged significant.

Results

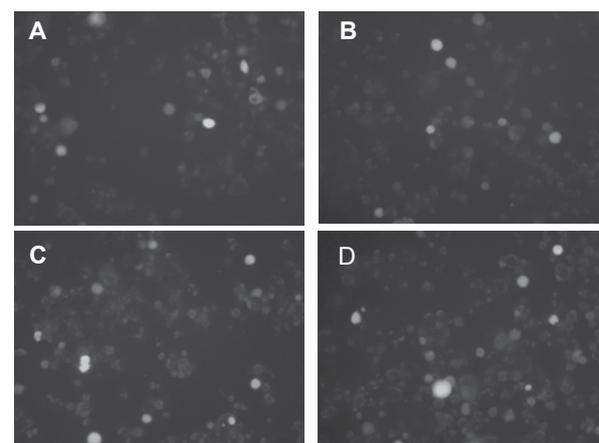


Figure 2. Fluorescent Micrographs of HHUA Cells Transfected with the p53 Gene inserted pIRES-hrGFP II. GFP-producing recombinant plasmid; p53R (A), p53P (B), EREp53R (C), or EREp53P (D) was transfected in HHUA cells and cultured for 7 days before being photographed with a 488-nm emission wave and a 530/40 bandpass filter on an inverted fluorescent microscope (IX71; Olympus, Tokyo, Japan)

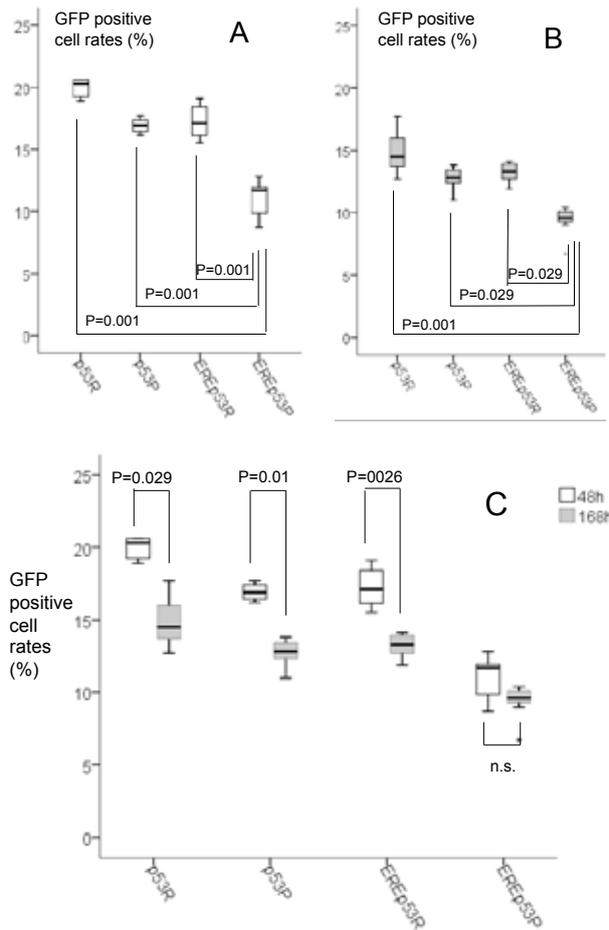


Figure 3. GFP-positive Rates in HHUA Cells Transfected with the p53 Gene Inserted pIRES-hrGFP II. HHUA cells at 48 h (A) or at 168 h (B) after transfection with p53R, p53P, EREp53R, or EREp53P were analyzed for GFP presence by flow cytometry, and differences of the GFP-positive cell rates in HHUA cells between at 48 h and at 168 h were shown (C). The data from 7 wells per condition were analyzed by non-parametric methods

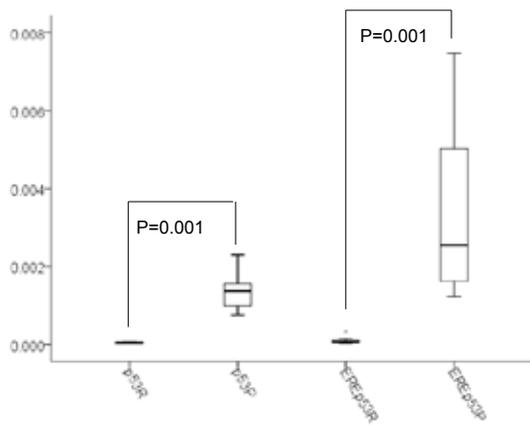


Figure 4. Relative mRNA Expression of p21 in GFP-positive HHUA cells Transfected with the p53 Gene Inserted pIRES-hrGFP II. RNAs were extracted from HHUA cells at 168 h after transfection with p53R, p53P, EREp53R, or EREp53P, and assayed for p21mRNA by real-time PCR. The data from 7 wells per condition were analyzed by non-parametric methods

Fluorescence microscopy

On the second day after transfection with the p53 genes inserted pIRES-hrGFP II, presence of GFP fluorescence was observed in about 10–20% of HHUA cells, and the cell shapes appeared to be normal. On the seventh day after transfection, the number of GFP-positive cells appeared to be slightly decreased as compared to those on the second day, and some GFP-positive cells tended to be larger in the wells transfected with EREp53P (Figure 2D).

Flow cytometry

At 48 h after transfection, the GFP-positive rate in HHUA cells transfected with EREp53P was significantly lower than in cells transfected with the other plasmids ($p < 0.001$; Fig. 3A). At 168 h after transfection, the GFP-positive rates of HHUA cells transfected with p53R, p53P and EREp53R were significantly lower than those at 48 h (Fig. 3C), but they were still higher than cells transfected with EREp53P (Fig. 3B). However, the GFP-positive rates of EREp53P did not change between at 48 and at 168 h after transfection (Fig. 3C). We speculated that the transfection efficiency with polycationic lipid was reduced because of the inhibition against cell cycle by EREp53P.

RT-PCR assay for p21 mRNA

The expression of p21 mRNA was significantly higher in HHUA cells transfected with p53P than in those transfected with p53R ($p = 0.001$; Fig. 4), and also significantly higher in HHUA cells transfected with EREp53P than in those transfected with EREp53R ($p = 0.001$; Fig. 4). There was no significant difference in p21 mRNA expression between HHUA cells transfected with EREp53R and those transfected with p53R. The median value of p21 mRNA expression in HHUA cells transfected with EREp53P was higher than in those transfected with p53P, but not significantly so.

Discussion

The relationship between the codon 72 polymorphism of p53 and cancer risk is controversial. The ability of p53 to bind to the transcriptional machinery, activate transcription and induce apoptosis differs according to the state of the codon 72 variant; the induction of transcription was stronger for the proline variant, while the induction of apoptosis was faster for the arginine variant (Thomas et al., 1999). In an epidemiological study of cervical neoplasms, the majority of which are associated with long-term HPV infection, homozygotes for the arginine variant were significantly enriched in HPV 16-positive cervical cancer patients compared with HPV 16-positive pre-cancer or control subjects (Zehbe et al., 1999). Transfection of the p53 gene inhibits the replication of HPV with lowered expression levels of E1 or E2 (Lepik et al., 1998), suggesting that progression to neoplasm formation may be caused by the reduced activity of p53. p21, an inhibitor of cyclin-dependent kinase, is one of the major transcriptional targets of p53. Although intracellular metabolism continues under p21 expression, most tumor cell senescence in cancer therapy is accompanied by p21 induction (Roninson et al., 2003). However, as transfection

efficiency with a polycationic lipid depends on cell proliferation (Brunner, S. et al., 2000), transfection of the p53 gene may cause cellular senescence and attenuate transfection efficiency. The relatively lower GFP-positive cell rates in HHUA cells transfected with EREp53P are considered to reflect the stronger effects of EREp53P, which induced p21 mRNA more, thereby inhibiting the cell cycle more, as compared with the HHUA cells transfected with the plasmids.

Synergistic interactions between some target sequences of p53 and ER have been demonstrated in some human cancer cell lines, suggesting transcriptional cooperation due to their cognate response elements (Menendez et al., 2010). In our experiments, the interaction of ER with an ERE located upstream from the p53 gene may activate the transcription of the p53 gene. Even if ERE-linked p53P could increase p21 mRNA levels as compared to ERE-linked p53R in HHUA cells, the inhibition of the cell cycle, as a result of the increased p21 levels, limited the polycationic lipid-dependent gene transfection rate.

In conclusion, placing an ERE upstream of an exogenous p53 gene with proline variant at codon 72 differentiates the stronger inhibition on the cell cycle than that with arginine variant, reducing the transfection efficiency.

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