Estrogen response element enhances adenovirus-mediated transfer of the p53 gene according to codon 72 polymorphisms and cellular estrogen receptor expression

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Abstract

Background: Relations between response systems for estrogen receptors and p53 with codon 72 polymorphism in cancer cell lines are suggested through experiments for adenovirus-mediated p53 gene delivery.

Methods: Recombinant adenoviruses containing an upstream estrogen response element (ERE)-linked p53 gene (bases 166–1143 from the start codon) were prepared for the infection of HHUA endometrial cancer cells, KLE endometrial cancer cells, or SW48 colon cancer cells, and transduction of p53 gene was measured by real time PCR.

Results: The transduction of the ERE-linked p53 gene with a proline variant at codon 72 in HHUA cells or in SW48 cells, which express estrogen receptor β , was over ten-fold greater than the transduction of the unlinked p53 gene. The transduction of the ERE-linked p53 gene with an arginine variant at codon 72 in KLE cells, which do not express estrogen receptor, was over ten-fold greater than the transduction of the unlinked p53 gene.

Conclusion: ERE-linked p53 gene may be efficiently transduced to cancer cells according to codon 72 polymorphisms and cellular estrogen receptor expression.

Keywords: Adenovirus vector, codon 72 polymorphism, estrogen receptor, estrogen response element, p53

Background

The proline-rich region (residues 64–93) of the p53 tumor suppressor protein regulates apoptosis and inhibits MDM2-mediated p53 degradation [1,2]. Polymorphisms in codon 72 of p53 (proline or arginine) are represented by the nucleotide sequences, CCC or CGC, at nucleotides 214–216. Storey *et al.* suggested that women with the homozygous CGC variant were seven times more susceptible to cervical cancer in comparison to CCC homozygotes [3]. Reports that cancer patients with the arginine variant at codon 72 of the p53 gene have a better prognosis are controversial [4,5], and so other factors may affect p53 gene function. Menendez *et al.* showed that p53-mediated transcription is increased by the estrogen receptor acting as a transcription factor in cultured cells transfected with a recombinant gene containing an estrogen responsive element (ERE) and a p53 responsive element [6]. ERE is a DNA sequence, -GGTCAnnnTGACC-, to which estrogen receptors (ERs) bind in order to activate the transcription of target genes [7]. We expected that the relation between response systems for estrogen receptors and p53 could be applied to

promote adenovirus-mediated p53 gene transfer in ER-expressing cells and there would be differences in cellular response according to codon 72 polymorphisms of p53. In this study, upstream ERE-linked p53 genes were inserted into adenovirus vectors and used to infect cancer cell lines, supposing selective transduction of ERE-linked p53 gene to the cells expressing ERs. The effects of ERE on the cellular reaction against the recombinant adenoviruses seemed to be quite different between the proline variant and arginine variant p53 genes, and also different between in ER-expressing cells and in ER-not expressing cells.

Materials and methods

Plasmids

Human cDNAs were amplified by RT-PCR from total RNA purified from chorionic tissue obtained at 11 weeks of gestation with informed consent [8]. The p53 gene (bases 166–1143 from the start codon, including the last non-coding 21 nucleotides) was amplified with the forward primer, GAAGACCCAGGTCCAGAT, and the reverse primer, TTTATGGCGGGAGGT. To

incorporate an *Eco*R1 site together with the ERE and the start codon at the 5' terminal, the forward primer, GAATTCGGTCATAGTGACCATATGGAAGACCCAGGTCCAGAT, and the reverse primer, AGTGTGATGGATATCTGCAGAATTCTTTATGGCGGGAGGT, were used to amplify the ERE-linked p53 gene [8]. To incorporate an *Eco*R1 site together with the start codon but without the ERE at the 5' terminal, the forward primer, AGGAATTCATGGAAGACCCAGGTCCAGAT, and the reverse primer, CAGAATTCTTTATGGCGGGAGGT, were used to amplify an ERE-unlinked p53 gene. These PCR products were gel purified, ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), and transfected into competent *E. coli* DH5 α cells (Competent High, Toyobo, Osaka, Japan). The amplified plasmids were digested with *Eco*RI and ligated into pIRES-hrGFP-1 (Agilent Technologies). For the polymorphic variants at codon 72 of p53, the arginine variant (CGC) cDNA isolated from chorionic tissue was changed to the proline variant (CCC) by using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) with the primers, GCCAGAGGCTGCTCCCCC and CGTGCAAGTCACAGACTT.

Recombinant adenoviruses

The ERE-linked or ERE-unlinked p53 genes and their arginine variants (EREp53R or p53R) or proline variants (EREp53P or p53P) at codon 72 were excised by digestion with *Eco*RV and *Xho*I, and inserted into the pShuttle-IRES-hrGFP-1 shuttle vector (Agilent Technologies) (Figure 1). After linearization by *Pme*I digestion and gel purification without dephosphorylation, the p53 gene constructs were electroporated into BJ5183-AD-1 cells pre-transformed with E1-deficient adenovirus vector. The recombinant adenoviruses were amplified in XL-10 ultracompetent cells, digested with *Pac*I and used to infect AD-293 cells. The adenovirus inserted with arginine variant or proline variant was indicated as Ad-p53R or Ad-p53P, and the corresponding ERE-linked adenovirus was indicated as Ad-EREp53R or Ad-EREp53P.

To prepare viral stocks, AD-293 cells that were fully GFP-positive at 3 days after infection were collected, and the adenoviruses were extracted from the supernatants by centrifugation of the cells after four rounds of rapid freezing in methanol cooled with dry ice and thawing in a 37°C water bath. The adenoviruses were purified using a Virabind Adenovirus Miniprep kit (Cell Biolabs Inc., San Diego, CA, USA) and stored in 10% glycerol at -80°C

SDS gel electrophoresis of purified recombinant adenoviruses

Proteins in the purified adenovirus solutions were separated on any kD-precast TGX polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and stained with Coomassie brilliant blue R. The hexon protein was identified by western blotting with an anti-hexon mouse antibody and anti-mouse IgG in the adenovirus titration kit (Agilent Technologies).

Quantification of shuttle vectors or recombinant adenoviruses containing the p53 gene by real time PCR

To isolate adenovirus DNA (the isolation step can be omitted for real time PCRs), viruses in solution or in cultured cells were lysed with 0.6% SDS containing 10 mM EDTA, followed by protein precipitation with 0.3 volumes of 5 M NaCl, and the DNA was precipitated with 3 volumes of cold ethanol and dissolved in water. For TaqMan real-time PCR, the forward primer, GAATTGCTATTATTTGTCGTCATCA, the reverse primer, AGGTAGACTGACCCTTTTGGACTT, and the probe (CCTTGTAGTCCTCGAGTTA conjugated with FAM (6-carboxy fluorescein) on the 5' end and with minor groove binder (MGB) on the 3' end; Applied Biosystems, CA, USA) corresponding to the junction region between the 3' end of the p53 insert and the *Xho*I site of the shuttle vector, were used to quantify the copy number of adenovirus containing p53 constructs [9]. The real time PCRs were performed to analyze the absolute copy numbers of transduced recombinant adenovirus in the cells in each well on a 7500 Fast real time PCR System (Applied Biosystems).

Cell culture

The HHUA human endometrial cancer cell line, which expresses ER β [10], was supplied by Riken Laboratories (Wako, Saitama, Japan). The SW48 human colon cancer cell line [11] and the KLE human endometrial cancer cell line [12] were supplied by DS Pharma Biomedical Co. (Osaka, Japan). The SW48 cells were cultured in minimal essential medium supplemented with 10% FBS. In preliminary experiments, the HHUA cells and SW48 cells were found to express the arginine variant at codon 72 p53 gene without any mutations in bases 166-1203 from the start codon. HHUA cells were found to express ER β but not ER α [13]. SW48 cells were found to express ERs by real time PCR (data not shown). KLE cells, which have a mutation at codon 213 of the p53 gene [12], were found not to express ERs.

The HHUA cells and KLE cells were cultured in F-12/minimal essential medium (1:1) (Invitrogen, Carlsbad, CA, USA) supplemented with 15% charcoal-stripped FBS (HyClone Laboratories, Logan, UT, USA) at 37°C in a humidified incubator containing 5% CO₂. The concentration of estradiol in the medium supplemented with FBS was about 10⁻¹⁰ M.

For viral infection, cultured cells were dispersed with 0.25% trypsin/1 mM EDTA, washed, and inoculated into 12-well (24-mm diameter) tissue culture plates (BM Equipment Co. Tokyo, Japan). For infection with the same copy number of adenovirus DNA for each insert, the volume of adenovirus stock was adjusted according to the quantification data by TaqMan real-time PCR assay, corresponding to a multiplicity of infection of about 10.

Titration of recombinant adenoviruses

AD293 cells in 24-well tissue culture plates (BD Biosciences, San Jose, CA, USA) were infected with 50 μ l of 10-fold

serially-diluted recombinant adenovirus. The cells were fixed with methanol and the adenoviral hexon protein was stained with an anti-hexon mouse antibody followed by horseradish peroxidase-conjugated goat secondary antibody, and bound antibodies were detected by reaction with diaminobenzidine (AdEasy Viral Titer kit, Agilent Technologies). The number of hexon-positive cells was counted under a microscope and the number of infectious units (IFU) per ml was calculated.

RT-PCR assay

Total RNA was extracted from the HHUA cells at 72 h after adenovirus infection using an RNeasy Mini kit (Qiagen Sciences, Valencia, CA, USA). High Capacity cDNA Reverse Transcription kit (Applied Biosystems) were used to synthesize cDNA from the RNA. The relative expression levels of target mRNAs, compared to the level of 18S ribosomal RNA, were analyzed by real time PCR with the corresponding TaqMan MGB probes and primers (Hs00153349_m1 for p53, Hs00154661_m1 for coxsackievirus and adenovirus receptor (CAR), Hs00914223_m1 for p300 using the multiplex threshold method on a 7500 Fast Real Time PCR System (Applied Biosystems).

Treatment of HHUA cells with estradiol or radiation and flow cytometry

We studied the effects of 10^{-9} M β -estradiol treatment or 10 Gy radiation at 24 h after adenovirus infection on the expression of p53 and p300, which accelerates p53 turnover by polyubiquitination, and on the annexin V binding, as an indicator of early phase apoptosis, and propidium iodide (PI) staining, as an indicator of late phase apoptosis in HHUA cells.

For the estradiol-treated group, the HHUA cells were incubated in medium containing 10^{-9} M β -estradiol from the start of adenovirus infection. β -estradiol (Wako Pure Chemicals) was first dissolved at 10^{-5} M in ethanol, and then diluted 1:10,000 in culture medium. For the untreated group and the radiation group, 0.01% ethanol was included in the culture medium.

For the radiation group, the HHUA cells were exposed to 10 Gy X-ray irradiation at 150 kV on the X-ray apparatus (MBR-1320R, Hitachi, Tokyo, Japan) 24 h after adenovirus infection.

Forty-eight hours after infection with recombinant adenoviruses, the HHUA cells were dispersed with 0.25% trypsin/1 mM EDTA, washed with PBS and stored at -80°C for RT-PCR assay, or suspended in 50 mM Tris-HCl (pH 8.0), 80 mM NaCl, 0.2% BSA, 2.5 mM CaCl_2 , and then incubated with phycoerythrin-conjugated annexin V (BD Biosciences). The cells were analyzed by flow cytometry, and cells expressing GFP were detected at 515-545 nm, and cells binding to annexin V were detected at 564-606 nm, and the double-positive rates in GFP positive cells were counted.

Seventy two hours after infection with recombinant

adenoviruses, the HHUA cells were dispersed with 0.25% trypsin/1 mM EDTA, washed with PBS and stored at -80°C for RT-PCR assay, or stained with PI (BD Biosciences) (detected at >650 nm), and the double-positive rates in GFP positive cells were counted.

Statistical analysis

Copy numbers of recombinant adenoviruses in the cells, relative mRNA expression levels, annexin V binding rates, and propidium iodide staining rates were analyzed by nonparametric methods using PASW Statistics 18 Software (SPSS, Chicago, IL, USA), and results with $p < 0.05$ were judged as significant.

Results

SDS gel electrophoresis of purified recombinant adenoviruses

By SDS gel electrophoresis, the protein composition of each recombinant adenovirus constructed from corresponding p53 gene-inserted shuttle vector shown in **Figure 1** was identical, including the hexon confirmed by Western blotting (**Figure 2**).

Quantification of shuttle vectors and recombinant adenoviruses containing p53 gene inserts by real-time PCR

The quantification curves for Shuttle-p53R, Shuttle-p53P, Shuttle-EREp53R and Shuttle-EREp53P were identical (**Figure 3**). The copy numbers of adenoviruses containing p53 gene inserts in the isolated virus solution or in the cell lysate were calculated from the corresponding amplification cycle numbers on the quantification curve.

Analysis of GFP positive cells infected with recombinant adenoviruses by fluorescence microscopy

A greater number of HHUA cells was infected with Ad-EREp53P (**Figure 4D**) compared to Ad-p53R, Ad-p53P and Ad-EREp53R (**Figure 4A, 4B, 4C**). However, a greater number of KLE cells was infected with Ad-EREp53R (**Figure 4G**) compared to Ad-p53R, Ad-p53P or Ad-EREp53R (**Figure 4E, 4F, 4H**).

Titration of recombinant adenoviruses in AD293 cells

In accordance with the manufacturer's protocol, a significantly higher titer of Ad-EREp53P compared to Ad-p53R, Ad-p53P and Ad-EREp53R was noted (**Figure 5**). The difference was considered to depend on the greater transduction rates of Ad-EREp53P than other recombinant adenovirus into AD293 cells, which were found by real time PCR to express ERs (data not shown).

High transduction for Ad-EREp53P in HHUA cells or SW48 cells, and high transduction for Ad-EREp53R in KLE cells

The transduction in HHUA cells were over ten-fold higher

for Ad-EREp53P than for Ad-p53R, Ad-p53P or Ad-EREp53R (**Figure 6A**).

The transduction in SW48 cells at 72 h after infection were about ten-fold higher for Ad-EREp53P than for Ad-p53R, Ad-p53P or Ad-EREp53R (**Figure 6B**).

The transduction in KLE cells at 72 h after infection were about ten-fold higher for Ad-EREp53R than for Ad-p53R, Ad-p53P or Ad-EREp53P (**Figure 6C**).

Real-time PCR analysis of p53 mRNA expression in HHUA cells and KLE cells

The expression level of p53 mRNA in HHUA cells infected with Ad-EREp53P was about ten-fold higher than in cells infected with Ad-EREp53R, ERE-unlinked Ad-p53R or ERE-unlinked Ad-p53P (**Figure 7A**).

The expression level of p53 mRNA in KLE cells infected with EREp53R was about ten-fold higher than in cells infected with Ad-EREp53P, ERE-unlinked p53R or ERE-unlinked Ad-p53P (**Figure 7B**).

Real-time PCR analysis of CAR mRNA expression in HHUA cells and KLE cells

The expression level of CAR mRNA in HHUA cells infected with Ad-EREp53R was significantly higher than in cells infected with ERE-unlinked Ad-p53R or ERE-unlinked Ad-

p53P (Figure 8A).

The expression levels of CAR mRNA in KLE cells infected with Ad-EREp53R or ERE-unlinked Ad-p53P were significantly higher than in cells infected with ERE-unlinked Ad-p53R (Figure 8B).

Real-time PCR analysis of p53 mRNA and p300 mRNA expression in HHUA cells treated with 10^{-9} M estradiol or 10 Gy radiation

We found no differences in the levels of p53 mRNA expression between the untreated group, the estradiol-treated group and the radiation group (Figure 9A).

In the untreated group and estradiol-treated group, the p300 mRNA levels in HHUA cells infected with Ad-EREp53P were lower than in cells infected with the ERE-unlinked p53P gene (Figure 9B). In cells in the radiation group, the p300 mRNA expression levels were lower in cells infected with Ad-EREp53P than in cells infected with Ad-EREp53R (Figure 9B).

Flow cytometric analysis of annexin V binding and propidium iodide staining in HHUA cells treated with or without estradiol or 10 Gy radiation

We also studied the effects of 10^{-9} M β -estradiol or 10

Gy radiation at 24 h after adenovirus infection on early phase apoptosis, as demonstrated by annexin V binding, and late phase apoptosis, as demonstrated by PI staining, in HHUA cells.

At 48 h after infection of HHUA cells with recombinant adenoviruses, the annexin V binding rate was higher in cells infected with Ad-p53P than in cells infected with Ad-p53R. Also, the annexin V binding rate was higher in cells infected with Ad-EREp53P than in cells infected with Ad-p53P or with Ad-EREp53R (Figure 10A). No differences in annexin V binding in HHUA cells were found between the untreated group, the estradiol-treated group and the radiation group.

At 72 h after infection of HHUA cells with recombinant adenoviruses, the PI staining rate was higher in cells infected with Ad-EREp53P than in cells infected with Ad-EREp53R, Ad-p53P or Ad-p53R (Figure 10B). No differences in the PI staining of HHUA cells were found between the untreated group, the estradiol-treated group and the radiation group.

Discussion

It has been suggested that crosstalk exists between the estrogen receptor and the transcriptional network via the p53 gene. It has also been shown that p53 can mediate

the transactivation of responsive elements by synergizing with ER at locations in the DNA sequence near to the ERE and p53 responsive element [14]. In contrast, ERE can be used as a promoter to induce the transcriptional activation of adenovirus genes to obtain restricted replication by complementation of the E1a protein [15]. In our study, the transduction efficiencies of adenovirus gene containing an upstream ERE linked-exogenous p53 gene and the expression levels of p53 mRNA in cultured cells were greater than those not linked to ERE.

In our previous study [8], HHUA cells transfected with a plasmid vector containing an ERE-linked p53 gene with the proline variant polymorphism at codon 72 showed increased p21 expression. Although infection of recombinant adenovirus containing ERE-linked p53 genes didn't make no difference in p21mRNA induction (data not shown), the transduction rates of recombinant adenoviruses containing the ERE-linked proline variant p53 gene were over ten-fold greater than the unlinked p53 genes in ER-expressing HHUA cells derived from endometrial cancer and also in ER-expressing SW48 cells derived from colon cancer. Surprisingly, the transduction rates of recombinant adenoviruses containing the ERE-linked arginine variant p53 gene were over 10-fold greater than the transduction rates of recombinant adenoviruses containing the unlinked p53 gene in KLE cells, which are derived from endometrial cancer but do not express ER. Unknown intracellular changes may occur after transduction with these constructs.

CAR is an important entry site factor, and the expression level of CAR was higher after infection with EREp53R in both HHUA cells and KLE cells. We hypothesized that the function of CAR in promoting adenovirus entry was negated in HHUA cells in which stronger factors promote the transduction of the proline variant p53 gene. Cyclic AMP dependent protein kinase (PKA) is known to play a role in the nuclear transport of infected virus genes [16]. PKA mRNA expression was detected at higher levels in HHUA cells and in SW48 cells than in KLE cells by real time PCR (data not shown). ER is activated by cAMP even in the absence of exogenous hormone, and ERE is necessary for cAMP dependent gene expression via ER β [17].

For the role of codon 72 polymorphisms in the p53 gene, some functional differences between the proline and arginine variants of p53 have been suggested [18]. The apoptotic potential of the arginine variant has been shown to be related to its greater localization in the mitochondria [18]. The higher efficiency of mitochondria-targeted p53 gene delivery in xenografted human tumor models compared to nuclear delivery suggests the importance of mitochondrial transfer of p53 [19].

The inclusion of 10^{-9} M β -estradiol in the culture medium made no obvious difference to the responses of HHUA cells to recombinant adenovirus infection. The concentration (10^{-9} M) of estradiol in the culture medium of cells in

the estradiol group was 10-fold greater than the basal concentration of estradiol (10^{-10} M) in the medium of cells in the untreated group. The p300 mRNA levels in HHUA cells infected with Ad-EREp53P were lower than in cells infected with the ERE-unlinked p53P gene. Because there was no differences between the untreated group and the estradiol group, the increased response of HHUA cells to the recombinant adenovirus containing an ERE-linked proline variant p53 gene is considered to be hormone-independent.

There was also no difference in the responses of HHUA cells to recombinant adenoviruses between untreated group and the 10 Gy irradiated group. The p53 gene product promotes cell sensitivity to low energy radiation [20,21], and it has been reported that adenoviral-mediated delivery of the p53 gene *in vitro* and *in vivo* increased cancer cell sensitivity towards radiation [22]. Radiation caused no additional effects on the p53 mRNA level in HHUA cells in our study, and the expression of p300 mRNA was kept when the cells were infected either with Ad-p53P or with Ad-EREp53R. However, suppression of p300 mRNA expression was observed following infection with Ad-EREp53P, suggesting that the codon 72 polymorphism rather than the upstream ERE is more important for the effect of p53 on p300 expression. As cyclic AMP response element binding protein (CREB) and p300 contribute to the ability of estrogen receptor to trans-activate target genes [23], and p300 play roles with CREB-binding protein to induce apoptosis by ionizing radiation [24]. After transduction of the proline variant p53 gene in HHUA cells, the low expression of p300 may attenuate p53 degradation because p300 exhibits intrinsic ubiquitin ligase activity [25,26].

As transcriptional effects of PKA are mediated through CREB, lower p300 mRNA level in HHUA cells may be related to the interaction with CREB-binding proteins. Neither PKA mRNA nor p300 mRNA were detected in ER-negative KLE cells (data not shown). We are considering that future study using arginine-variant p53 gene which induces greater p53 mRNA in ER-negative KLE cells by other indicators may help to prepare an efficient ERE-linked p53 gene therapy for ER-negative neoplasia.

Conclusions

An ERE-linked p53 gene with codon 72 polymorphism of proline variant or arginine variant are over 10-fold greater transduced than unlinked one in the ER-expressing cells or ER-not expressing cells, respectively. These relations may be applied to the efficient adenovirus-mediated transfer of p53 gene.

Competing interests

The Authors' declare that they have no competing interests.

Authors' contributions

KK, KH, HT, MA, TF, HY, TY, TS, OI were all involved in designing the study. KK and KH wrote the first draft

of the manuscript. All authors reviewed the data and were involved in the final analysis and conclusions.

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