A/T gap tolerance in the core sequence and flanking sequence requirements of non-canonical p53 response elements

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Bi-He Cai^{1,2}, Chung-Faye Chao¹,
Hwang-Chi Lin³, Hua-Ying Huang¹,
Reiji Kannagi^{2,4} and Jang-Yi Chen^{1,*}

¹Department of Biology and Anatomy, National Defense Medical Center, Taipei, Taiwan, ²Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, ³Division of Plastic Surgery, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, Republic of China and ⁴Research Complex for Medical Frontiers, Aichi Medical University, Aichi, Japan

*Jang-Yi Chen, Department of Biology and Anatomy, National Defense Medical Center, Taipei 114, Taiwan, Republic of China. Tel: +886 2 87923100 ext 18712, Fax: +886 2 87923159, email: jychen01@ndmctsgh.edu.tw

The canonical core sequence of the p53 response element, CATG, has a two-base A/T gap. Previously, we found that p53 can also activate a non-canonical four-base A/T gap CATATG core sequence. In this study, we investigated the possible number of A/T bases used by p53 and showed that a six-base A/T gap CATATG core sequence was the maximum A/T gap in the p53 response element that could be upregulated by p53 and p63. Canonical and non-canonical p53 response elements also have three-base flanking sequences. A/T bases could be substituted by G/C bases, including CACG and CGTGTG, but not CGCGCG. We found that the SV40 promoter with functional six- and two-base A/T gap core sequences could be activated by TAp63 γ and that TAp63 γ could upregulate SV40 small and large T antigens expression in COS7 cells. We also found that the distal region of PUMA promoter with functional two six-base A/T gap core sequences could be activated by TAp63 γ in 293T cells. These new findings could provide novel rules for the non-canonical p53 family response element and could extend the entire p53 family regulation network.

Keywords: flanking sequence/non-canonical/p53/p63/response element.

Abbreviations: ChIP, Chromatin immunoprecipitation; DAPA, DNA affinity purification assay; Pu, purine; Py, pyrimidine; RT-PCR, reverse transcription-PCR; SDS-PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis.

The canonical p53 response element can be represented as PuPuPuC(A/T)(A/T)GPyPyPy{0–13}PuPuPuC(A/T)(A/T)GPyPyPy (1, 2) where C(A/T)(A/T)G is the core sequence, and the purine (Pu) and pyrimidine (Py) bases are the flanking sequence. The motif PuPuPuC(A/T)(A/T)GPyPyPy represents a half-site, and PuPuPuC(A/T) or (A/T)GPyPyPy are quarter

sites. {0–13} represents a spacer with variable 0–13 bases. Although the sequences CATG, CTAG, CAAG and CTTG are all functional core sequences, the sequence CATG is the most effective core sequence used by p53 (3, 4). In our previous study, the CATTAG core sequence on the 14-3-3 σ promoter was shown to be a functional and non-canonical p53 core sequence of the p53 response element (5). By screening one- to five-base A/T gap core sequences, we found that only two- and four-base A/T gap core sequences can be used by p53 and that CATATG is more effective than CATTAG. The greatest length of A/T bases used by p53 was further clarified in this study. Furthermore, p63, another p53 family member, can also regulate the 14-3-3 σ promoter (6). The 5' purine and 3' pyrimidine flanking sequences are present as three-base motifs in the canonical p53 response element (1, 2). The identification and characterization of the novel rules of the non-canonical p53 family response elements has been an important topic for p53 family biology in recent years (7–12). Consequently, in this study, we also investigated how p63 regulated the non-canonical p53 response element and clarified the number of bases in the flanking sequences of the non-canonical p53 response element. It has been reported elsewhere that a two-base A/T gap with one base C or G substitution, such as CACG and CGTG, can also be used by p53 and p63 (13, 14). In this study, the influence of possible A/T to G/C substitutions in the non-canonical p53 response element was further examined.

Materials and Methods

Construction of reporter vectors with different non-canonical p53 response elements

Different non-canonical p53 response elements were synthesized and annealed to a SmaI-digested pGL3 promoter vector (Promega). The vector and insert were ligated at a 1:4,500 ratio at 22°C for 15 min and were transformed at 37°C overnight. The positive clones were verified by DNA sequencing.

Construction of reporter vectors with PUMA distal promoter region reporter

PUMA distal promoter region was amplified from 293T Cell genomic DNA using the following primers: AAAATTGCAGGTAGAAGTTGTGG and TCCCAACCAACTTGCGCTTTA. Linearized pGL3 promoter vector was digested by SmaI restriction enzyme. Fifteen base pair extension homologous to vector ends was amplified from PUMA distal promoter region using the following primers: TAGCGGTGCTAGCCCAAATTGCAGGTAGAAGTTGTGG and GCAGATCTCGAGCCCTCCCAACCAACTTGCGCTTTA.

pGL3 promoter vector with PUMA distal promoter region reporter was created by in-fusion cloning according to the instructions of the manufacturer (In-Fusion HD Cloning Kits, Clontech) and was verified by DNA sequencing.

Site-directed mutagenesis of the pGL3 promoter mutants

The pGL3 promoter vectors with point mutations or deletion clones were created by site-directed mutagenesis according to the

instructions of the manufacturer (Phusion Site-Directed Mutagenesis Kit, Finnzymes). 6g mutant (mt) was created using the following primers: AAGCCTAGGCTCGAAAAACCTCCTCAC and T TGCAAAAAGCTTGGCATTCCGGTA. 2g mt was created using the following primers: GCCAAGCTTTTGGCAAAAAGCGTACGCT and ATTCCGGTACTGTTGGTAAAGCCACCA. 4g mt was created using the following primers: GCCAAGCTTTTGGAAAA CCCTAGGCCTC and ATTCCGGTACTGTTGGTAAAGCCACCA. 6.2.4g del was created using the following primers: GCCAAGCTTTTGGCTCCTCACTACTTC and ATTCCGGTACTGTTG GTAAAGCCACCA. 6.2g mt was created using the following primers: AAGCGTACGCCTCGAAAAACCTCCTCAC and TTG CAAAAAGCTTGGCATTCCGGTA. All pGL3 promoter mutants were verified by DNA sequencing.

Luciferase activity assay

H1299 cells were grown in 12-well tissue culture dishes to 60% confluence and were co-transfected with 0.5 µg of pcDNA 3.0 (Invitrogen), pcDNA 3.0 p53 or pcDNA 3.0 Tap63γ in the presence of 0.5 µg of pGL3 promoter firefly luciferase (Promega) or pGL3 promoter with non-canonical p53 response elements or pGL3 promoter mutants and 10 ng of pCMV *Renilla* luciferase plasmids (Promega). Twenty-four hours after transfection, the cells were harvested in 0.25 ml of reporter lysis buffer and were subjected to a dual luciferase assay according to the protocol of the manufacturer (Dual-Luciferase Reporter Assay System, Promega). The firefly luciferase activity was normalized to the *Renilla* luciferase activity, and the data are represented as the mean plus or minus the standard deviation of three independent experiments with each performed in triplicate.

Western blot analysis

Twenty micrograms of total protein was prepared from pcDNA3.0 or p53 or Tap63γ overexpressing COS7 or 293T cells 48 h post-transfection and was run on 10% sodium dodecylsulphate (SDS)–polyacrylamide gels. Proteins were transferred onto a polyvinylidene difluoride membrane and blocked with 3% skim milk–phosphate-buffered saline–Tween 20. The primary antibodies were specific for p53 (DO-1 mouse monoclonal, Santa Cruz), p63 (4A4 mouse monoclonal, Santa Cruz), SV40 T antigen (Pab419 mouse monoclonal, Santa Cruz), PUMA (H-136 rabbit polyclonal, Santa Cruz), Tubulin (Rabbit Polyclonal, GeneTex) and β-actin (AC-15 mouse monoclonal, Sigma). Goat anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase was used as the secondary antibody (Santa Cruz), and chemiluminescent signals were detected using an enhanced chemiluminescence (ECL) kit (Amersham).

DNA affinity purification assay

Nuclear extract was prepared from 5×10^7 H1299 cells transfected with 16 µg of p53Δ364-393. The 100 µg nuclear extracts were pre-cleared by incubation with 40 µl streptavidin–agarose beads (4%) with a 50% slurry at 4°C for 1 h with rotation and subsequently centrifuged at $4,000 \times g$ for 1 min at 4°C. The supernatant was collected as the pre-cleared nuclear extract. The binding reaction was performed in a 1 ml total solution volume by mixing the pre-cleared nuclear extract proteins with the annealing probe (0.3 nmol), 5× binding buffer (100 mM Hepes; pH 7.6, 5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM DTT, 1% Tween 20 (w/v), and 150 mM KCl), 10 µl sonicated salmon sperm DNA (1 µg/µl) and the 40 µl streptavidin–agarose beads (4%) with a 50% slurry and incubating the mixture at room temperature for 1 h with rotation. The beads were washed three times with 1× phosphate-buffered saline (PBS). The binding proteins were eluted with 20 µl of 5× loading dye and separated by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blot analysis with protein locations probed with p53 antibody (p53 Ab-2, Lab Vision). Nuclear extract (10 µg) was mixed with 20 µl 5× loading dye as a 10% ‘input’ sample.

Real-time reverse transcription–polymerase chain reaction

Total RNA was extracted using a Trizol reagent, and 1 µg of RNA was synthesized into cDNA using a first-strand cDNA synthesis kit for reverse transcription according to the manufacturer's instructions (SuperScript III Reverse Transcriptase cDNA Synthesis Kit, Invitrogen). The cDNA samples were pre-mixed with Maxima SYBR Green qPCR Master Mix (2×; Fermentas) and the following

specific paired-primers: the SV40 large T antigen (+) 5'-TGCCCTT GGACAGGCTGAAC-3' and (–) 5'-TGGACTCCCCTCCAGTG CCC3'; the SV40 small T antigen (+) 5'-TGCAAAACAATGGCCT GAGTGTGCA-3' and (–) 5'-CGAAGCAGTAGCAATCAACCC ACA-3'; β-actin (+) 5'-GGCATGGGTCAAGGATT-3' and (–) 5'-GAAGGTGTGGTGCCAGATTT-3'. The product sizes were as follows: SV40 large T antigen, 93 bp; SV40 small T antigen, 136 bp; β-actin, 135 bp. The real-time polymerase chain reaction (PCR) amplification and data acquisition were carried out using the instructions from Applied Biosystems 7500, and the following cycling parameters were used: 50°C for 2 min, 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. The real-time fold difference is expressed as $2^{-(\Delta\Delta Ct)}$ where $\Delta\Delta Ct = [Ct(\text{larger or small T antigen}) - Ct(\beta\text{-actin}) \text{ of TAp63}\gamma\text{-transfected sample}] - [Ct(\text{larger or small T antigen}) - Ct(\beta\text{-actin}) \text{ of pcDNA3.1-transfected sample}]$. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay method was modified from the EZ chip kit (Upstate) as follows: 48 h post-transfection, p53 or TAp63γ over-expressing COS7 or 293T cells were grown to 90% confluence in a 100 mm culture dish containing 10 ml of fresh media. Fresh 18.5% formaldehyde (540 µl) was added to the 10 ml of growth media to crosslink DNA–protein complex, and the dish was gently swirled to mix the solution. The mixture was incubated at room temperature for 10 min. One millilitre of 10× glycine was added to each dish to quench any unreacted formaldehyde. The dish was swirled to mix the solution, incubated at room temperature for 5 min, and placed on ice. The medium was aspirated, removing as much medium as possible and taking care to not to disturb the cells. Ten millilitres of cold 1× PBS was added to wash the cells. The PBS was removed, and the wash was repeated. One cubic centimetre of 1× PBS plus protease inhibitor was added. The cells were scraped from each dish into a microfuge tube and spun at $700 \times g$ at 4°C for 5 min to pellet the cells. The cell pellet was resuspended in 5 ml of ice-cold NP-40 lysis buffer and incubated on ice for 5 min. The nuclei were pelleted at $1,200 \times g$ at 4°C for 10 min, and the supernatant was removed and discarded. The nuclei were washed with 2.5 ml of Micrococcal Nuclease (MNase) digestion buffer (10 mM Tris–HCl pH 7.4, 15 mM NaCl and 60 mM KCl) plus protease inhibitor. The nuclei were pelleted at $1,200 \times g$ at 4°C for 10 min, and the supernatant was carefully discarded. The nuclei were resuspended in 1 ml of MNase digestion buffer with 1 mM CaCl₂. Fifty microlitres of the resuspended liquid composed the MNase untreated sample, and 500 U of MNase (USB) was added to 950 µl of the resuspend liquid. The samples were incubated at 16°C for 10 min. The MNase untreated and the MNase digested samples were sonicated. The MNase digest sample (40 µl) served as the input, and 50 µl of the MNase digest sample was a chromatin-size check sample. The following solutions were added in a 1.5 cc Eppendorf tube: 500 µl of ChIP dilution buffer (16.7 mM Tris pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA), 400 µl of MNase digest sample and 60 µl of protein A/G (Santa Cruz). The solutions were incubated for 1 h at 4°C with rotation. The agarose was pelleted by brief centrifugation at $4,000 \times g$ for 1 min. The supernatant was collected into fresh microfuge tubes. Two micrograms of immunoprecipitating antibody IgG (rabbit, sigma) or p53 (DO-1 mouse monoclonal, Santa Cruz) or p63 (4A4 mouse monoclonal, Santa Cruz) were added, and the mixture was incubated overnight at 4°C with rotation. The next day, 60 µl of protein A/G agarose was added, and the solution was incubated for 1 h at 4°C with rotation. Protein A/G agarose was pelleted by brief centrifugation ($4,000 \times g$ for 1 min), and the supernatant was removed. The protein A/G agarose–antibody/chromatin complex was washed by resuspending the beads in 1 ml each of a series of cold buffers. First, one wash was completed with a low salt immune complex wash buffer (20 mM Tris pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA). Next, another wash was performed with a high salt immune complex wash buffer (20 mM Tris pH 8.1, 500 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA). Next, a LiCl immune complex wash buffer (10 mM Tris pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% NaDeoxycholate and 1 mM EDTA) was used to complete one wash. Finally, two washes were performed with TE buffer. During each wash, incubation occurred for 3 min on a rotating platform followed by brief centrifugation ($4,000 \times g$ for 1 min) and careful removal of the supernatant. For the input sample, 200 µl of elution buffer (1% SDS and 0.1 M NaHCO₃) was added, and the sample was set aside at room temperature. For the elution steps, 100 µl of elution buffer was

added to each tube containing the antibody/agarose complex. The tubes were gently flicked to mix the samples and incubated at room temperature for 15 min. The agarose was pelleted by brief centrifugation (3,000–5,000 × g for 1 min), and the supernatant was collected into new microfuge tubes. The elution steps were repeated and the eluates were combined to reach a total volume of 200 µl. Eight microlitres of 5M NaCl was added to immunoprecipitations (IPs) and input samples. Two microlitres of 5M NaCl were added to the chromatin size check sample. All of the samples were capped with parafilm and incubated at 65°C overnight to reverse-crosslink. The next day, 1 µl of RNase A was added to each sample, and the samples were incubated for 30 min at 37°C. Next, 4 µl of 0.5M EDTA, 8 µl of 1M Tris–HCl and 1 µl proteinase K add to the IPs and input samples. To the chromatin size check sample, 1 µl of 0.5M EDTA, 2 µl of 1M Tris–HCl and 1 µl of proteinase K were added, and the sample was incubated at 45°C for 1–2 h. For chromatin size checking, the chromatin of MNase digest sample appears at <200 bps (Fig. 8A). To the IPs and the input samples, 200 µl of phenol/chloroform (1:1) were added, and the samples were vortexed 30 s. The samples were centrifuged at 15,000 × g for 15 min, and the upper layer was carefully collected into fresh microfuge tubes. Next, 1M NaCl was added to a 4× dilution, for a final concentration of 250 mM of NaCl in the samples. One volume of isopropanol was added, and the samples were mixed and incubated at –20°C overnight. The next day, the samples were centrifuged at 15,000 × g for 15 min, and the supernatants were carefully discarded. Next, 1 cc of 70% ethanol was added, the samples were centrifuged at 7,500 × g for 5 min and the supernatants were carefully discarded. The pellets were dried for 10 min at 65°C on a hot plate with the microfuge cap open. The pellets were resuspended with 50 µl of ddH₂O for use in a 25 µl PCR consisting of 12.5 µl of 2× PCR pre-mix (Fermentas), 5 µl of sample, 1 µl of pre-mix primers and 6.5 µl of ddH₂O. The was performed as follows: 35 cycles at 95°C for 40 s, annealing at 60°C for 40 s and 72°C for 40 s. The PCR products were loaded into a 2% agarose gel. The PCR primers used (CCAGTTCGCCATTCTC and TTGCAAAAGCCTAGGCCTCCAA) targeted the SV40 promoter. The product size of the SV40 promoter was 131 bp. The PCR primers used (AAAA TTGAGGTAGAAGTTGTGG and TCCCAACCAACTTGCGCT TTA) targeted the PUMA promoter. The product size of the PUMA promoter was 129 bp.

Results

Six-base, but not eight-base, A/T gaps act as core sequences of the p53 response element

To evaluate the maximum permissible number of bases of the A/T gap in the core sequence for a functional p53 response element, four-, five-, six-, seven- and eight-base A/T gaps were synthesized and cloned into the 1.5× half-site (Fig. 1A), and the p53 2× half-site and 14-3-3σ RE served as the positive control. The promoter activities were examined by a luciferase activity assay. Four- and six-base, but not five-, seven- and eight-base of A/T gaps could be activated by p53 (Fig. 1A). The construct p53Δ364–393, lacking the C-terminal non-specific binding domain (15), was used to investigate the p53-binding character of six- to eight-base A/T gaps (Fig. 1B). Synthetic biotin-labelled positive strand annealed to unlabelled negative strand as probes with six- to eight-base of A/T bases within the p53 1.5× half-site were used to examine the binding capacity of p53Δ364–393. The p53Δ364–393 could only bind to six-base gap CATATATG core sequences but not to the seven-base gap CATATATAG or eight-base gap CATATATATG core sequences (Fig. 1B). Therefore, the maximum permissible number of bases of the A/T gap in the p53 core sequence is six. Both p53 and TAp63γ could also upregulate 2× half-sites as a whole site with a CATATATG core sequence (Fig. 2).

A four-base A/T gap p53 core sequence must be flanked by three-base 5' purine or 3' pyrimidine sequences

Because the canonical p53 response element is the 10-base PuPuPuC(A/T)(A/T)GPpPyPy sequence repeated as a 20-base sequence, the 5' purine or 3' pyrimidine flanking sequences must always be present as three-base stretches to fit the 20-base rule (1, 2). If the 20-base rule must be followed in cases with four-, six- or eight-bases within the A/T gap of p53 core sequences, the flanking sequence must accordingly comprise 2 or 1 or 0 bases, respectively. This requirement might explain why an eight-base A/T gap could not be activated by p53, due to the lack of any flanking sequence. In order to test this hypothesis, we created three-, two-, and one-base flanking sequences around four-base A/T gap whole sites (Fig. 3). Surprisingly, only three-base flanking sequences that were combined with four-base A/T gaps could be activated by p53 (Fig. 3). Therefore, even though the A/T base numbers can be changed, the flanking sequence must remain a three-base sequence.

The tolerance of C or G bases within a four-base A/T gap is also activated by p53 and p63

Several reports have mentioned that the two-base A/T gap of the canonical core sequence can be replaced by C or G bases. The sequence PuPuPuC(A/G)(A/T)GPpPyPyPuPuPuC(A/T)(C/T)GpyPyPy is a p63 response element, the underline is the meaning of non-A or T bases (13). GGGCGTGCTCGGGCACGCCC can also be bound by all members of the p53 family, p53, p63 and p73 (14). In this study, we designed two four-base A/T gap core sequences with C or G base substitutions, CACACG and CGTGTG, and an all G/C replacement CGCGCG as a control. A luciferase promoter activity assay indicated that CATATG, CACACG and CGTGTG, but not CGCGCG, could be activated by p53 and TAp63γ (Fig. 4).

The SV40 promoter with six-base and two-base A/T gap core sequence is specifically activated by TAp63γ

Although we co-transfected the p53 or TAp63γ with the pGL3-promoter as a normalization control as 1 in Fig. 4, we also co-transfected p53 or p63 isoforms with pGL3-promoter and found that p53 or TAp63 isoforms could repress and activate the pGL3-promoter separately (Fig. 5A). According to the official sequence map of the pGL3 promoter vector from Promega (GenBank: U47298.2), bases 48–250 comprise the SV40 promoter sequence, which contains six-, two- and four-base A/T gap core sequences within bases 217–240 (Fig. 5A). It has been demonstrated that p53 can repress the SV40 promoter (16, 17). In order to investigate how TAp63 isoforms could activate the SV40 promoter, we co-transfected the highest activation form TAp63γ with different core sequence mutants with C-to-G or G-to-C mutation within bases 217–240 of the pGL3-promoter to verify the functional A/T gap core sequence on SV40 promoter (Fig. 5B). Six- or two-base A/T gap core sequence mutants could be activated by TAp63γ lower than non-mutation controls and four-base A/T gap core sequence mutants, and TAp63γ could not activate both

Fig. 1 The promoter activity in six- to eight-base A/T gap core sequences of p53 promoter response element. (A) The p53 2× half-site is a canonical sequence of the p53 response element. 14-3-3σ RE is a p53 response element on the 14-3-3σ promoter. The design of various non-canonical A/T gap core sequences of p53 response elements is shown. m4-4g: fourth quarter-site mutation (m4) and first half-site core sequence CATATG (4g); m4-5g: fourth quarter-site mutation (m4) and first half-site core sequence CATATAG (5g); m4-6g: fourth quarter-site mutation (m4) and first half-site core sequence CATATATG (6g); m4-7g: fourth quarter-site mutation and first half-site core sequence CATATATAG; m4-8g: fourth quarter-site mutation and first half-site core sequence CATATATATG. All of the canonical and non-canonical response element constructs were co-transfected with the p53 expression vector into H1299 cells, and the luciferase promoter activity was compared, normalized to the activity with pcDNA3.0 as 1. Only the p53 2× half-site, 14-3-3σ RE, m4-4g or m4-6g but not m4-5g, m4-7g, or m4-8g could be activated by p53. (B) In a DAPA assay, biotin-labelled positive strand and non-biotin-labelled negative strand were annealed as probes. p53Δ364–393 could bind to CATATATG core sequences but could not bind to CATATATAG or CATATATATG core sequences. DAPA, DNA affinity purification assay.

the pGL3-promoter with base 217–240 deletion (as a 6.2.4g del) and six- and two-base A/T gap core sequence mutants (as a 6.2g mt) (Fig. 5B). Therefore, six- and two-base A/T gap core sequences on the SV40 promoter are the functional response elements for Tap63γ activation.

Tap63γ but not p53 can activate five bases between two core sequences

There are only five bases between six- and two-base A/T gap core sequences on the SV40 promoter (Fig. 5B),

2X ATATAT ACCCTGTAGCATATATGCCAGACATATGTCCCTACT

Fig. 2 p53 and Tap63γ activated six-base A/T gap core sequences of p53 response element. p53 or Tap63γ were co-transfected with six-base A/T core sequence p53 promoter response elements into H1299 cells, and the relative induction of luciferase promoter activity was measured and normalized to the activity with pcDNA3.0 as 1. Both p53 and Tap63γ can activate a six-base A/T gap core sequence of the p53 promoter response element.

2X ATAT ACCCTGTAGCATATGCCAGACATATGTCCCTACT
 2X ATAT F2 ACCCTAGCATATGCCAGACATATGTCTACT
 2X ATAT F1 ACCCTGCATATGCACATATGTACT

Fig. 3 The promoter activity in one- to three-base 5'- and 3'-flanking sequence of p53 promoter response element with four-base A/T gap core sequences. The design of four-base A/T gap core sequence p53 response elements with flanking sequence of different lengths is shown. 2× ATAT: CATATG core sequence with three-base flanking sequence as a whole-site candidate; 2× ATAT F2: CATATG core sequence with two-base flanking sequence as a whole-site candidate; 2× ATAT F1: CATATG core sequence with one-base flanking sequence as a whole-site candidate. The four-base constructs described in A were co-transfected with p53 expression vector into H1299 cells, and the relative luciferase promoter activity was determined, normalized to the activity with pcDNA3.0 as 1. Only 2× ATAT but not 2× ATAT F2 or 2× ATAT F1 could be activated by p53.

and it only can be activated by p63 but not p53 (Fig. 5A). Crystal structure of p63 DNA-binding domain tetramer can bind to 1-bp overlapping response element (18). This means that only five bases between two core sequences could be activated by p63. We found that p53 and Tap63γ could activate six bases between two core sequences, but only Tap63γ could activate five bases between two core sequences (Fig. 6). This phenomenon could explain why only p63 but not p53 could activate the SV40 promoter.

SV40 small and large T antigens are upregulated by Tap63γ in SV40 infected COS-7 cells

COS-7 is a SV40 virus-infected cell line, and it contains with whole virus genome incorporation within its

Fig. 4 p53 and TAp63 γ activated the four-base A/T gap with G or C mismatch in the core sequences of p53 response element. p53 or TAp63 γ were co-transfected into H1299 cells with a four-base A/T gap containing G or C mismatches in the core sequence of the p53 response element, and the relative luciferase promoter activity was determined, normalized to the pGL3 promoter as 1. Both p53 and TAp63 γ could activate the core sequences CATATG and CACACG, but not CGTGTG.

chromosome with only SV40 ori site mutation (19). The SV40 ori site is located in bases 149–212 of the pGL3-promoter (20); therefore, the functional response elements for TAp63 γ activation are not mutations in COS-7 cells. The SV40 promoter can transcribe an early mRNA, which can translate small and large T antigens (21), and COS-7 can express both the small and large T antigens (22). We transfected pcDNA 3.0 or TAp63 γ to COS-7 cells and found that TAp63 γ could upregulate both protein and mRNA levels of SV40 small and large T antigens (Fig. 7A and B). These data indicated that the endogenous SV40 small and large T antigens could be activated by TAp63 γ . In addition, TAp63 γ could bind to the SV40 promoter region *in vivo* by a ChIP assay in TAp63 γ overexpressing COS7 cells (Fig. 8B).

PUMA and SV40 T antigens are upregulated by TAp63 γ in 293T cells

It had been found that approximate promoter region –1400 and –2300 on p21 promoter have two p53 binding sites (23). In addition, approximate distal promoter region –4500 of p21 promoter with another p53 binding site binding site (24). In the previous report, there are two p53 binding sites in the first intron of PUMA gene (25). And we found in the distal region of PUMA promoter sequence, which contains two six-base A/T gap core sequences within bases of –2955 to –2827. Over-expression p63 could activate the PUMA distal promoter region reporter in 293T cells, and this activation was dramatically reduced in core sequence mutant with C-to-G or G-to-C (PUMA 6.6g mt) (Fig. 9A). Over-expression TAp63 γ but not p53 could activate protein level of both PUMA and SV40 T antigens (Fig. 9B). We also found TAp63 γ could bind to the both PUMA distal promoter and SV40 promoter region *in vivo* by ChIP assay in TAp63 γ overexpressing 293T cells (Fig. 9C), but p53 couldn't bind to these two sites in p53 overexpressing 293T cells.

Discussion

According to the rules determining the canonical p53 response element parameters, p53-specific regulation sites consisting of 20 bases can be found by *in silico* tools (26–29). One report has described a functional, non-canonical 1.5 $\times$ half-site p53 response element (7). In addition, functional, non-canonical single half-sites only have also been identified (30, 31). Thus, a great amount of effective information from sequences might currently be ignored by *in silico* tools following a strict 20 bases rule. In this study, we found more complex rules of p53 regulation: p53 and p63 can activate not only the canonical two-base A/T gap, but also a non-canonical four- or six-base A/T gap. This finding could extend the entire p53 family regulation network.

Another challenge arises from this new set of extended parameters: how do we design a good *in silico* tool covering the non-canonical 1.5 $\times$ half-sites or single half-sites and recognizes candidates with different A/T gaps? In addition, the A/T gap can be partially substituted by G or C bases (13, 14). Together, these new findings indicate that the entire family of p53 response elements is more complex than previously suspected, and the results certainly increase the difficulty of functional sequence prediction.

p63 had been reported to influence viral transcripts. Human papillomavirus (HPV) early and late transcripts are decreased in p63 knockdown HPV-positive CIN612 cells (32). In this study, we found that TAp63 γ could activate both the SV40 small and large T antigens, and TAp63 γ could activate the SV40 promoter through six- and two-base A/T gap core sequences. The SV40 small and large T antigens are both the oncoproteins in cells. The SV40 small T antigen can activate the cell proliferation and survival signals PI3K and AKT (33), and the SV40 large T antigen can inactivate the tumour suppressors p53 and Rb (34). The SV40 virus is most likely to infect fibroblast cells, such as COS cell lines (19). SV40 infects only a fraction of human fibroblasts (~20%) compared with 100% of monkey cells, so human cell infection has been sometimes referred to as 'semipermissive' (35). In addition, it has been reported that mesothelioma, brain tumours, osteosarcoma and non-Hodgkin lymphoma contain SV40 DNA sequences, and these sequence has been introduced into humans by the SV40-contaminated vaccines (36). Scar and keloid are dermal fibro-proliferative disorders (37), and keloid specimens can express the Δ Np63 γ (38). The p53 amounts in fibroblast cells are highest in normal skin, lower in scars, and lowest in keloids, but the Δ Np63 amounts in fibroblast cells are higher in keloids, lower in scars and lowest in normal skin (39). Although TAp63 amounts are the same in fibroblast cells from normal skin or scars or keloids (39), the upregulated Δ Np63 (especially the γ isoform) may play a role for the disease progression in fibro-proliferative disorders. Δ Np63 isoforms could upregulate specific skin markers much more than TAp63 isoforms, such as Keratin 5 and Keratin 14 (40, 41). In addition, Δ Np63 γ could upregulate the IPTG3 enhancer with all p53 half-site mutation linked to the SV40 promoter at a higher level than TAp63 γ (30). Because some benign

scars or keloids can progress to malignant fibrosarcoma (42, 43), the abnormal expression of Δ Np63 γ in a scar or keloid may promote the SV40-infected fibroblast to express a greater amount of small or large T antigens to become a sarcoma-prone fibroblast.

PUMA is an apoptosis-related gene named as p53-upregulated modulator of apoptosis (44), but we found

that p63 but not p53 bind to PUMA distal promoter in 293T cells (Fig. 9C). This is because 293T cells carry a stably integrated copy of the SV40 genome and stable expression of large T antigen (45), and SV40 large T antigen could bind to p53 DNA binding domain to block its DNA binding activity (46). p63 didn't interact with SV40 large T antigen (47), so p63 still have

Fig. 5 TAp63 γ activated SV40 promoter through six- and two-base A/T gap core sequence. (A) The pGL3 promoter vector with a multiple cloning site and a SV40 promoter located at base 1–41 and bases 48–250 (GenBank: U47298.2). p53, TAp63 α , TAp63 β or TAp63 γ were co-transfected with a pGL3 promoter into H1299 cells, and the relative induction of luciferase promoter activity was measured and normalized to the activity with pcDNA3.0 as 1. p53 can repress the pGL3 promoter, but all of the TAp63 isoforms can activate the pGL3 promoter. The activation function for the pGL3 promoter is strongest for TAp63 γ , weaker for TAp63 β , and weakest for TAp63 α . (B) Bases 217–240 on the pGL3 promoter contain six-, two- and four-base A/T gap core sequences. Various core sequence mutants with C-to-G or G-to-C mutation served as the different A/T gap core sequence mutants. 6g mt: CTTTTTG is mutated to GTTTTTTC; 2g mt: CTAG is mutated to GTAC; 4g mt: CTTTTG is mutated to GTTTTC. All of the promoter mutants were co-transfected with the TAp63 γ expression vector into H1299 cells, and the luciferase promoter activity was compared, normalized to the activity with pcDNA3.0 as 1. TAp63 γ could activate non-mutation control and 4g mt higher than 6g mt and 2g mt, and TAp63 γ could not activate both 6.2.4g del and 6.2g mt. This finding indicated that TAp63 γ could activate the SV40 promoter through six- and two-base A/T gap core sequences.

2X AT 6bp b/w core TCGATAGGCATGTCTAGGCATGTCTAAGCT
 2X AT 5bp b/w core TCGATAGGCATGTCTAGGCATGTCTAAGCT

Fig. 6 TAp63γ could activate both five and six bases between two core sequences, but p53 could only activate six bases between two core sequences. p53 or TAp63γ were co-transfected into H1299 cells with five or six bases between two core sequences of the p53 response element, and the relative induction of luciferase promoter activity was measured and normalized to the activity with pcDNA3.0 as 1. Both p53 and TAp63γ could activate six bases between two core sequences, but only p53 could activate five bases between two core sequences.

Fig. 7 The SV40 small and large T antigens were upregulated by TAp63γ in COS7 cells. (A) After 48 h of transfection time with the pcDNA3.0 or TAp63γ expression vector in COS7 cells, total protein was isolated to assay using a western blot. TAp63γ could be detected by the p63 antibody in TAp63-transfected cells, and the protein expression of the SV40 small and large T antigens in TAp63γ-transfected cells was higher than pcDNA3.0-transfected cells. β-Actin expression was the internal control, and each well was loaded with 20 μg of total protein. (B) In a real-time RT-PCR assay, the mRNA expression of the SV40 small and large T antigens in TAp63γ-transfected COS7 cells was higher than pcDNA3.1-transfected COS7 cells.

Fig. 8 TAp63γ could bind to the SV40 promoter *in vivo* by ChIP assay. (A) Only sonication of the chromatin of TAp63γ-transfected COS7 cells did not match the expected size of chromatin for ChIP assay. The TAp63γ-transfected COS7 cells were treated with MNase, and the chromatin was sonicated. All of the detected chromatin molecular sizes were <200 bp. After these treatments, the chromatin of TAp63γ-transfected COS7 cells were used to perform the ChIP assay. (B) The input was the PCR product of the 10% MNase-treated chromatin of TAp63γ-transfected COS7 cells for IP analysis, and only the p63 antibody but not IgG could precipitate the fragment of the SV40 promoter.

Fig. 9 PUMA was upregulated by Tap63γ in 293T cells. (A) Tap63γ was co-transfected into 293T cells with PUMA distal promoter region reporter, and the relative induction of luciferase promoter activity was measured and normalized to the activity with pcDNA3.0 as 1. Tap63γ could activate PUMA distal promoter region reporter but not core sequence mutant with C-to-G or G-to-C mutation of PUMA distal promoter region. (B) After 48 h of transfection time with the pcDNA3.0 or p53 or Tap63γ expression vector in 293T cells, total protein was isolated to assay. The protein expression of the p21, PUMA and SV40 T antigens in Tap63γ-transfected cells was higher than both pcDNA3.0 and p53-transfected cells. Tubulin expression was the internal control, and each well was loaded with 20 μg of total protein. (C) p63 antibody but not IgG could precipitate the fragment of the both PUMA distal promoter and SV40 promoter in Tap63γ-transfected 293T cells. p53 and IgG could not precipitate the fragment of the both PUMA distal promoter and SV40 promoter in p53-transfected 293T cells.

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Conflict of Interest

None declared.

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