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Advances in Brief

A Promoter that Acquired p53 Responsiveness During Primate Evolution[1]

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Abstract

The tumor suppressor p53 activates the transcription of human *PIG3* through direct interaction with a polymorphic microsatellite sequence, $(TGYCC)_n$. Here, the evolution of this p53-responsive element was recapitulated. Comparison between primate species revealed that the *PIG3* promoter acquired this sequence element in its full length only in *Hominoidea* (apes and humans), whereas the number of TGYCC repeats is far lower in monkeys. Accordingly, only the *PIG3* promoters from *Hominoidea* respond efficiently to p53, whereas those from monkeys respond poorly or not at all. In parallel, the *PIG3* gene was strongly induced by p53 in human and chimpanzee cells but was unaffected by p53 in cells derived from a common marmoset monkey. Thus, a novel p53 target gene appeared as recently as during the evolution of primates. This suggests that mechanisms of tumor suppression are subject to ongoing evolution in humans and their closest relatives.

Introduction

p53 suppresses the formation of malignant tumors through its ability to induce programmed cell death. At least to a large extent, this biological activity is accomplished by the activation of target genes. p53 interacts with the DNA of its target promoters and enhances the transcription of the corresponding genes (1, 2). Human *PIG3* is activated by p53 and was suggested to function in p53-induced apoptosis through accumulation of reactive oxygen species (3). In accordance, p53 mutants that selectively fail to induce apoptosis but not cell cycle arrest also fail to activate *PIG3* but not other p53-responsive genes (4–6).

We have shown previously that a microsatellite within the *PIG3* promoter, $(TGYCC)_n$, with Y = C or T, mediates the induction of *PIG3* by p53 (7). p53 interacts directly and specifically with this sequence element *in vitro* and *in vivo*. This p53-responsive element is unusual not only for its relatively loose correspondence to the canonical p53-responsive consensus $RRRCWWGYYYN_{(0-13)}RRRCWWGYYY$, with *r* = A or G, W = A or T, Y = C or T, and N = any nucleotide. Curiously, the *PIG3* microsatellite is polymorphic in humans, the number of pentanucleotide repeats being 10, 15, 16, and 17. The size of the microsatellite correlates directly with the extent of promoter activation by p53 (7).

Microsatellites are prone to instability in the course of evolution (8). Therefore, we hypothesized that the p53-responsive element within the *PIG3* promoter may differ from the human sequence even in closely related species. To test this, we analyzed the sequence and function of *PIG3* promoters in apes and monkeys. It was found that *PIG3* evolved its p53 responsiveness at the transition from monkeys to apes. These analyses allowed, for the first time, to recapitulate the occurrence of a p53-responsive promoter element during evolution.

Materials and Methods

Plasmids and Adenovirus Vectors. Expression plasmids for p53 (9), as well as a reporter plasmid with the p53-responsive portion of the *PIG3* promoter controlling the expression of luciferase (7), have been described previously.

We obtained reporter constructs containing the *PIG3* promoter of different primate species by PCR amplification of the promoter region, using the primers CTC AGA TCT CAC GGA CAA GTG GGA ATG TAT AGC and CTC TAA GCT TTG CAC GGC TAA CAT ATT GTC TG, from the genomic DNA of different animals, followed by treatment with *Bgl*II and *Hind*III and ligation into pGL3-Basic (Promega).

We generated recombinant adenoviruses to express p53 or control proteins using the AdEasy system (10) as described (11).

Cell Culture and Transfections. H1299 (a human $TP53^{-/-}$ cell line derived from an adenocarcinoma of the lung), CP132 cells (derived from chimpanzee fibroblasts), and G3SV1 cells (derived from ovarian granulosa cells of *Callithrix jacchus*; Ref. 12) were maintained in DMEM (Life Technologies, Inc.), supplemented with 10% FCS. For transfections, 2.5×10^5 H1299 cells were seeded on each well of a six-well dish (Greiner) and transfected with Lipofectamine 2000 (Life Technologies). Cells were harvested 24 h after transfection, followed by luciferase assays (Promega).

Semiquantitative Reverse Transcription-PCR. To analyze the induction of the endogenous *PIG3* by p53, G3SV1 cells were transduced with adenovirus vectors to express either p53 or a control vector expressing the green fluorescent protein. Similar experiments were done with H1299 and CP132 cells. The amount of virus and time of incubation were adjusted in each case to assure that 100% of the cells were transduced, as determined by green fluorescent protein expression. After 24 h (G3SV1 and H1299 cells) or 48 h (CP132 cells), total RNA was prepared (TRIzol Reagent; Life Technologies), followed by reverse transcription with Superscript II polymerase (Life Technologies), using the "RT" primers, and PCR amplification with Expand HiFi DNA polymerase (Roche), using the "forward" and "reverse" primers indicated below. We stopped the reaction after different numbers of temperature cycles and visualized the PCR product by ethidium bromide on an agarose gel. For the analysis of the different transcripts, the following primers were used:

RT MDM2: AAC ATC TGT TGC AAT GTG ATG G
MDM2 forward: TCA GGA TTC AGT TTC AGA TCA G
MDM2 reverse: CAT TTC CAA TAG TCA GCT AAG G
RT p73Δ*N*: CAG GTG GCT GAC TTG GCC GTG CTG,
p73ΔN forward: CGC CTA CCA TGC TGT ACG TCG GTG
p73ΔN reverse: TGC TGG AAA GTG ACC TCA AAG TGG
RT Apaf-1: AGA TCT GAT GTC TTC TCT GAG C
Apaf-1 forward: GTG TTA CAG ATT CAG TAA TGG G
Apaf-1 reverse: CTG AAG CTT CCC AGC GAT TGG G
RT GAPDH:[3] GGT TCA CAC CCA TGA CGA ACA TG
GAPDH forward: TGA AGG TCG GAG TCA ACG GAT TTG GT
GAPDH reverse: GCA GAG ATG ATG ACC CTT TTG GCT C

For the analysis of the *PIG3* expression on *C. jacchus*, the primer GCG CCT TGC TGT GCT GGT CTC C was used for reverse transcription, and the primers TGC TAC TGG GAC CCG CAA GAG C and CGT GCT CCT GCC TGG GAG TTC C were used for PCR amplification. For the amplification of the *PIG3* transcript from H1299 and CP132 cells, we used the primer CGG

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[3] The abbreviation used is: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

***Homo sapiens* (15)**	TGCCC TGTCC TGTCC TGCCC TGCCC TGTCC TGTCC TGCCC TGCCC TGCCC TGTCC TGTCC TGCCC TGCCC TGCCC
***Pan troglodytes* (16)**	TGCCC TGCCC TGCCC TGCCC TGCCC TGCCC TGTCC TGTCC TGTCC TGCCC TGCC**A** TGTCC TGTCC TGCCC TGCCC TGCCC
***Pan paniscus* (19)**	TGCCC TGCCC TGCCC TGCCC TGCCC TGCCC TGCCC TGCCC TGTCC TGTCC TGTCC TGCCC TGCC**A** TG**G**CC TGTCC TGCCC TGCCC TGCCC TGCCC
***Gorilla gorilla* (17)**	TGCCC TGTCC TGCCC TGCCC TGCCC TGCCC TGCCC TGTCC TGTCC TGTCC TGTCC TGCCC TGTCC TGCCC TGCCC TGCCC TGCCC
***Pongo pygmaeus* (14)**	TGCCC TGCCC TGCCC TGCCC TGCCC TGTCC TGTCC TGTCC TGTCC TGCCC TGTCC TGTCC TG**A**CC TGCCC
***Hylobates lar* (16)**	TGCCC TGTCC TGTCC TGCCC TGCCC TGCCC TGCCC TGTCC TGCCC TGTCC TGCCC TGTCC TGCC**T** TGCCC TGCCC TGCCC
***Macaca mulatta* (6)**	TGCCC TGC**T**C TGCCC TGTCC TGT**T**C TGCCC
***Colobus guereza* (6)**	TGCCC TGC**T**C TGCCC TGTCC TGT**T**C TGCCC
***Callicebus cupreus* (5)**	TGCCC TGCCC T**T**TCC TGTCC TGCCC
***Cebus apella* (5)**	TGCCC TGCCC T**T**TCC TGTCC TGCCC
***Callimico goeldii* (5)**	TGCCC TGCCC T**T**TCC TGT**T**C TGCCC
***Callithrix jacchus* (5)**	TGCCC TGCCC T**T**TCC TGT**T**C TGCC**T**

Fig. 1. Sequence of the *PIG3* microsatellite in different primates. The *PIG3* promoter region was amplified from the genomic DNA obtained from one individual of each indicated species, followed by cloning into a luciferase reporter plasmid (pGL3-Basic) and determination of the sequence. The repetitive sequences contained within the *PIG3* promoters are shown. The number of TGYCC repeats is indicated in *brackets*. Nucleotides that deviate from the TGYCC consensus are shown in *bold*.

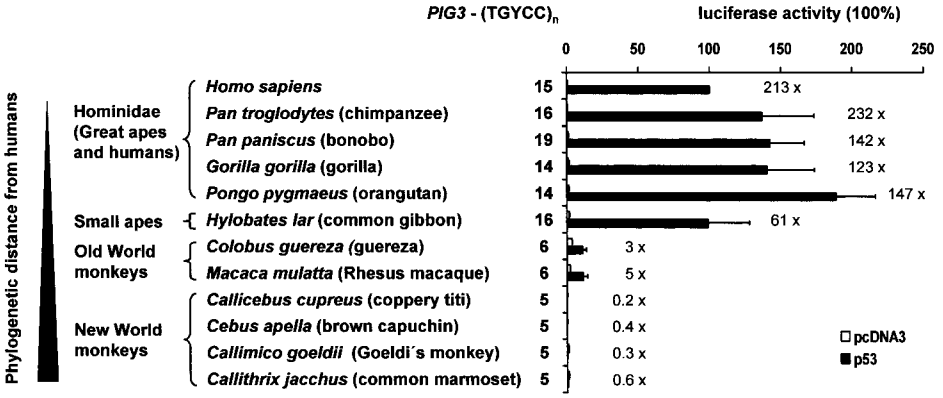

Fig. 2. The *PIG3* promoter and its induction by p53 in primates. The species of primates studied are indicated, in order of increasing distance to humans,[4] along with the corresponding TGYCC repeat numbers. The 15 repeats indicated for *Homo sapiens* correspond to the most frequently found allele (7). The reporter constructs described in the legend to Fig. 1 were transfected into H1299 cells ($TP53^{-/-}$), along with an expression plasmid for p53 (*filled bars*) or a corresponding empty vector construct (pcDNA3, *open bars*), as described previously (7). The luciferase activity obtained with p53 and the human *PIG3* promoter (with 15 TGYCC repeats) was set at 100%, and the other values were normalized accordingly. The average of at least three independent experiments is shown along with the SE in each case. The ratio of luciferase activities in the presence *versus* the absence of p53 (fold activation) is indicated at each *row*.

TGA GCA GGC CTC TGG GAT GGC for reverse transcription and the primers GTG CAC TTT GAC AAG CCG GGA GGA and CAG CCT GGG TCA GGG TCA ATC CCT for PCR amplification.

Results and Discussion

The *PIG3* Microsatellite Sequence in Apes and Monkeys. As the number of TGYCC repeats varies even within one single species (human), we investigated the diversity of this number in related species. We amplified and sequenced the *PIG3* promoter region of several primate species (Fig. 1). Great apes (*Pan troglodytes, Pan paniscus, Gorilla gorilla,* and *Pongo pygmaeus*) and the small ape *Hylobates lar* showed 14–19 TGYCC repeats, a number comparable with the one present in humans. In contrast, only 6 repeats were found in Old World monkeys (*Macaca mulatta,* and *Colobus guereza*) and 5 repeats in New World monkeys (*Callicebus cupreus, Cebus apella, Callimico goeldii,* and *C. jacchus*). Furthermore, the number of mismatches (pentanucleotide elements containing one nucleotide that does not correspond to the TGYCC consensus) was higher in Old and New World monkeys. These results indicate that the size of the *PIG3* microsatellite decreases rapidly with the phylogenetic distance to humans.

Response of *PIG3* Promoters from Monkeys and Apes to p53. A reduced number of TGYCC repeats results in a weaker interaction with p53 (7). Therefore, we addressed the question whether the shorter *PIG3* microsatellite observed in Old and New World monkeys also corresponds to a weaker promoter response to p53. The induction of the *PIG3* promoter by p53 was quantified by reporter assays. As shown in Fig. 2, transactivation by p53 was strong when the *PIG3* promoters of human, great ape, and gibbon species were analyzed. In contrast, the promoters from both Old World monkeys were only weakly inducible, and those from New World monkeys were not responsive at all. Thus, the response of the *PIG3* promoter to p53 was acquired during the evolution of *Hominoidea*.

Response of *PIG3* to p53 in Monkey Cells. To test if p53 also fails to induce the endogenous *PIG3* of a New World monkey, we used a granulosa cell line derived from the marmoset *C. jacchus* (12). These cells were transduced to overexpress p53, and we then analyzed the levels of several transcripts by reverse transcription-PCR. As shown in Fig. 3, the p53-responsive genes *p73ΔN* and *MDM2* were induced in the presence of p53, and the same was found for the p53-responsive and apoptosis-related gene *Apaf-1*. In contrast, no induction was observed for *PIG3,* as in the *GAPDH* control. When using human H1299 cells, or CP132 cells from the chimpanzee *P. troglodytes, PIG3* was clearly activated by p53. We conclude that the endogenous *PIG3* in marmoset cells is not p53 responsive.

Concluding Remarks and Perspectives. p53 and its functions as a regulator of transcription and inducer of programmed cell death are conserved from *Caenorhabditis elegans* to humans (13). Many target genes, including *CDKN1A* (*p21*), *MDM2, TNFRSF6* (*Fas*), *bax, Apaf-1,* and *PIG8,* respond to p53 in murine as well as in human cells (14–16). In contrast, *PIG3* evolved its p53 responsiveness during the

[4] Internet address: http://warprc.org/psic/taxonomy.asp.

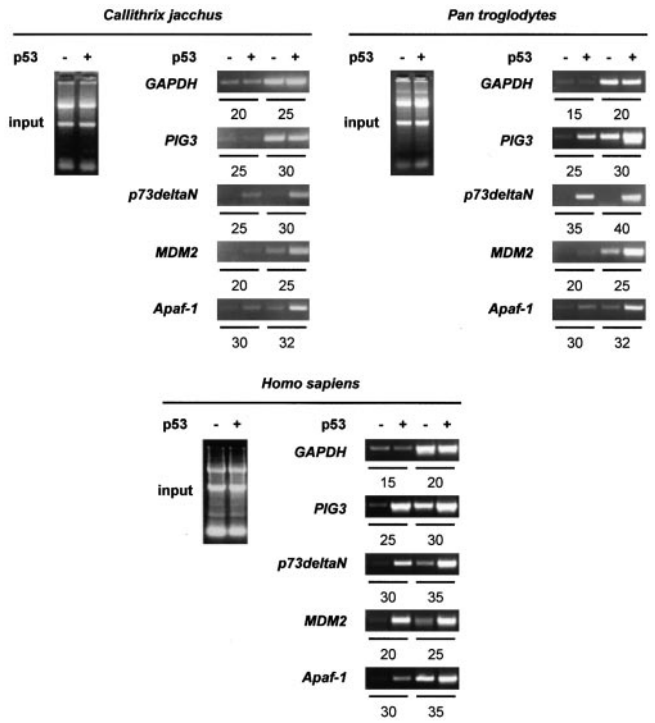

Fig. 3. Inducibility of the endogenous *PIG3* gene in marmoset cells. A cell line derived from granulosa cells of *C. jacchus* (G3SV1), the human cell line H1299, or the cell line CP132 from a chimpanzee (*P. troglodytes*) was transduced by adenovirus vectors with (+) and without (−) an expression cassette for p53. Total RNA was then prepared and analyzed by agarose gel electrophoresis (*input*). The indicated mRNA species was reverse transcribed, followed by semiquantitative PCR amplification, as described (7, 21). The reaction was stopped after the indicated number of temperature cycles, and the PCR products were analyzed by agarose gel electrophoresis.

most recent stages of evolution and, to our current knowledge, represents the phylogenetically youngest p53 target gene. Its regulation by p53 is unique to humans and apes, raising some caution about the analogies drawn between most animal models and human cancer. Differences in gene expression levels, rather than the structure of gene products, have long been proposed to be largely responsible for the different biological properties of distinct primate species (17, 18). However, examples of genes that are differentially regulated among primates are rare (19, 20), and the mechanistic basis of the differences in regulation is poorly understood. To our knowledge, *PIG3* represents the clearest available example of a mechanistically explained difference in promoter regulation between primate species. Mechanisms of growth regulation have been established since the earliest stages of life, and molecular functions of tumor suppression are well established throughout mammalian species. Nonetheless, it appears that such mechanisms are still subject to ongoing evolution in humans and their closest relatives.

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