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p53 regulates the expression of human angiotensin II AT_2 receptor interacting protein (ATIP1) gene

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Abstract. Angiotensin II AT_2 receptor interacting protein 1 (ATIP1) has been recently identified as a tumor suppressor. In the present study, a 2.2 kb fragment of the 5' flanking region of the human ATIP1 gene was cloned, and its promoter activity was confirmed. Two putative p53 binding sites were identified in the minimal promoter. Cisplatin treatment and ectopic expression of p53 led to enhanced ATIP1 expression. Knockdown of p53 reduced the ATIP1 expression. The direct interaction of p53 and the ATIP1 promoter was confirmed by reporter gene and chromatin-immunoprecipitation assays. When the p53 sites were mutated, the effect of p53 on ATIP1 promoter was eliminated. The results suggest that the ATIP1 gene is regulated by p53 at the transcriptional level, and that it may play an important role in cancer initiation and progression.

Introduction

Angiotensin II AT_2 receptor interacting protein (ATIP, also known as mitochondrial tumor suppressor gene 1, or MTUS1) is known to be a tumor suppressor gene that resides in the LOH region (8p21.3-p22) frequently observed in a number of cancer types (1-6). Alternative exon utilization leads to the generation of four known transcript variants and four different protein isoforms. Based on the observed differential tissue distribution of the ATIP variants, it has been hypothesized that the transcription of the variants are controlled by different gene promoters, and that their expression may be regulated by different molecular mechanisms. This hypothesis has been confirmed in a recent study, in which four independent gene promoters were cloned and functionally validated (7).

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ATIP1 is the first transcript variant of the ATIP gene to be cloned in two separate laboratories. ATIP1 is widely expressed in various tissues (8), and is transiently up-regulated during the initiation of cell differentiation and quiescence (6). Functional analyses indicate that ATIP1 is an early component of the growth-inhibiting signaling cascade that interacts with the angiotensin II AT_2 receptor. It inhibits EGF-mediated ERK kinase activation and cell proliferation in an AT_2 receptor-dependent manner (6,9,10). Although the tumor suppressor function of this gene has been defined, little is known about the molecular mechanisms that regulate the expression of this tumor suppressor gene. We previously cloned a 0.7 kb fragment of the 5' flanking region of the ATIP1 gene and demonstrated its promoter activity in oral cancer cell lines (7). In the present study, we aim to further characterize this gene promoter to gain a better understanding of the regulatory mechanisms of the tumor suppressor gene.

Materials and methods

Cell culture. Oral squamous cell carcinoma cell lines (UM1 and UM2) and an immortalized oral keratinocyte NOK16B cell line were maintained in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). WI38 cells were maintained in EMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37˚C in a humidified incubator containing 5% CO_2.

Luciferase reporter assay. Serial deletion of the ATIP1 promoter fragments were produced by PCR amplification using a common antisense primer located at 117 bp downstream of the transcription starting site (5'-TGTCCTCGAGCCAAGTGTTTCTGGCTTCCGT-3'), and the sense primers: 5'-CTGAGGTACCACCAGGAGGCAGACTATGTT-3' (-2070 construct), 5'-CTGAGGTACCTGTGAGGTCCTCAGCATGTG-3' (-733 construct), 5'-CTGAGGTACCTGTGAGGTCCTCAGCATGTG-3' (-448 construct), and 5'-CTGAGGTACCGCGATCTTTCAAGTGGTTACA-3' (-124 construct). The PCR products were then cloned into the *Kpn*I/*Xho*I sites of the pGL4.10 vector. The

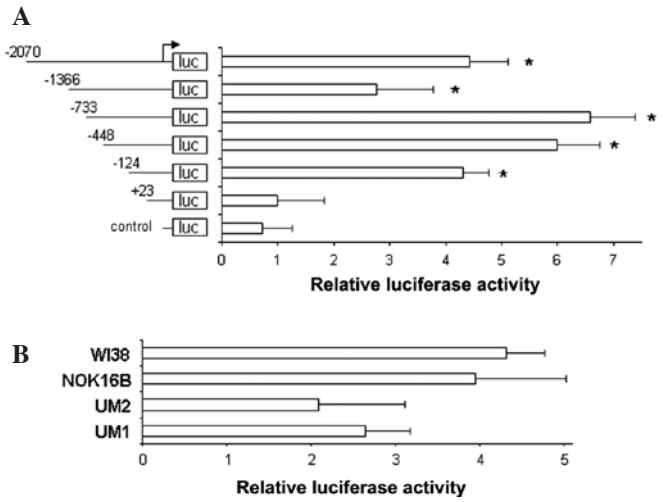

Figure 1. The promoter activity of human ATIP1 gene. (A) Serial deletions of the 5' flanking sequence of the ATIP1 gene were inserted into the luciferase reporter constructs as described in Materials and methods. Numbers indicate the length of the respective constructs in the base pairs. The constructs were transiently transfected into WI38 cells and the luciferase reporter assays were performed to test relative promoter activities. *p<0.05, statistically significant difference in the promoter activity when compared to the control (empty vector). (B) The luciferase reporter construct containing the fragment from -124 to +117 bp of the ATIP1 gene promoter and an empty vector (as reference) were transfected into WI38, NOK16B, UM1 and UM2 cells and the luciferase reporter assays were performed to test relative promoter activities. Data represent at least three independent experiments with similar results.

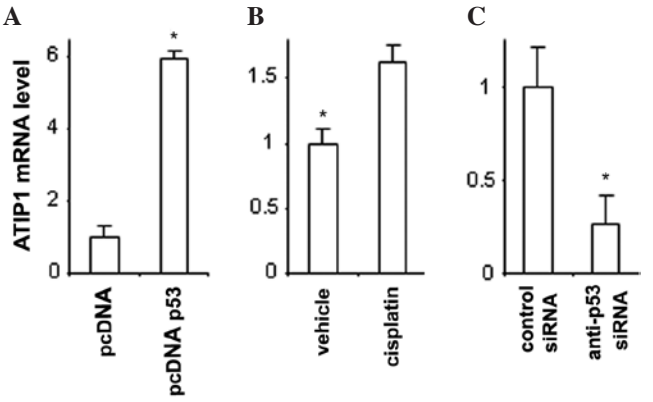

Figure 2. ATIP1 gene expression is regulated by p53. WI38 cells were transfected with either (A) p53 expression vector or an empty vector (pcDNA), (B) incubated with cisplatin or vehicle alone, or (C) treated with anti-p53 or control siRNA. Quantitative RT-PCR was performed to measure the expression of the ATIP1 gene. Data represent at least three independent experiments with similar results. *p<0.05.

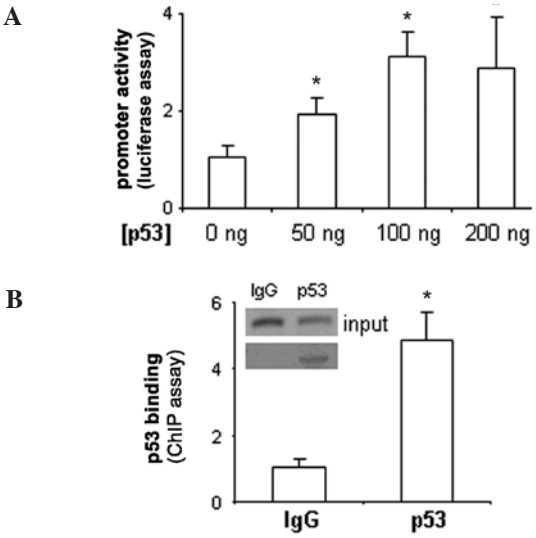

Figure 3. p53 interacts with the ATIP1 gene promoter. (A) The luciferase reporter construct containing the fragment from -124 to +117 bp of the ATIP1 gene promoter was co-transfected into WI38 cells with increasing concentrations of a p53 expression vector. The luciferase reporter assays were performed to assess the relative promoter activities. (B) Chromatin-immunoprecipitation (ChIP) assays were performed using either p53 antibody or control IgG. Quantitative PCR was performed to measure the relative enrichment of the DNA fragment representing the ATIP1 gene promoter in the IP products. Insert: gel images of the PCR products that terminated in the log phase of the amplification (semi-quantitative PCR analysis of the ChIP products). Data represent at least three independent experiments with similar results. *p<0.05.

+23 construct was created by annealing synthesized sense and antisense oligonucleotides (Sigma-Genosys, St. Louis, MO, USA) corresponding to +23 to +117 of the promoter fragment and then inserted into the *Kpn*I/*Xho*I sites of the pGL4.10 vector. The -1366 construct was created by PCR amplification of the -1366 to +117 bp of the promoter fragment (sense primer: 5'-CAAGGTTTTGGGAGGTAGAGG-3' and antisense primer: 5'-CCAAGTGTTTCTGGCTTCCGT-3'). The PCR products were cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA), then subcloned into the pGL4.10 vector. The p53 site mutation constructs were created based on the -124 construct in which the predicted p53 site (from -7 to +3) was mutated to TCTTCGAACG (p53m1 construct), the second p53 site (from +12 to +21) was mutated to GAGTCGATTC (p53m2 construct), or both p53 sites were mutated (p53m12 construct). The constructs were verified by sequencing and transiently transfected into cells using lipofectamine 2000 (Invitrogen). The pGL4.74 vector (Promega, Madison, WI, USA) was co-transfected as an internal control for the normalization of transfection efficiency. After 48 h, transfected cells were harvested with ice-cold phosphate-buffered saline (PBS), and dual luciferase assays were performed according to the manufacturer's protocol using a Glomax 20/20 luminometer (Promega).

Effect of p53 on ATIP1 expression. To test the effect of p53 on ATIP1 expression, a p53 expression vector (Addgene plasmid 10838, Addgene Inc., Cambridge, MA, USA), empty pcDNA3.0 vector (Invitrogen), siRNA specific to p53 (Cell Signaling, MA, USA) or control siRNA were transfected as previously described (11). To test the effect of DNA damage on ATIP1 expression, 50 nm of cisplatin (cis-diamminedichloroplatinum II; Sigma-Aldrich, St. Louis, MO, USA) or vehicle alone was incubated with the WI38 cells for 4 h. Total RNA from the cells was isolated using an RNeasy mini kit (Qiagen, Germany). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed as previously described (12) to evaluate the expression of ATIP1 gene. In brief, RNA was converted to first-strand cDNA using

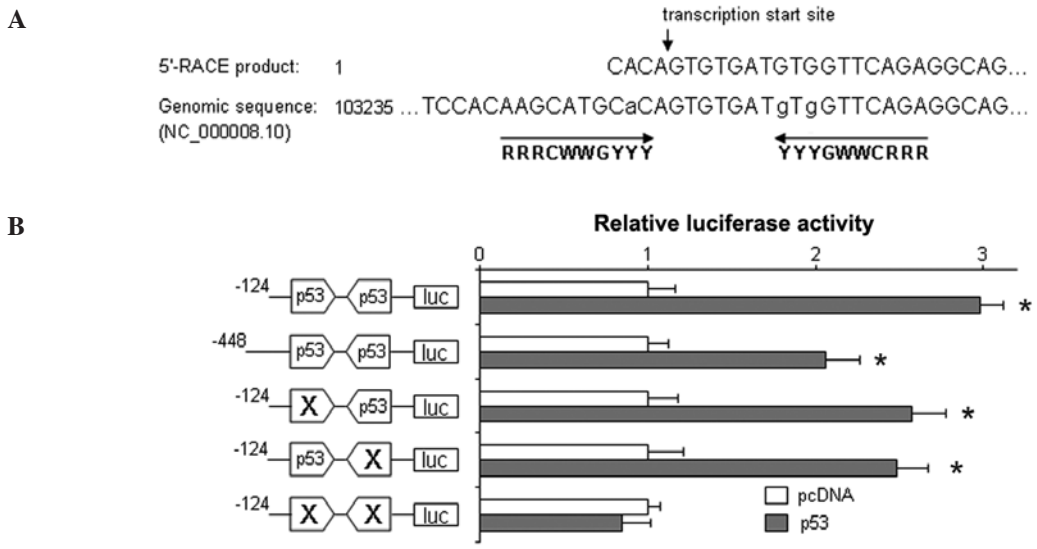

Figure 4. ATIP1 promoter activity is regulated by two p53 binding sites located in the minimal promoter. (A) Two putative p53 binding sites (underline) were identified in the minimal promoter of the ATIP1 gene. The first binding site overlaps with the intron/exon junction (-7 to +3). The second binding site is located in the exon (+12 to +21). The consensus sequence was shown below the identified p53 sites (R=A/G, W=A/T and Y=C/T). 5' RACE assay was carried out to confirm the start of the exon (transcription start site). (B) The mutation luciferase constructs were created by mutating one or both p53 binding sites from the -124 construct. These mutant constructs and wild-type -124 and -448 constructs were co-transfected into the cells with a p53 expression vector or an empty vector (pcDNA). The luciferase reporter assays were performed to test relative promoter activities. Data represent at least three independent experiments with similar results. *$p<0.05$.

Thermoscript reverse transcriptase and random hexamers (Invitrogen). The quantitative PCR was performed using an ABI 7900HT Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primer sets specific to ATIP1 (forward: 5'-TCCATGGAAATTGCCTCTTC-3', and reverse: 5'-AGTCGAAGGTTTCGAAGCAA-3') were used. Reactions were performed in triplicate. The melting curve analyses were performed to ensure the specificity of the qRT-PCR reactions. The data analysis was performed using $2^{-\Delta\Delta Ct}$ as previously described (13), where β-actin was used as the reference gene.

ChIP assays. The chromatin-immunoprecipitation (ChIP) assays were performed with a SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling) according to the manufacturer's protocol with a TransCruz reagent for p53 ChIP analysis (rabbit polyclonal p53 antibody) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit IgG was used as the control. Quantitative PCR was performed as described above using the primers: forward: 5'-TCCTGAGACCACCCACTTTC-3', and reverse: 5'-AATCTCCCTCCTGCAGGTCT-3', which were located around the predicted p53 sites in the ATIP1 promoter The input chromatin samples obtained prior to the IP experiment were used as a reference. Semi-quantitative PCR analyses were also performed to assess the recruitment of p53, in which PCR reactions were terminated at the log phase of the amplification (25 cycles). The resulting PCR products were separated and visualized on a gel.

5' RACE. To characterize the 5' untranslated region (5'-UTR) of the ATIP1 transcript, 5' RACE assays were carried out using mRNA isolated from NOK16B cells and a FirstChoice® RLM-RACE kit (Ambion Inc., Austin, TX, USA) as previously described (7). Gene-specific outer (5'-GCAGGTGGCGAGATT TCACA-3') and gene-specific inner primers (for nested PCR: 5'-CCAAGTGTTTCTGGCTTCCGT-3') were used. Outer and inner forward primers supplied in the FirstChoice RLM-RACE kit were used for 5' RACE. The RACE products were PCR amplified, gel purified and then sequenced.

Results and Discussion

We cloned a 2.2 kb fragment of the 5' flanking region of the ATIP1 gene, from 2070 bp upstream of the transcription start site (-2070) to 117 bp downstream of the transcription start site (+117). As shown in our serial deletion experiment (Fig. 1A), when the five constructs (the -2070 to +117 bp, -1366 to +117 bp, -733 to +117 bp, -448 to +117 bp, or the -124 to +117 bp sections of this fragment) were inserted into the luciferase reporter gene constructs, compatible promoter activities were observed for the five constructs. The minimal promoter appears to be located at the -124 to +117 bp section of this fragment. A significant reduction in promoter activity was observed when the -124 to +22 segment was truncated from the minimal promoter. This minimal promoter is functional in WI38 cells (embryonic lung fibroblasts), UM1 and UM2 cells (oral squamous cell carcinoma cell lines) and NOH16B cells (an immortalized oral keratinocyte cell line) (Fig. 1B). The ATIP1 promoter activity has also been independently confirmed in a breast cancer cell line (MCF7) and a human endothelial cell (EA.hy926) (14). These results suggest that the promoter for the ATIP1 gene is ubiquitous (independent of cell type). This finding correlates with the previous observation that ATIP1 is widely expressed in a variety of tissues (8).

As shown in Fig. 2A, the ectopic expression of p53 led to up-regulation of the ATIP1 gene expression. Similarly, when cells were treated with the DNA-damaging agent, cisplatin, an enhanced ATIP1 expression was observed (Fig. 2B). In contrast, knockdown of p53 with siRNA led to a reduced

ATIP1 gene expression (Fig. 2C). Fig. 3A shows a p53-induced dose-dependent increase of promoter activity in cells transfected with the luciferase construct containing the minimal promoter of the ATIP1 gene. The direct interaction of p53 and the ATIP1 promoter was further confirmed by the ChIP assay (Fig. 3B). To the best of our knowledge, this is the first study showing the direct interaction of p53 with the ATIP1 gene promoter.

Two putative p53 binding sites were identified in the minimal promoter of the ATIP1 gene (Fig. 4A). The first p53 binding site overlaps with the predicted transcript start site (-7 to +3). The second binding site is a reversed p53 binding site, and is located immediately downstream of the transcript start site (+12 to +21). We have already confirmed the predicted transcript start site of the ATIP1 gene, which is orthologously conserved (7). An additional transcription start site has been detected in cardiac cells (left ventricle), which is located 10 bp upstream of the conserved transcription start site (14). However, this alternative transcription start site was not detected in our cell line using 5' RACE. This difference may be cell-type-specific. Thus, to functionally confirm these p53 sites located in the minimal promoter, we constructed a series of mutation constructs. As shown in Fig. 4B, when either of the p53 sites was mutated, the effect of p53 on promoter activity was largely preserved. However, when the two sites were mutated, the effect of p53 on the promoter was eliminated.

The results from our study provide evidence suggesting a potential correlation between p53 and the signaling pathway(s) mediated by the angiotensin II AT_2 receptor. ATIP1 has been shown to be involved in the trans-inactivation of the EGF receptor and the subsequent inhibition of extracellular-regulated ERK kinase activity and cell proliferation (6,9,10). The present study indicated that p53 may also be involved in the regulation of crosstalk between EGF signaling and angiotensin II AT_2 receptor signaling. Further in-depth analysis is required to thoroughly assess the functional relevance of these interactions and their contributions to tumorigenesis.

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