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Research article

The Alzheimer's disease-associated *TREM2* gene is regulated by p53 tumor suppressor protein

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ABSTRACT

TREM2 mutations evoke neurodegenerative disorders, and recently genetic variants of this gene were correlated to increased risk of Alzheimer's disease. The signaling cascade originating from the TREM2 membrane receptor includes its binding partner TYROBP, BLNK adapter protein, and SYK kinase, which can be activated by p53. Moreover, *in silico* identification of a putative p53 response element (RE) at the *TREM2* promoter led us to hypothesize that *TREM2* and other pathway elements may be regulated in p53-dependent manner. To stimulate p53 in synergistic fashion, we exposed A549 lung cancer cells to actinomycin D and nutlin-3a (A + N). In these cells, exposure to A + N triggered expression of TREM2, TYROBP, SYK and BLNK in p53-dependent manner. *TREM2* was also activated by A + N in U-2 OS osteosarcoma and A375 melanoma cell lines. Interestingly, nutlin-3a, a specific activator of p53, acting alone stimulated *TREM2* in U-2 OS cells. Using *in vitro* mutagenesis, chromatin immunoprecipitation, and luciferase reporter assays, we confirmed the presence of the p53 RE in *TREM2* promoter. Furthermore, activation of *TREM2* and *TYROBP* by p53 was strongly inhibited by CHIR-98014, a potent and specific inhibitor of glycogen synthase kinase-3 (GSK-3). We conclude that *TREM2* is a direct p53-target gene, and that activation of *TREM2* by A + N or nutlin-3a may be critically dependent on GSK-3 function.

1. Introduction

Genetic variants of *TREM2* modulate the risk of Alzheimer's disease and other neurodegenerative disorders. Under physiological conditions, the cell surface receptor TREM2, is expressed on immune cells including microglia. Upon ligand binding, TREM2 connects to TYROBP, inducing TYROBP phosphorylation and activation of a SYK kinase [1]. Activated SYK phosphorylates variety of substrates that promote cell survival [2], at times guided by the adapter protein, BLNK [3]. Thus, TREM2, TYROBP, SYK and BLNK comprise part of a signaling pathway. The *SYK* gene can be upregulated by p53 [4], a pleiotropic transcriptional regulator [5], and the *TREM2* gene contains a potential p53 RE, detected *in silico,* close to the transcription start site [6]. Hence, we hypothesized that p53 could stimulate expression of *TREM2* and other genes in this pathway. As the p53-activating stimulus, we employed the co-treatment of cells with actinomycin D and nutlin-3a, which in our previous experiments demonstrated synergistic activation of p53 [7].

2. Material and methods

2.1. Cell culture and treatment

A549 (lung adenocarcinoma, American Type Culture Collection [ATCC]), U-2 OS (osteosarcoma, ATCC) and A375 (melanoma, ATCC) cells were grown as previously described [7].

The stock solutions of chemicals were prepared in DMSO: actinomycin D (10 μM; Sigma-Aldrich, St. Louis, MI, USA), camptothecin (10 mM; Calbiochem-Merck, Darmstadt, Germany), nutlin-3a (10 mM; Selleck Chemicals LLC, Houston, TX, USA), CHIR-98014 (5 mM, Selleck Chemicals LLC). Stock solutions were diluted in culture medium to concentrations: 5 nM actinomycin D, 5 μM nutlin-3a, and 5 μM camptothecin. CHIR-98014 was diluted to concentrations indicated in Results. Control cells were mock-treated with medium containing DMSO. Control and knockdown p53 A549 cells were prepared previously utilizing lentivirus-delivered shRNA molecules [8].

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2.2. Semi-quantitative real-time PCR

Total RNA samples were prepared using RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized with MuLV reverse transcriptase and random hexamers (Applied Biosystems, Foster City, CA, USA). Measurements of *TREM2* and *ACTB* (internal reference) mRNA levels were performed using Real-Time 2 × PCR Master Mix SYBR (A&A Biotechnology, Gdynia, Poland) and the following oligonucleotide primers: TREM2-Q1 (5′-ACGCTGCGGAATCTACAACC), TREM2-Q4 (5′-CAGGAGGAGAAGGATGGAAGT) (Genomed, Warsaw, Poland), β-actin (5′-GCAAGCAGGAGTATGACGAG) and (5′-CAAATAAAGCCATGCCAATC) (BioTeZ Berlin-Buch, Germany). Amplification was performed on CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). In each PCR run, cDNA samples were amplified in triplicate. Relative quantitation of mRNA was performed using the ΔΔCT method with β-actin as a reference. Means and standard deviations were calculated from three independent treatments.

2.3. Western blotting

Whole-cell lysates were prepared using IP buffer, supplemented with protease and phosphatase inhibitors as described previously [7]. Lysates (35–50 μg) were separated by SDS-PAGE on 8% or 13% gels and electrotransferred onto PVDF membranes. The membranes were incubated for 1 h at room temperature in blocking solution (5% skim milk in PBS with 0.1% Tween-20 or 5% bovine serum albumin in PBS with 0.1% Tween-20 for detection of TYROBP). The following primary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA): anti–phospho-Ser46 p53, anti-phospho-Ser392 p53, anti-BLNK (D3P2H), anti-TYROBP (DAP12) (D7G1X), anti-SYK (D3Z1E), anti-TREM2 (D8I4C). Anti-p53 (DO-1), and loading control anti-HSC70 (B-6) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). All incubations with primary antibodies were performed overnight at 4 °C in blocking solution. HRP-conjugated secondary antibodies were detected by chemiluminescence (SuperSignal West Pico or SuperSignal West Femto Chemiluminescent substrate, Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Molecular cloning, in vitro mutagenesis and luciferase reporter assay

The promoter of *TREM2* was cloned into the pGL3-Basic vector, which encodes firefly luciferase (Promega, Madison, WI, USA). The relevant DNA fragment was amplified by PCR from a genomic DNA sample (A549 cells) using primers (5′-TTTTGAGCTCTGCTCCTGATAACCCTTTGC) and (5′-TTTTCTCGAGCAGAAGCAG AGTGCCTTGT) with attached restriction sites (underlined) for SacI and XhoI, respectively. Amplified DNA was ligated into the SacI and XhoI sites of pGL3-Basic. PCR was performed with PfuPlus! DNA polymerase mix (EURx, Gdańsk, Poland) to ensure high fidelity DNA amplification. The inserted DNA was sequenced to ensure that the clone contained no mutations.

Mutations in the p53 RE within the *TREM2* promoter were introduced with complementary forward (5′-GTGGGCAGCGCCTGAGCGTCCTGATCCTCTCTTT) and reverse (5′-AAAGAGAGGATCAGGACGCTCAGGCGCTGCCCAC) primers using the GeneArt Site-Directed Mutagenesis PLUS kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions.

U-2 OS cells were seeded onto 24-well plates. The next day, the cells were co-transfected using FuGene6 (0.6 μl per well, Promega) with a combination of pGL3-Basic containing the *TREM2* promoter (wild-type or mutant, 0.1 μg), and the expression vector pC53-SN3, encoding wild-type p53 (0.1 μg), pC53-SCX3 encoding Val143Ala p53 mutant (a gift from Bert Vogelstein and Kenneth W. Kinzler from Johns Hopkins University, Baltimore, MD, USA, [9]) or pC53-190 encoding Pro190Arg p53 mutant created by us as described previously [10]. As a negative control, the p53 plasmid was replaced by empty vector. The transfection mixture also contained pRL-TK (0.01 μg), encoding *Renilla* sp. luciferase under the control of the herpes simplex virus thymidine kinase promoter, which served as an internal control. The test was performed as described previously [10].

2.5. ChIP-PCR assay

A549 cells were mock-treated or incubated with A + N for 30 h and subsequently washed with PBS and fixed with 1% formaldehyde in 1 x PBS for 10 min at room temperature. Fixation was quenched by glycine (125 mM) and nuclei were isolated using buffers and protocol from iDeal ChIP-seq Kit for Transcription Factors (Diagenode, Seraing, Belgium). Chromatin was re-suspended in 200 μl and sheared using the Bioruptor® PLUS combined with the Bioruptor® Water cooler & Single Cycle Valve (at HIGH power setting) with 15 cycles of 30 s shearing followed by 30 s of standby; chromatin fragments with approximate length 100–600 bp were obtained. Chromatin immunoprecipitation was performed using the iDeal ChIP-seq Kit for Transcription Factors (Diagenode) with 3 μl anti-p53 polyclonal antibody (C15410083, Diagenode) or control rabbit IgG (C15410206, Diagenode), according to the manufacturer protocol. To verify efficiency of immunoprecipitation, the human *CDKN1A* gene promoter was amplified by Q-PCR using specific primers covering the known p53 motif: forward, 5′-GACACCACTGGAGGGTGACT; reverse, 5′-CAGGTCCACATGGTCTTCCT. The following primers were used to amplify the *TREM2* promoter fragment with the tested p53 binding site: TREM2-CHIP-F1 (5′-CCAGACCCCAGTCCTGACTATT) and TREM2-CHIP-R1 5′-TTGTGCAAGATCTCGTC TTTCC.

3. Results

3.1. A + N treatment stimulates TREM2, TYROBP, SYK and BLNK genes in a p53-dependent manner

A549 cells were treated for 48 h as shown in Fig. 1A. Actinomycin D and nutlin-3a synergistically induced phosphorylation of p53 on Ser46, a marker of strong p53 activation [7] and on Ser392, which stabilizes the tetramer formed by p53 molecules [11] and may promote the expression of a subset of p53-regulated genes [5]. Consistent with our hypothesis, A + N treatment resulted in strong upregulation of TREM2 and other pathway elements, including TYROBP, SYK and BLNK. To determine whether upregulation of these proteins was p53-dependent, we performed a time-course experiment using p53 knockdown A549 cells [8]. Both p53 knockdown and control cells were exposed to A + N for 12–48 h (Fig. 1B). Neither TREM2, TYROBP, SYK nor BLNK were upregulated in p53-knockdown cells following A + N treatment. Thus, upregulation of these proteins in A549 cells exposed to A + N is p53-dependent.

Next, we focused on the expression of *TREM2*, which encodes the most upstream component of the signaling pathway. Quantitative RT-PCR demonstrated that *TREM2* mRNA accumulated in response to p53 stimulating agents (Fig. 1C). Thus, even actinomycin D alone resulted in 100-fold upregulation of *TREM2* mRNA expression levels. It must be stressed that actinomycin D, at the concentration used here, does not induce general inhibition of transcription, but rather generates nucleolar stress that activates p53 [7]. To further test the hypothesis that *TREM2* is upregulated in p53-dependent manner, we examined RNA levels in p53 knockdown cells and corresponding controls (Fig. 1D). Consistently, p53 knockdown prevented the strong accumulation of *TREM2* mRNA typically mediated by A + N or camptothecin treatment.

3.2. TREM2 accumulates following A + N treatment in cells of various origins

We selected U-2 OS and A375 cell lines with wild type *TP53* to test whether other cells could upregulate *TREM2* following A + N treatment (Fig. 2). In all three cell lines TREM2 strongly accumulated following