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Research Article

The WRAP53α gene undergoes both transcriptional and post-transcriptional regulation in response to DNA damage

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ABSTRACT

The Wrap53α mRNA transcript regulates expression of the p53 tumor suppressor gene by binding to the 5'-untranslated region of the p53 mRNA transcript. The binding of Wrap53α mRNA, which we demonstrate here is induced in response to a variety of DNA damaging agents, stimulates translation of the p53 mRNA, which increases the levels of active p53 protein in the cell. This allows the cell to respond to DNA damage through a p53-mediated cell cycle arrest or apoptosis. In order to determine whether the Wrap53α gene is regulated at the transcriptional and/or post-transcriptional level we carried out two sets of experiments. In one, we cloned a region of the Wrap53α gene predicted to carry the promoter and transcriptional regulatory elements of Wrap53α and tested for alterations in its activity. In addition, we carried out a series of experiments designed to measure the stability of the Wrap53α mRNA. Our results indicate that while there is a clear transcriptional response to treatment of cells with agents that damage DNA, some treatments also give rise to a post-transcriptional response leading to changes in mRNA stability.

Introduction

The p53 tumor suppressor gene is one of the most frequently mutated genes identified in human cancers [1, 2]. The p53 protein is responsible for a cell-cycle checkpoint in response to DNA damaging agents or cell stressors, leading to either cell-cycle arrest or apoptosis [3-7].

In 2009, a gene oriented in the antisense direction relative to p53 was identified by Mahmoudi et al. and designated Wrap53α [8, 9]. This gene is located on the complementary strand of DNA to the p53 gene and overlaps with its first exon. In the study, it was determined that the Wrap53α mRNA transcript regulates p53 expression by binding to the 5' untranslated region of the p53 mRNA transcript [8]. In cooperation with the protein CTCF, the binding of Wrap53α mRNA induces translation of the p53 mRNA, which increases the levels of active p53 protein in the cell [10]. This allows the cell to respond to DNA damage through cell cycle arrest or apoptosis. Recent studies of the expression of the Wrap53α gene have determined that the level of Wrap53α mRNA transcript increases in response to the DNA damaging agent cisplatinum (*cis*-diamineplatinum (II) dichloride) [11]. This increase in the expression of Wrap53α mRNA was able to induce p53-dependent apoptosis in cisplatinum-treated human U2OS cells [11]. However, the mechanism of induction of the Wrap53α gene in response to DNA damage remains unknown.

In order to determine whether the Wrap53α gene is regulated at the transcriptional and/or post-transcriptional level we carried out two sets of experiments. In one, we cloned a region of the Wrap53α gene predicted to carry the promoter and transcriptional regulatory elements of Wrap53α and tested for alterations in its activity. In addition, we carried out a series of experiments designed to measure the stability of the Wrap53α mRNA. Our results indicate that while there is a clear transcriptional response to treatment of cells with agents that damage DNA, some treatments also give rise to a post-transcriptional response leading to changes in mRNA stability.

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Materials and Methods

Cell culture and drug treatments

The human osteosarcoma cell line U2OS, the breast cancer cell line MCF-7, and the colon cancer cell lines HCT116 and HCT116KO were grown at 37°C with 6% CO_2. They were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 2 mM L-glutamine, 3.5 g/L glucose, 100 U/uL penicillin and 100 ug/uL streptomycin (DMEM). U2OS, MCF-7 and HCT116 cells all express wild-type p53. HCT116KO cells contain a targeted mutation in the p53 gene and are p53-null. These cells were generously provided by Dr. Bert Vogelstein. To induce DNA damage, when cells were approximately 70% confluent, they were treated with the following drugs for 24 to 72 hrs: Camptothecin (15mM), Doxorubicin (1mM), Etoposide (10mM), Cisplatinum (35mM) or Actinomycin D (10nM).

PCR and qPCR analysis

At the respective time-points, cells were harvested, and washed twice with PBS. RNA was purified from the cell pellets using Norgen Biotek's Total RNA Purification Kit. The concentration of the purified RNA was determined with the NanoDrop 2000c from Thermo Scientific. 1.0 ug of the purified RNA was used in a 10 uL reverse transcription reaction to create cDNA using the RETROscript Kit from Ambion.

PCR amplifications were performed in a total volume of 50µl. The samples were subjected to various amplification cycles, ranging from 25-30, in order to obtain a linear response. The PCR products were detected by electrophoresis. The sequences of the PCR primers used were: Wrap53α: (F) 5'-cgg agc cca gca gct acc-3'; (R) 5'-TTG TGC CAG GAG CCT CGC A-3'; GAPDH (F) 5'-TGA AGG TCG GAG TCA ACG GCA TTT GGT-3'; (R) 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'.

qPCR was carried out using the Applied Biosystems 7300 Real-Time PCR System. The 20 mL reaction/well included 10 mL of 2X Power SYBR Green Master Mix from Life Technologies, 1.0 mL of cDNA, 4.0 mL of H_2O, and 5.0 mL of 0.8 mM primers from Life Technologies. The sequences of the qPCR primers are given below: Wrap53α: 5'-TGG CAC AAA GCT GGA CAG T-3' (F) and 5'-GCT GGG TCC TGG TCT GAA G-3' (R); GAPDH: 5'-ACA TCG CTC AGA CAC CAT G-3' (F) and 5'-TGT AGT TGA GGT CAA TGA AGG G-3' (R). The Ct values were used to determine the fold change in the level of Wrap53α mRNA using the ΔΔCt method.

Luciferase reporter gene assays

Cells were plated at $1x10^5$ cells/well in 24-well plates and allowed to adhere for 18 hours after plating. The cells were transiently transfected using Promega's lipid-based TransFast Transfection Reagent with 0.1 ug of either the Wrap53α promoter luciferase vector, or a Bax promoter luciferase vector as a positive expression control since Bax is known to be induced in response to DNA damage. A 1400-bp region carrying the Wrap53α promoter was cloned from normal human genomic DNA by PCR. The primers used for cloning were: Forward: 5'-ctg gaa ctg gaa tgg cct agc c-3'; Reverse 5'-GAG AAT CCT GAC TCT GCA CCC TCC-3'. Each well was also transfected with 25 ng of TK *Renilla*. This vector expresses a reporter gene from the Herpes simplex virus thymidine kinase promoter and functions as an internal control for transfection efficiency. The cells were lysed using Passive Lysis Buffer from Promega and a Bradford Assay was used to determine the protein concentration for each sample. 10 ug of protein were used in the Dual-Luciferase Reporter Assay System from Promega to determine the firefly and *Renilla* luciferase activity through the use of a luminometer. All experiments were carried out in duplicate and repeated at least twice, and results were normalized to the renilla activity.

Inhibition of Transcription and mRNA half-life measurements

For experiments on the half-life of Wrap53α mRNA, an additional actinomycin D-mannitol treatment was required. Once the cells incubated for 18 hours after the 10 nM actinomycin treatment or 24 hours after the 20 µM cisplatinum treatment, they were treated with 4 µM actinomycin. Actinomycin at 4µM inhibits transcription by interfering with RNA Polymerase II. Cells that had not been previously treated with DNA damaging agents were also treated with 4 µM actinomycin to determine the Wrap53α half-life under normal conditions. At various time points after inhibition of transcription, RNA was harvested, and subjected to reverse transcription followed by qPCR.

Results

Induction of Wrap53α after treatment of cells with DNA damaging agents

Results previously reported by Yuan et al. demonstrated that there was a 3- to 40-fold increase in the level of Wrap53α mRNA after treatment of U2OS cells with 5 to 20 mM cisplatinum [11]. In order to confirm and extend these findings we treated a series of cell lines with a variety of DNA damaging agents. We tested the following cells lines, all of which express wild-type p53: U2OS, MCF-7 and HCT116. Cells were treated with DNA damaging agents for 24 and 36 hrs and assayed for Wrap53α levels by standard RT-PCR followed by gel electrophoresis. As shown in (Figure 1), there was a clearly detectable increase in the level of Wrap53α product after treatment of HCT116 cells with a series of DNA damaging agents. Similar results were obtained when U2OS or MCF-7 cells were tested (data not shown). Since these RT-PCR experiments are difficult to quantify, we carried out a subset of the DNA damage experiments in U2OS and measured the extent of induction of Wrap53α by qPCR. These results were confirmed when after 36 hours of incubation, the level of Wrap53α had increased ~8X (Figure 2) after treatment with 20mM cisplatinum. The ability of another DNA damaging agent, 10nM actinomycin, to induce the Wrap53α gene was also tested in U2OS cells. The results showed that 10 nM actinomycin increased Wrap53α mRNA levels ~11X after 18 hours of incubation (Figure 3).

The Wrap53α promoter is induced in response to DNA damage

In order to determine whether the Wrap53α gene is induced at the transcriptional level in response to DNA damage, we cloned a 1400-bp region of the Wrap53α gene that overlaps the identified transcriptional start site by 100bp. This element was cloned into a luciferase reporter

vector and the ability of this element to function as a promoter was evaluated by transient transfections and reporter gene assays in U2OS and HCT116 cells (data not shown). These experiments demonstrate that we successfully cloned the Wrap53α promoter. Having cloned the promoter, we next determined whether the Wrap53α promoter is modulated in its expression after treatment of cells with agents that damage DNA. Cells were transfected with the WRAP53α-lucifease vector and after 24hrs were further treated by the addition of varying amounts of the drugs. These cells were then harvested after an additional 24hr in the presence of the drugs. As shown in Figure 3, all the DNA damaging agents used induced the WRAP promoter but to varying degrees. Treatment with actinomycin D and cisplatinum led to an approximately 2-fold elevation in promoter activity, camptothecin to a 3-fold elevation and doxorubicin and etoposide to a 6.5- and 4.5-fold elevation, respectively. Therefore, although the WRAP**α** promoter is induced in response to DNA damage, the extent of the response depends on the type of DNA damage or the type of DNA-damaging drug. That a drug like cisplatinum induced an 8-fold elevation in the level of WRAP**α** RNA yet only a 2-fold elevation in promoter activity suggests that other regulatory mechanisms may be at play. One possibility is that regulation occurs post-transcriptionally by altering the level of Wrapα mRNA stability.

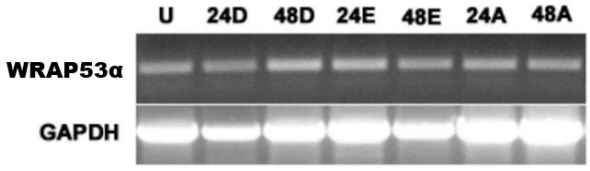

Figure 1: Elevated levels of Wrap53α RNA after treatment of U2OS cells with DNA damaging agents. U2OS cell were treated with the drugs indicated and harvested after either 24 or 48 hrs. RNA was purified, reverse transcribed and assayed by PCR. Top panel shows the Wrap53α product; bottom panel shows the control GAPDH, U = untreated U2OS. Cells were treated with doxorubicin (D), etoposide (E) or Actinomycin D (A).

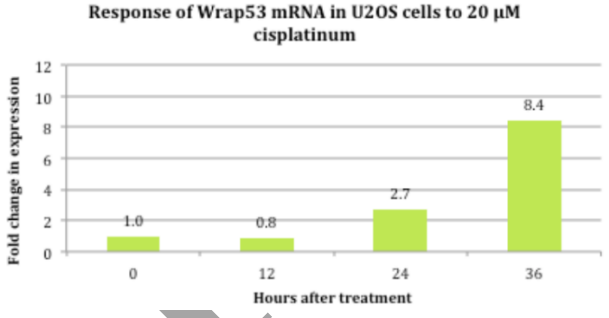

Figure 2: Fold changes in Wrap53α mRNA in response to treatment of U2OS cells with 20 µM cisplatinum. Cells were treated with cisplatinum and harvested at the indicated timepoints. RNA was purified and subjected to qPCR analysis.

The Wrap53α transcript is regulated post-transcriptionally in response to DNA damage

To determine whether the induction of the Wrap53α gene in response to DNA damage by cisplatinum may also be regulated through modification in the half-life of the mRNA, the post-transcriptional stability of Wrap53α mRNA was studied. In a time, course experiment with U2OS cells that were not induced by DNA damage, the half-life of Wrap53α mRNA was determined to be 5.3 hours (Figure 5). When U2OS cells were treated with 20 µM cisplatinum for 24 hours, the half-life of Wrap53α mRNA increased to 10.1 hours (Figure 6). These results indicate that post-transcriptional regulation could potentially account for the induction of Wrap53α mRNA in response to cisplatinum.

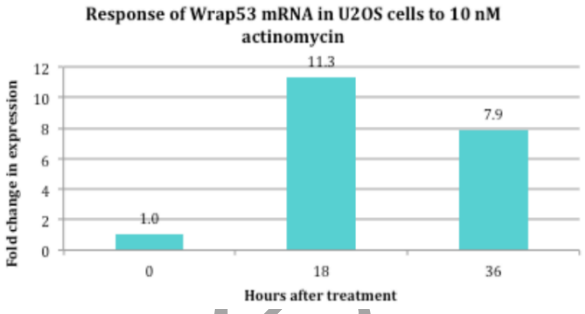

Figure 3: Fold changes in Wrap53α mRNA in response to treatment of U2OS cells with 10nM actinomycin D. Cells were treated with actinomycin D and harvested at the indicated timepoints. RNA was purified and subjected to qPCR analysis.

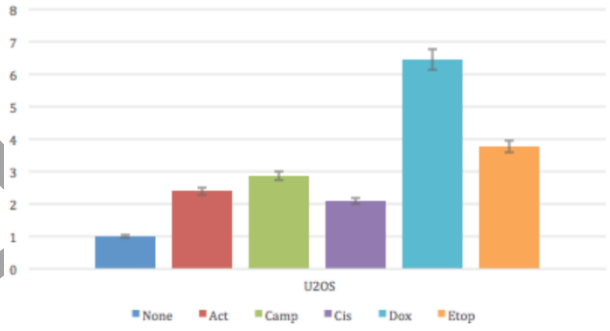

Figure 4: Transient transfections with WRAP promoter-luciferase reporter vectors reveal elevated transcription after treatment of cells with agents that damage DNA. Cells were transfected with the reporter vectors and after 18hrs were further treated with the drugs listed. After an additional 24hrs, cells were harvested and processed for luciferase assays. The expression relative to cells not treated with any drugs is illustrated by the graph.

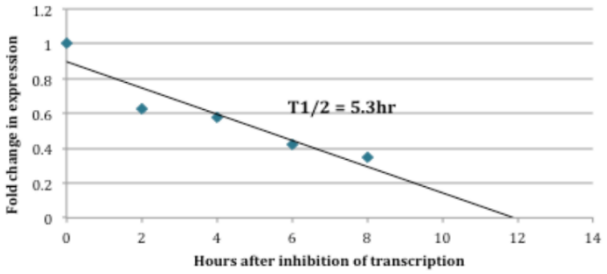

Figure 5: Graph of the fold change in the abundance of WRAP53A RNA over time after the inhibition of transcription, as determined by qRT-PCR. The linear trendline is $y = -0.0755x + 0.898$ with an R^2 value of 0.8902. From this trendline, the half-life of Wrap53α mRNA under untreated conditions was calculated to be 5.3 hours.

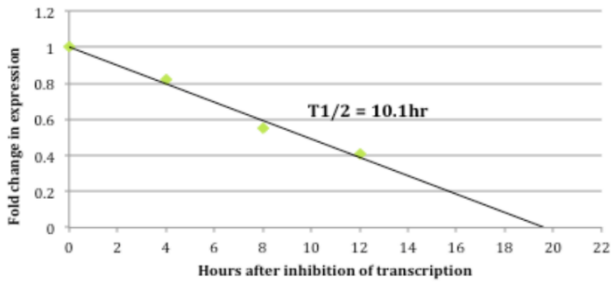

Figure 6: Graph of the fold change in the abundance of WRAP53A RNA over time after the inhibition of transcription. The linear trendline was determined to be $y = -0.051x + 1.001$ with an R^2 value of 0.9866. From this trendline, the half-life of Wrap53α mRNA after treatment with cisplatinum was determined to be 10.1 hours.

Discussion

In 2009, an antisense gene to p53 was identified by Mahmoudi et al., and designated Wrap53α [8]. This gene is located on the complementary strand of DNA to the p53 gene and overlaps with its first exon. In the study, it was determined that the Wrap53α mRNA transcript regulates p53 expression by binding to the 5' untranslated region of the p53 mRNA transcript [8]. The binding of Wrap53α mRNA induces translation of the p53 mRNA, which increases the levels of active p53 protein in the cell. This allows the cell to respond to DNA damage through cell cycle arrest or apoptosis.

Recent studies of the expression of the Wrap53α gene have determined that the level of Wrap53α mRNA transcript increases in response to the DNA-damaging agent cisplatinum [11]. An increase in the expression of Wrap53α mRNA was able to induce p53-dependent apoptosis in cisplatinum-treated human U2OS cells [11]. However, until now the mechanism of induction of the Wrap53α gene in response to DNA damage was unknown.

The present work focused on determining the mechanism of Wrap53α induction in response to DNA damage through analysis of the post-transcriptional and transcriptional regulation of the Wrap53α gene. The experiments on transcriptional regulation were carried out using a previously created plasmid which has the promoter of the Wrap53α gene cloned into a luciferase reporter vector. The Wrap53α vector was introduced into cells and used to determine the activity of the Wrap53α promoter in response to treatment with DNA damaging agents. In the studies on post-transcriptional regulation, time course experiments were used to determine the Wrap53α mRNA half-life after inhibition of RNA synthesis. Our findings indicate that the WRAP gene can be regulated at both the transcriptional and post-transcriptional levels and the choice of which pathway is employed may depend on the type of DNA damage induced; for example, while treatment of cells with doxorubicin leads to a 6-7-fold elevation in WRAP promoter activity, treatment of cells with cisplatinum leads to only a 2-fold elevation in promoter activity. However, the cell may compensate for the minimal 2-fold transcriptional response by a post-transcriptional pathway resulting in a 5hr increase in the stability of the WRAP mRNA. We now plan to embark on a more detailed examination looking at the relative degrees of transcriptional and post-transcriptional regulation as a function of a complete series of DNA damaging agents and ultimately the molecules that participate in these pathways.

Since Wrap53α regulates p53 expression and p53 is essential for the proper response to DNA damage, our work will begin to define the numerous mechanisms by which p53 is ultimately controlled. As a part of the p53 DNA damage response, mutations in the Wrap53α gene may be an independent cause of cancer. This idea has been supported in studies by which found that variants of the Wrap53α gene are associated with an increased risk of ovarian and rectal cancer [12]. Radiation, carcinogens, or accumulated mutations due to natural inborn processes are all sources of DNA damage in humans that can lead to cancers. A better understanding of the Wrap53α gene's involvement in the cell's response to DNA damage would add to the current knowledge of the pathways responsible for the response to DNA damage and for keeping tumors in check. Increased knowledge of Wrap53α would also create the possibility of modulating the activity of the Wrap53α gene as an anti-cancer therapy.

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