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Stability of p53 mRNA Isoforms in MCF7 Cells

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ABSTRACT

Tumor protein 53 (p53) is a tumor suppressor gene that has two key functions. This protein regulates cell cycle and induces apoptosis, or programmed cell death. TP53 mRNA isoforms differ in lengths of the 5'-leader sequence. Longer isoforms (p53 mRNA-L) contain a putative upstream open reading frame, not present in shorter 5' leaders (p53 mRNA-S). We hypothesize p53 mRNA-L is subject to nonsense-mediated mRNA decay (NMD). Treatment with cycloheximide, caffeine, and wortmannin diminish NMD. Our objective was to chemically inhibit NMD in MCF7 cells concurrently treated with Actinomycin D. Cellular proteins were subjected to SDS-PAGE and western analyses for p53. Isolated RNA samples were synthesized into cDNA, then subjected to qRT-PCR analyses of p53 mRNA isoforms. p53 mRNA-L/ p53 mRNA-S isoform ratios (L/S) were calculated from Relative Quantification (RQ) values obtained from p53 mRNA isoforms, by comparing treated to untreated samples and were reported as mean L/S ratios and standard deviations. Actinomycin D treatment, without inhibitors, resulted in a L/S = 1.070 ($\pm$0.05). Actinomycin D co-treatment with cycloheximide, caffeine or wortmannin resulted in L/S means of 1.159 ($\pm$0.07), 1.181 ($\pm$0.18) and 1.279 ($\pm$0.15), respectively. Western blot analyses were consistent with reduced translation of p53 protein in cycloheximide treated cells. Caffeine and wortmannin treated cells contained a prominent p53 protein band consistent with hypo-phosphorylated p53. In conclusion, chemical treatment effectively inhibited translation and kinase activity. p53 mRNA-L is partially rescued in cells treated with inhibitors of translation and kinase activity.

1 INTRODUCTION

The TP53 gene plays a vital role in cellular DNA repair and apoptosis by forming a tetramer and binding the DNA as a transcription factor (Stockklausner et al., 2006). Strudwick et al. (2003) reported that two major isoforms of p53 mRNA were detected, one with a longer 5'-leader (p53 mRNA-L) and shorter 5' sequence (p53 mRNA-S) (Strudwick et al., 2003). Those were transcribed from different transcription initiation sites. It was previously reported that p53 mRNA isoforms differed in: (1) p53 mRNA-L was more prevalent in normal cells/tissues, while p53 mRNA-S was more abundant in tumor tissue/tumor-derived cell lines, and (2) the p53 mRNA-L 5'-leader represses translation efficiency of downstream report genes in chimeric constructs. The p53 mRNA-S 5'- leader does not affect translational efficiency (Strudwick et al., 2003).

The p53 mRNA-L isoforms containing the longer 5' leader encodes a putative upstream open reading frame (uORF), while the p53 mRNA-S isoforms does not (Figure 1). Unpublished work in Michael Carastro's lab is consistent with the uORF in p53 mRNA-L 5'-leader is translatable in human cells. It was speculated that p53 mRNA-L uORF translation could trigger NMD.

NMD is a mRNA degradation process in eukaryotic cells that requires translation termination at a premature termination codon (PTC) (Pal et al., 2001). PTC can be a result from genomic mutation, alternative splicing or RNA damage, triggering an NMD response to target and degrade the mRNA (Brown et al., 2011). Some uORF termination codons are reportedly recognized as PTCs and trigger NMD (Hofseth et al., 2004; Kuzmiak & Marquat, 2006; Lee &Schedl, 2004; Messenguy et al., 2002). When NMD is initiated, phosphorylation of hUPF1 protein located at the PTC occurs by the phosphatidylinositol-3 kinase-like kinase (PI3K), hSMG-1 (Hofseth et al., 2004). Following the phosphorylation of hUPF1, SMG5 and SMG6 are recruited leading to the termination of the protein translation and degradation of the abnormal mRNA sequence (Fatscher et al., 2014). hSMG-1 and ATM phosphorylate hUPF1 and p53. These PIKK members are inhibited by caffeine (CAFF) and wortmannin (WORT) (Ruiz-Echevarria & Peltz, 2000). Other chemical treatments that will be used in this study are Actinomycin D (Act D), which blocks transcription by inhibiting RNA polymerase II and mRNA synthesis (Cervantes-Gomez et al., 2009) and Cycloheximide (CHX), which is a protein synthesis inhibitor that blocks translation (Ito et al., 2011). Our hypothesis is that translation of an uORF triggers p53 mRNA-L degradation through the NMD mechanism in cells. Chemical inhibition of translation and kinase activity can selectively rescue p53 mRNA-L from degradation.

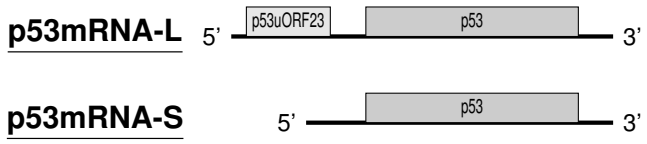

Fig. 1. Two Major p53 mRNA Isoforms. p53 mRNA-L contains the putative upstream open reading frame that does not exist in the p53 mRNA-S.

2 MATERIALS AND METHODS

Cell Culture

The p53-postive breast cancer cell line, MCF7 (American Type Culture Collection), was utilized for chemical inhibition studies. The cultured cells were maintained in 5% CO_2 at 37 °C in a ThermoScientific incubator. Cells were maintained in DMEM 10% Fetal Bovine Serum, 100 units/mL penicillin and 100 µg mL^{-1} streptomycin.

RNA Isolation, Quantitation, and cDNA Synthesis

RNA samples were isolated using the RNeasy Plus Mini kit (Qiagen). RNA samples were analyzed quantitative and qualitatively with the SmartSpec Plus spectrophotometer (Bio-Rad). RNA samples (1.0 µg) were used to synthesize cDNA following the DyNAmo cDNA Synthesis Kit (Thermo) protocol.

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qRT-PCR

Custom TaqMan primer probe sets were designed previously to detect the 5'leader p53 mRNA isoforms, p53 mRNA-L and p53 mRNA-S. The p53 mRNA isoform specific primer probe sequences were p53 mRNA-S forward primer, 5'-CAG ACC TAT GGA AAC TAC TTC CTG AAA-3', p53 mRNA-S reverse primer, 5'-ACA GCA TCA AAT CAT CCA TTG C-3', p53 mRNA-S probe, 5'-Vic-TCT GTC CCC CTT GCC GTC CCA-3BHQ_1-3'; p53 mRNA-L forward primer 5'-GGT TTT CCC CTC CCA TGT G-3', p53 mRNA-L reverse primer 5'- GAC GGT GGC TCT AGA CTT TTG AG-3', p53 mRNA-L probe 5'-6-FAM-AAG ACT GGC GCT AAA AGT TTT GAG C-3BHQ_1-3'. Endogenous gene expression controls GAPDH (Hs03929097_g1) and β-actin (Hs99999903_m1) (Applied Biosystems) were used. The cDNA samples were diluted 10 fold and used to assemble RT-PCR reactions using the TaqMan Gene Expression Master Mix (Applied Biosystems). Reactions were performed using a StepOne Plus RT-PCR system (Applied Biosystems) with Amperase activation (2 min, 50 °C), Taq activation (10 min, 95 °C), and then 40 cycles of 15 s, 95 °C and 1 min, 60 °C. Data was analyzed using the pre-installed software (Applied Biosystems). Three independent experiments were analyzed for statistical significance using One Way Anova computer software.

Protein Quantitation, SDS-PAGE, Western, and ECL

Cellular proteins were isolated by lysing the cell pellets with RIPA Buffer (Pierce), which included Halt Phosphatase inhibitor (1X)(Pierce) and Halt Protease Cocktail Mix inhibitor (1X)(Pierce). A BCA Protein Assay Kit (Thermo) was used for protein quantitation using a SmartSpec Plus Spectrophotometer (Bio-Rad). Protein samples (20 µg) were denatured in 1× SDS Sample Buffer (Boston BioProducts) at 95 °C for 3 min, and proteins were resolved on 10% SDS-PAGE. Transfer Buffer was made with 20% methanol, 1X TG (Bio-Rad) and water. The SDS gels were then transferred to PVDF membranes for 3 h. The membrane was then blocked with PBS (1X), 5% non-fat milk, 0.1% Tween20, and 0.02% NaN_3 for one hour. The membrane was then probed for an hour using monoclonal anti-p53 antibody (DO-1) 1:1000 dilution, PBS (1X), 5% non fat milk, and 0.1% Tween20. The membrane was washed using PBS (1×), 5% non-fat milk, and 0.1% Tween20. The secondary Horseradish Peroxidase anti-mouse IgG (A9044) (Sigma-Aldrich) was then probed for an hour using a 1:5000 dilution. Multiple washes using dH_2O of the membrane was completed and then western blot was taken to the developer. Enhanced chemiluminescence (ECL Prime) (Amersham GE) was used to detect the immunofluorescence of the samples. Quantitation was performed with ImageJ computer software (NIH) and blot was normalized to β-actin.

3 RESULTS

Translation of an uORF could trigger p53 mRNA-L degradation through the NMD mechanism in cells, chemical inhibition of translation and kinase activity can selectively rescue p53 mRNA-L from degradation. Two treatment methods were tested: (1) Media containing Actinomycin D (Fisher Scientific) (5 µg mL^{-1}) to inhibit transcription was applied 4 h prior to initiating chemical treatments to inhibit translation or kinase activity. Wortmannin (MP Biomedicals) (10 µM) or caffeine (MP Biomedicals) (10 mM), and cycloheximide (Fisher Scientific) (20 µg mL^{-1}) were used in three separate chemical inhibition treatments and were applied four hours after actinomycin D treatment. (2) Cells were individually treated for a total of 8 continuous hours with chemical inhibitors. At the end of the 8-hour chemical treatments, cells were harvested and cell samples were isolated for protein analysis. Three independent experiments were subjected to Real Time-PCR with custom TaqMan Assays. The RQ values were calculated using the StepOne automated software (Applied Biosystems). p53 mRNA-L isoform levels following treatment were obtained with non-treated cells standardized to 1.000. Concurrent Act D treatments with Act D, CHX, CAFF, and WORT yielded RQ values of 0.8156 ± 0.064, 1.0682 ± 0.261, 0.8169 ± 0.139, 0.9217 ± 0.124 respectively (Figure 2). p53 mRNA-S isoform followed the same calculations and resulted in RQ values of 0.7629 ± 0.063, 0.9154 ± 0.181, 0.6926 ± 0.077, and 0.7216 ± 0.075 respectively (P value < 0.05) (Figure 3).

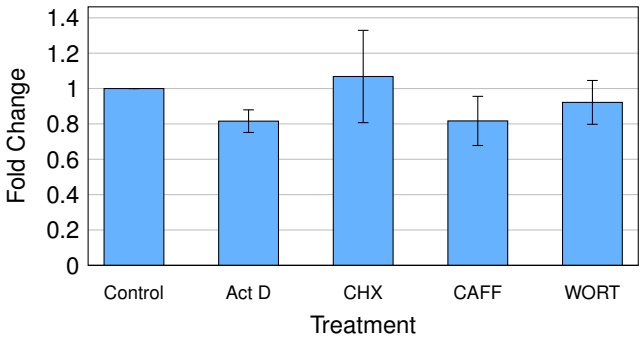

Fig. 2. p53 mRNA-L Isoform RQ values from TagMan RT-PCR. Cells were Treated with Act D (5 µg mL^{-1}), CHX (20 µg mL^{-1}), CAFF (10 mM) or WORT (10 µM), Non-treated was normalized to 1.

p53 mRNA-L & -S isoforms levels were then taken as a ratio (-L/-S) and reported (Figure 4). The non-treated cells ratio was normalized to 1.000. The average of the three independent experiments treating with Act D/Act D ratios was 1.0700 ± 0.047, Act D/CHX was 1.1591 ± 0.074, Act D/CAFF was 1.1808 ± 0.175, and Act D/WORT 1.2794 ± 0.145 (P Value < 0.05).

The western blot following the ECL shows protein levels following both single treatment using Act D, CHX, CAFF, and WORT and co-treatment samples, using Act D/Act D, Act D/CHX, Act D/CAFF, and Act D/WORT (Figures 5 and 6). The β-actin endogenous control was then blotted for following stripping of the p53 antibody from the membrane. Quantification of the blots were preformed using ImageJ computer software. Normalizing the western blot to β-actin, fold induction ratios were determined (Figure 6). Non-treated (lane 1) was set to 1.0, as it serves as the control group. Lane 2 p53 increased 125%. CHX single treatment resulted in a decreased of 52%, CAFF decreased 6%, and WORT increased 30%. Lanes 6–9 were co-treatments resulting in 193%, 176%, 161%, and 163% inducing compared to untreated respectively.

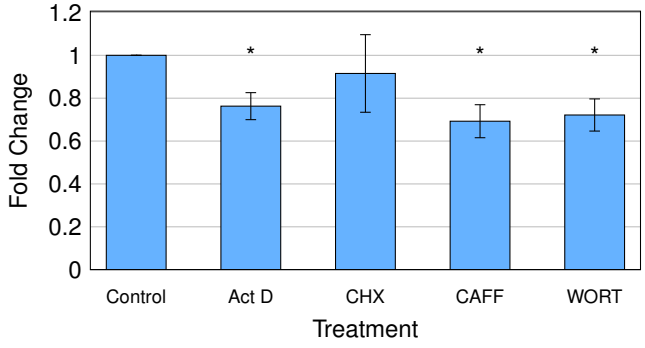

Fig. 3. p53 mRNA-S Isoform RQ values from TagMan RT-PCR. Cells were Treated with Act D ($5\,\mu g\,mL^{-1}$) for 4 hours, followed by a 4 hour treatment of Act D ($5\,\mu g\,mL^{-1}$), CHX ($20\,\mu g\,mL^{-1}$), CAFF (10 mM) or WORT (10 µM), Non-treated was normalized to 1.00. *denotes P value < 0.05

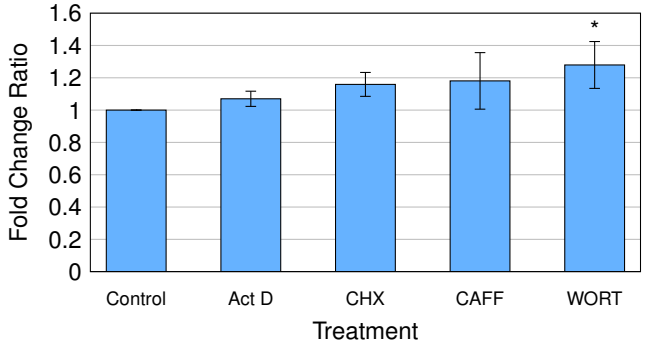

Fig. 4. p53 mRNA-L/-S Isoform RQ values from TagMan RT-PCR. Cells were Treated with Act D ($5\,\mu g\,mL^{-1}$) for 4 hours, followed by a 4 hour treatment of Act D ($5\,\mu g\,mL^{-1}$), CHX ($20\,\mu g\,mL^{-1}$), CAFF (10 mM) or WORT (10 µM), Non-treated was normalized to 1.00. *denotes P value < 0.05

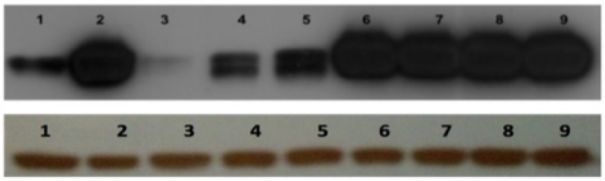

Fig. 5. p53 and β-Actin Westerns (Lanes 1: Untreated, 2: Act D, 3: CHX, 4: CAFF, 5: WORT, 6: Act D/Act D, 7: Act D/CHX, 8: Act D/CAFF, 9: Act D/WORT)

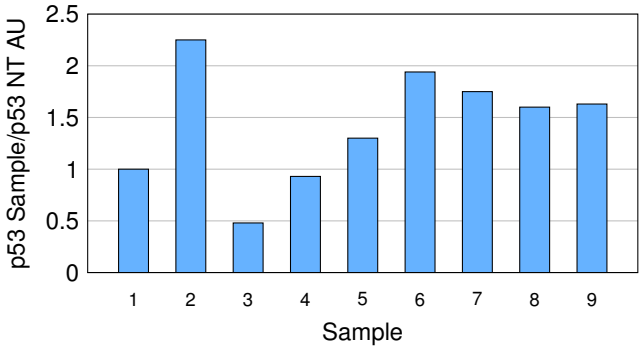

Fig. 6. p53 fold induction compared to non-treated and normalized to β-Actin (Lanes 1: Untreated, 2: Act D, 3: CHX, 4: CAFF, 5: WORT, 6: Act D/Act D, 7: Act D/CHX, 8: Act D/CAFF, 9: Act D/WORT)

4 DISCUSSION

RT-PCR analyses and protein analyses were combined to determine effectiveness of known chemical inhibitors of translation and kinase activity. By examining the p53 mRNA-L/p53 mRNA-S ratios resulting from RT-PCR and protein expression in western blot, effects of chemical inhibition were analyzed. By inhibiting transcription using actinomycin D, levels of transcription were halted, and examination of translation and kinase activity necessary for NMD was looked at. NMD is believed to target the uORF of the p53 mRNA-L isoform. Comparing co-treated cells with Act D and one of the select NMD inhibitors, p53 mRNA-L & -S isoforms RQ values could be determined. In both the p53 mRNA isoforms the RQ values decrease for cells treated with Act D/Act D, Act D/CAFF, and Act D/WORT. In the three treatment groups, p53 mRNA-S isoform decrease was statistically significant ($P < 0.05$). This is a result from the inhibition of transcription. In Act D/CHX the RQ values increase, although not significant. CHX inhibits translation, and using another chemical inhibitor of transcription, a buildup of mRNA occurred, as it cannot be transcribed.

Ratios of the two isoforms could be calculated. The Act D/Act D ratio was 1.070 ($\pm$0.048), Act D/CHX was 1.159 ($\pm$0.074), Act D/CAFF was 1.181 ($\pm$0.179), and Act D/WORT was 1.279 ($\pm$0.145), $p < 0.05$. Wortmannin is the only individual data set that is statistically significant. As a whole the data is considered to be significant because the increases in the three treatments of translation or kinase activity are larger than that of actinomycin D and the non-treated.

In the western blot, Lane 1 (Figure 5) is the non-treated cell sample. Lane 2 is the actinomycin D single treatment where the p53 band is bright. This is due to the actinomycin D treatment, which is an inducer of p53 by inhibiting MDM2 and resulting in stabilization of p53 (Chen et al., 2014). This resulted in 125% increase in p53 protein. Lane 3 shows the inhibition of translation, there is only trace amounts of p53 present. β-actin was used as the endogenous control since it has a longer half-life than p53, explaining why p53 diminished but Act-B did not when treated with CHX. Lanes

4 and 5 are the two PIKK inhibitors; they both show two bands of p53. The higher band is the hyper-phosphorylated p53 and the lower band is the hypo-phosphorylated p53. With kinase inhibitors, phosphorylation was inhibited while previously phosphorylated p53 remained in the cell. p53 protein for caffeine was relatively the same as the non-treated, however wortmannin co-treated cells showed p53 induction of 30%. Lanes 6, 7, 8, and 9 are all from the co-treatment and had similar results with p53 increases of 193%, 176%, 161%, and 163% respectively. The NMD inhibitors act in separate pathways than Act D mechanism. It is speculated that the even though p53 protein is induced the inhibitors are still able to effectively inhibit translation and kinase activity.

In conclusion, inhibition of transcription and translation (Act D/CHX) partially rescued p53 mRNA. Inhibition of transcription and PIKK activity (Act D/CAFF and Act D/WORT) partially rescued p53 mRNA-L. Western blot analyses data are consistent with the inhibition of translation with cycloheximide treatment and inhibition of kinase activity with caffeine and wortmannin. The use of this co-treatment process is able to increase the L/S ratio and show that is can partially rescue the p53 mRNA-L isoform from NMD degradation.

Future work for this experiment can include knockdown of NMD specifically. Using small interference RNA (siRNA), hUPF1 and hUPF2 genes can be knocked down; this will inhibit the ability of the mRNA surveillance mechanism of NMD. Panelli et al. (2012) reports that knockdown of the hUPF1, results in the loss of function of NMD mechanism. But when hUPF2 is knocked down and not hUPF1, hUPF1 is up regulated and does not result in loss of NMD function (Panelli et al., 2012). Maquat designed constructs to serve as an endogenous control for NMD (Zhang et al., 1998). The constructs consisted of a β-globin reporter gene in exon-2 of the mRNA. This gene does not affect mRNA abundance (Panelli et al., 2012). Knocking down the hUPF1 gene and transfecting an endogenous control into MCF7 cells for NMD, can allow direct analysis to whether the chemical inhibition of translation or kinase activity is inhibiting NMD and partially rescuing p53 mRNA-L from degradation

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