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14. ABSTRACT

To investigate how gain of function p53 mutants exert their oncogenic effects, protein expression was compared between a p53 null cell line stably expressing vector alone or the p53 gain of function mutants, p53-R175H and -R273H. One protein that was upregulated in cells expressing the p53 gain of function mutants control cell lines was identified by mass spectrometry as translocator of the mitochondrial membrane 50 (Tim50). p53-R175H and -R273H, but not WT p53, upregulated the luciferase activity of a Tim50 promoter construct. Loss of Tim50 expression also reduced the growth rate and survival from paclitaxel treatment in breast cancer cells that harbor p53-175H. Taken together, this data suggests that one pathway by which mutant p53 may upregulate cell growth and chemoresistance in breast cancer is through induction of Tim50 protein.

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INTRODUCTION

The tumor suppressor protein p53 is a multifunctional transcription factor involved in the control of cell cycle progression, DNA integrity, and cell survival. Some p53 mutations, in addition to eliminating the normal functions of p53, impart additional oncogenic properties to cells and have therefore been deemed p53 gain of function (GOF) mutations (1-4). This is reflected in a poorer prognosis of patients with breast cancer containing p53 mutants than those lacking the p53 protein (5). Remarkably, even though p53 is one of the most extensively studied single proteins to date, little data exists regarding the mechanism of action of p53 GOF mutants. To investigate how gain of function p53 mutants exert their oncogenic effects, protein expression was compared between a p53 null cell line (H1299) stably expressing vector alone or the p53 gain of function mutants, p53-R175H and -R273H. One protein that was upregulated 2-3 fold in H1299 cells expressing the p53 gain of function mutants p53-R175H and -R273H compared to the vector control cell line was identified by mass spectrometry as translocator of the mitochondrial membrane 50 (Tim50). This study aims to (1) Determine the contribution of Tim50 to the p53 GOF phenotype in breast cancer cells harboring mutant p53; (2) Examine whether Tim50 is a transcriptional target of mutant p53 and if Tim50 and mutant p53 contribute to each other's stabilization in breast cancer cells; (3) Determine if Tim50 expression is altered in human breast cancer cells and tumors and determine its relationship to p53 status and (4) Study the effect of Tim50 expression on mitochondrial protein import or mitochondrial membrane potential.

BODY

Objective 1: Determine the contribution of Tim50 to the p53 GOF phenotype in breast cancer cells harboring p53.

Mutant p53 expression in cancer cells results in loss of chemosensitivity, by knocking down mutant p53 in those cells a resurgence of chemosensitivity has been shown (Unpublished results from Dr. Deb's laboratory). We next tested the idea that mutant p53-induced chemoresistance in H1299 lung cancer cells may be due to Tim50 by using RNAi directed against Tim50. Preliminary data

We wanted to test now whether that principle holds if we use a number of naturally occurring breast cancer cell lines expressing mutant p53. **Figure 1** shows colony formation assays with breast cancer cell lines MDA-MB-468 and SK-BR-3 after treatment with paclitaxel. Cells were treated with the drug for 48 h after being nucleofected with siRNA specific for p53 or Tim50. Data in **Figure 1** shows that Tim50 siRNA had a drastic effect on chemoresistance against paclitaxel in the colony formation assay. These results strengthen the hypothesis that Tim50 is at least one of the causative agents for chemoresistance induced by p53 mutants.

In addition, Tim50 expression was reduced by siRNA in SK-BR-3 cells that harbor the p53-175H mutation. Tim50 siRNA greatly reduced the growth of cells expressing mutant p53 but had little effect on cells lacking p53 (**Figure 2**).

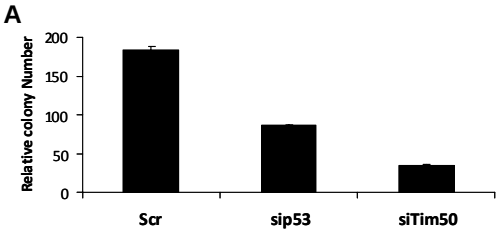
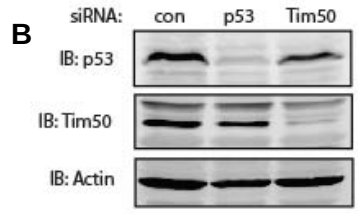


Figure 1. Loss of Tim50 expression reduces survival of mutant p53 expressing breast cancer cell lines to paclitaxel **(A)** SK-BR-3 cells were transfected with scrambled siRNA or siRNA directed against p53 or Tim50, treated with 25 nM paclitaxel for 48 hrs and colony survival assays performed. **(B)** Western analysis of the



siRNA treated cells. Cell lysates were harvested 48 hrs post transfection and immunoblotted with the indicated antibodies. The data shown are from three independent experiments and colony numbers were adjusted to account for plating differences based on control plates treated with vehicle (DMSO). Relative colony numbers are shown.

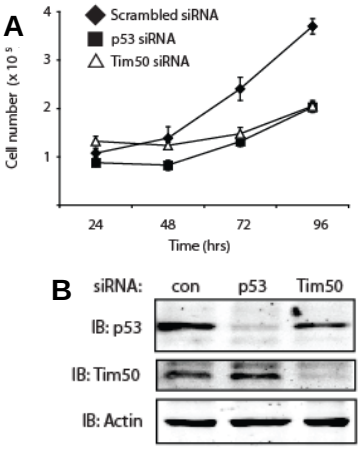


Figure 2. Loss of Tim50 expression impairs the growth rate of mutant p53 expressing H1299 cells. **(A)** SK-BR-3 cells were transfected with scrambled siRNA or siRNA directed against p53 or Tim50 and at the indicated times, cell numbers determined. Similar results were obtained in two additional experiments. **(B)** Western analysis of the siRNA treated cells. Cell lysates were harvested 48 hrs post transfection and immunoblotted with the indicated antibodies. The data shown are from three independent experiments and colony numbers were adjusted to account for plating differences based on control plates treated with vehicle (DMSO). Relative colony numbers are shown.

Objective 2: Examine whether Tim50 is a transcriptional target of mutant p53 and if Tim50 and mutant p53 contribute to each other's stabilization in breast cancer cells.

To test whether mutant p53 could affect Tim50 expression through upregulation of the Tim50 promoter, a Tim50 luciferase promoter construct was co-transfected along with WT p53, p53-R175H or p53-R273H. As shown in **Figure 3**, co-transfection with the p53 mutants, p53-R175H and –R273H, upregulated luciferase activity approximately 2.5 and 3 fold respectively. In addition, it is observed that p53 siRNA significantly reduced Tim50 mRNA levels (**Figure 4**). To confirm the interaction of mutant p53 with the Tim50 promoter mutant p53-R273H expressing cells were subjected to chromatin immunoprecipitation (ChIP) analysis. Binding of the Tim50 promoter to mutant p53 was detected (**Figure 5**).

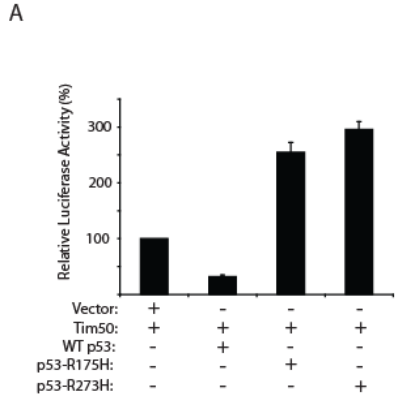


Figure 3. Mutant p53 up-regulates the Tim50 promoter. **(A)** Saos-2 cells were transfected with the pGL3 vector containing the Tim50 promoter upstream of the luciferase reporter gene, the β-galactosidase control plasmid, and empty FLAG vector or the indicated p53 plasmid for 48 hrs. After transfection, luciferase activity was detected using a luciferase reporter assay and values normalized to β-galactosidase values to control for transfection efficiency. **(B)** Cell lysates from the transfections were blotted with the indicated antibodies. Similar results were obtained in two additional experiments.

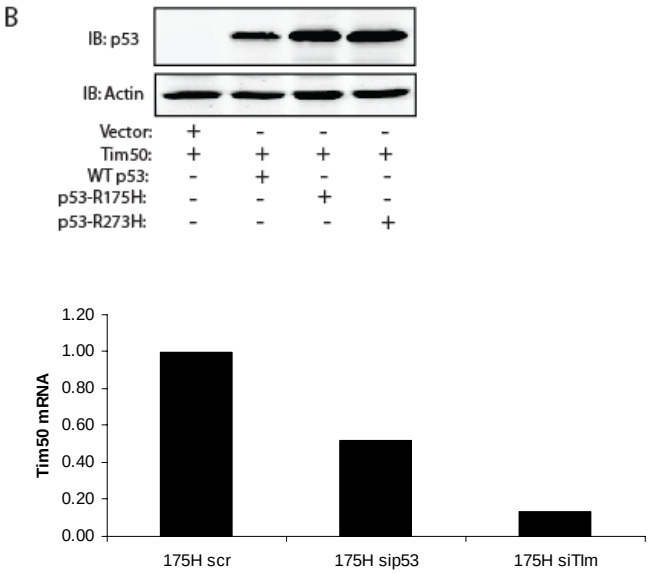


Figure 4. Loss of mutant-p53 expression results in the reduction of Tim50 mRNA. p53-R175H expressing cells were transfected with scrambled siRNA or siRNA directed against p53 or Tim50 (positive control). Total RNA was extracted from cells and following cDNA preparation, quantitative real-time PCR was performed to determine Tim50 mRNA levels. The amount of Tim50 expression was determined using a relative standard curve after normalization to the internal standard, GAPDH mRNA. The normalized Tim50 mRNA level in the control cell line, Scr, was set to 100 and the relative fold changes calculated.

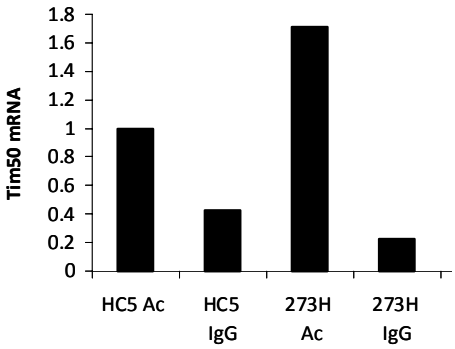


Figure 5. Mutant-p53 associates with Tim50 to augment transcription. Cells expressing p53-R273H compared to p53-null empty vector (HC5) were subjected to ChIP with antibodies against p53 or IgG as a negative control. The precipitated DNA was subjected to quantitative real-time PCR amplification. Relative amounts of Tim50 were normalized to the relative amounts of the pGEM(3z)f - DNA in that sample and the antibody/input ratio calculated using the normalized amounts.

Objective 3: Determine if Tim50 expression is altered in human breast cancer cells and tumors and determine its relationship to p53 status

3a. Determine the expression of Tim50 in breast cancer cell lines

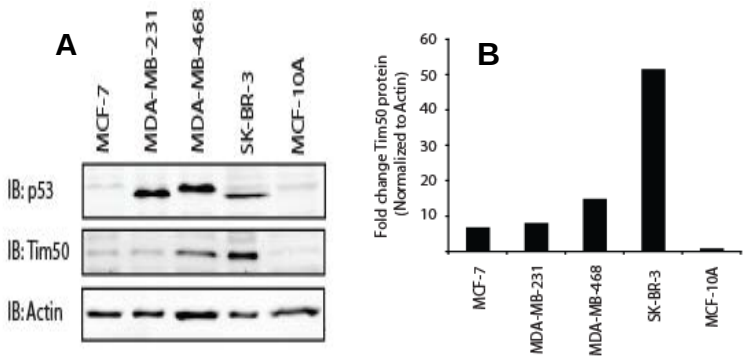


Figure 6. Expression of mutant p53 correlates with an increase in Tim50 protein levels in breast cancer cells. **(A)** Total cell lysates from the indicated breast cell lines were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Equal protein amounts were loaded and Tim50 protein levels normalized to actin. The fold change of Tim50 protein compared

to the level in MCF-10A cells is shown. A representative image is shown. The experiment was performed twice with similar results. **(B)** Quantitation of the data shown in panel A.

To explore the relationship between mutant p53 and Tim50, a panel of breast cancer cell lines was analyzed for p53 and Tim50 expression levels. It has been confirmed that Tim50 protein levels are observed to be higher in SK-BR-3 and MDA-MB-468 cells (which express mutant p53) than in MCF7 and MCF-10A cells (which express WT p53) after normalization to total actin levels (**Figure 6**). mRNA has also been collected for these cell lines and Tim50 mRNA levels will be examined by quantitative real-time PCR.

3b. Determine the expression of Tim50 in breast cancer patient tumor samples

Regulatory review and approval for studies involving human breast tumors has been obtained we are currently in the process of obtaining RNA from breast cancer patient tumor samples from the Tissue and Data Acquisition and Analysis Core at Virginia Commonwealth University in collaboration with Dr. Catherine Dumur and Dr. Carleton Garrett. Samples are currently being sequenced for p53 status.

Objective 4: Determine if Tim50 alters mitochondrial import or mitochondrial membrane potential

Using a novel assay utilizing a mitochondrial targeted GFP (mito-GFP) construct which only displays fluorescence upon import to the mitochondrial matrix mutant p53 expression had no effect on mito-GFP import (data not shown). Alternative hypotheses and approaches are being considered.

KEY RESEARCH ACCOMPLISHMENTS

- Inhibition of Tim50 by RNAi causes a significant reduction in chemoresistance in mutant p53-expressing breast cancer cells.
- siTim50 potently inhibits growth of human breast cancer cells expressing mutant p53.
- Mutant p53 could affect Tim50 expression through upregulation of the Tim50 promoter.

REPORTABLE OUTCOMES

Sankala, H., Harris, J.K., High, A., Mohanraj, L., Garrett, C., Dumur, C., Deb, S. and Graves, P. The Role of Tim50 in Mutant p53-Induced Chemoresistance and Growth Enhancement in Breast Cancer Cells (Manuscript in preparation).

Nesheiwat, I., Scian, M.J., Madduri, D, High, A.J., Ramamoorthy, M., Vaughan C.A., Graves, P. **Sankala H**, Yeudall, W.A., Deb, S.P., and Deb, S. Mutant p53 Induces the Loss of Chemosensitivity in Cancer Cells Using the NF-κB2 Pathway (Manuscript in preparation)

Sankala, H., Harris, J.K., High, A., Mohanraj, L., Vaughan, C., Deb, S and Graves, P.R. Tim50, a Component of the Mitochondrial Translocator, Contributes to Cell Growth and Survival in Mutant p53 Expressing Cells. The 15th International p53 Workshop. 2010. University of Pennsylvania School of Medicine, Philadelphia. PA.

CONCLUSIONS

To further explore the mechanism of action of mutant p53 we initiated a proteomics screen to identify mutant p53 specific protein interactors. One of the proteins we identified from the initial screen as a potential mutant p53 interacting protein was Tim50. During this analysis we noticed that the expression of mutant p53 correlated with a large increase in the level of Tim50 protein expression. Furthermore, we have shown that Tim50 expression is upregulated in mutant-p53 breast cancer cell lines. Moreover, promoter analysis and ChIP assays indicate that the *Tim50* gene is a direct transcriptional target of gain-of-function mutant p53. The upregulation of the Tim50 protein by mutant p53 may be a survival strategy for breast tumors that harbor p53 mutations since inhibition of Tim50 by RNAi causes a significant reduction in chemoresistance in mutant p53-expressing breast cancer cells and siTim50 potently inhibits growth of human breast cancer cells expressing mutant p53. Our data suggests that one pathway by which mutant p53 may upregulate breast cancer cell growth and chemoresistance is through induction of Tim50 protein expression.

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