Author's response to reviews

Title: Activation of endogenous p53 by combined p19Arf gene transfer and nutlin-3 drug treatment modalities in the murine cell lines B16 and C6

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Author's response to reviews: see over
Dr. Melissa Norton  
Editor-in-Chief  
BMC Cancer  

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Dear Dr. Norton,  

We were quite pleased to receive the comments from four Reviewers as well as the Associate Editor comments. To the best of our ability, we have modified the manuscript as suggested. Please find here our response to the Associate Editor and below our response to each of the Reviewers.  

Throughout the text, modifications have been highlighted in gray.  

The abstract has been modified to match the format of BMC Cancer. We have also included in the Methods section the protocol numbers given by our ethics committees upon approval of our animal procedures.  

As suggested, we examined mdm2 and mdmx for their alteration upon gene transfer or drug treatment (see figure below). For mdmx, a western blot did not reveal any alteration in protein level with or without treatment. For mdm2, we were not able to detect this protein in western blot due, most likely, to technical shortcomings. For this reason, we have altered the Discussion to reflect the fact that the mechanism for the resistance of B16 cells to nutlin-3 as well as the reason for p53 re-activation by the combination of p19Arf and nutlin-3 remain to be determined.  

In the original version of the manuscript we included our studies with doxorubicin treatment, an agent well known for its DNA-damaging activity as well as its ability to induce p53 function. Upon doxorubicin treatment, p53 was readily activated in both cell lines. Therefore, we believe that p53 can be activated in these cells and that their resistance to p19Arf and nutlin-3 must be due to some other, yet unknown, mechanism.  

In this work, we have presented an interesting comparison between two widely used cell lines that differ in their response to p19Arf gene transfer and nutlin-3 treatment. C6 cells served as a demonstration that the vector and drug treatments were reliable. We were surprised to find that B16 cells were quite resistant to these treatments. However, our true goal with this study was to show how our novel retroviral vector would perform in transformed cell lines that harbor endogenous p53wt. To this end, we have clearly shown that endogenous p53 could indeed drive expression from the pCLPG vector and that the inclusion of p19Arf resulted in increased vector expression. This is in agreement with the hypothesis that exogenous p19Arf would activate endogenous p53 and, as a result, vector expression would be elevated. This result is especially clear in Figure 3.
We are grateful for the time and effort that BMC Cancer has extended us. In agreement with two of the Reviewers, we feel that our work will be of interest to the readers of this journal who work with gene transfer in general and the p53 pathway in particular. C6 and B16, though they are murine cell lines, are widely used and the dissemination of information about their response to gene transfer and drug treatments may be helpful to many readers. In the spirit of open access publishing, and in the event of the acceptance of this manuscript, we welcome the chance for feedback directly from the readership.

Thank you so much for your attention and we would be happy to provide any additional information.

Bryan E. Strauss, PhD
Response to Reviewer Comments

Reviewer: Mauro Vaccarezza

The paper by Merkel et al. provides an interesting approach of p53 reactivation as a method to improve cancer therapy. The modulation of p53-related pathways to restore apoptosis on cancer cells is not new but the combined approach suggested by Merkel et al. makes sense and the "formal" proof of concept (activating p19arf by gene insertion through viral vectors and using a pharmacologic inhibitor of the p53-MDM2 complex) is attained convincingly.

The methods are appropriate and the data sufficiently solid; the discussion is complete.

Of course one would translate in a more physiological context (human cells) the message of the paper that is based on mouse melanoma and rat glioma cell lines, but the in vitro and in vivo data are a nice "scaffold" considering also the expression of the above proteins on human melanoma samples (as correctly reported by the authors)

Some minor mispelling is present throughout the English text.

We appreciate these positive comments. We also agree that testing in human cells would be of interest. However, we took great care to use the murine p19Arf cDNA and apply this in murine cells followed by an in vivo assay in an immunocompetent animal model. In order to test in human cells, we would have to construct new vectors and use an immunodeficient model, a situation that we consider to be beyond the scope of this manuscript.

The manuscript was carefully revised and spelling errors were corrected to the best of our ability.
Reviewer: Silvia Soddu

The ms by Merkel and coauthors describes the results obtained in B16 melanoma cells and in C6 glioma cells by p19Arf expression and concomitant treatment with the wtp53 reactivating compound Nutlin-3. The question is clearly posed by the authors but it is not very novel. Nutlin-3 is known to very strongly increase p53 expression, which, in turn should further increase the p19Arf levels in the system employed by the authors. However, no data regarding this relevant molecular aspect have been performed. The data reported in figure 6, 7, and 8 show mild effects of the combined treatment on B16 cells, only weakly supporting the authors’ conclusions.

Overall, this ms is clearly written, but the findings described are rather poor.

We have modified the manuscript in order to place greater emphasis on the use of the pCLPG vector, a novel aspect of this work. No other report in the literature has used a p53-responsive promoter to drive the expression of p19Arf in a gene transfer vector. Figure 3 clearly shows that the inclusion of p19Arf in the pCLPG vector resulted in increased levels of the viral transcript originating at the modified LTR. We interpret this assay as an indication that exogenous p19Arf did indeed activate endogenous p53 and, as a result, increased viral expression. An important aspect of the novelty that we would like to communicate with this work is the interaction between the regulatory mechanism of the vector, the activity of the transgene and function of endogenous p53 (shown most clearly in Figure 3). Especially in the case of C6, this combination seems to be effective in reducing cell proliferation. In the case B16, additional drug treatment was necessary. We feel that these findings would be of interest to the readers of BMC Cancer since these cell lines are commonly used in basic studies.
Reviewer: Charles Giardina

General comments

This manuscript describes a series of experiments aimed at determining the extent to which different p53 activators function independently and in combination to regulate the proliferation of cancer cells. Two cancer cell lines are compared in these studies; one is sensitive to p53 activation and growth regulation by p19Arf and the anticancer agents Nutlin-3 and doxorubicin (C6 cells). P53 in the other cell line appears to be more resistant to activation (B16 cells). Experiments show that p53 activation in the resistant B16 cell line requires two independent inducers. In particular the Nutlin-3, p19Arf combination appears to be particularly effective.

The findings reported here have potential implications for the use of viral vectors designed to control carcinogenesis through activation and/or accentuation of the p53 pathway (if such an approach ever becomes clinically feasible). These experiments also provide insight into the interplay between the endogenous p53 activator p19Arf and pharmacological agents. Although the experiments shown are convincing and carefully interpreted, some additional information would serve to make the findings more complete. Some suggestions are outlined below.

We appreciate the description of our experiments as convincing and carefully interpreted. As described below, we have taken the specific suggestion under advisement and modified the manuscript accordingly.

Specific comments

1. Some of the figures were difficult to interpret due to the use of numeric identifiers. The authors should consider replacing the number codes with direct labels that indicate the cell line and the viral vector employed.

   The figures were modified to avoid the use of numeric identifiers as suggested.

2. The legend for Figure 1 needs to include information on what the different regions of the virus are and what their purpose is.

   The legend to Figure 1 has been modified as suggested.

3. The data in Figure 4 is difficult to reconcile with Figure 2. The p19Arf infection appears to be sufficient to activate p21 expression, whereas it did not stabilize p53 in Figure 2. This could be due to transient p53 activation, or perhaps a p53-independent activation of p21. Additional discussion of these data is required.

   This is an interesting point and the text has been modified to reflect the possibility that p21 was activated due to p53-independent mechanism.
4. It would also seem logical to include additional time points examining p53 stabilization after the various treatments. This information would provide important insight into the mechanism by which the different treatments interact.

This is an interesting idea and we would like to pursue it. However, this new experiment has not yet been performed and may be an interesting point of departure for a future study.

5. It is somewhat surprising that p19Arf and Nutlin-3 provide an effective combination since theoretically they should be accomplishing the same thing: Mdm2 neutralization. The authors raise the possibility that p19Arf is inhibiting Mdm4 while Nutlin-3 neutralizes Mdm2. Data on Mdm4 and Mdm2 expression in the cell lines would therefore be of interest.

Yes, they both neutralize mdm2, but by different mechanisms. Nutlin-3 inhibits the interaction of mdm2 with p53. p19Arf inhibits the E3 ubiquitin ligase activity of mdm2. This latter point could have ramifications beyond p53 since mdm2 does interact with a variety of factors, such as Hif1α and PML.

We did attempt to study mdm2 and mdm4 protein levels by western blot (see figure below). We observed that mdm4 levels were unchanged by gene transfer or drug treatment. We were not able to detect mdm2 with either of the antibodies used. According to the literature, the failure of nutlin-3 may be due to mdm4, but we were not able to show this in our study. For this reason, the Discussion has been modified.

**Western blot for the detection of mdmx.** B16 and C6 cells were mock transduced or transduced with pCLPG or pCLPGp19 viruses. The next day, cells were treated with 100 ng/ml doxorubicin (D), 10 µM nutlin-3 (N) or no drug at all (-). After an additional 16 hours incubation, cells were harvested for western analysis (protocol as described in the manuscript). Mdmx was detected using rabbit polyclonal anti-mdmx (H-130, sc2822, Santacruz Biotechnologies), protein-G-HRP and ECL. We were unsuccessful in detecting mdm2 even after trying two different antibodies (sc812, Santa Cruz; SMP14, M4308, Sigma).
In this study, Merkel et al tried to show that either retroviral expression of p19ARF or treatment with nutlin-3, alone, failed to activate p53 in B16 mouse melanoma cells that are p53 wild type and p19ARF null. However, a combination of p19ARF overexpression and nutlin-3 can activate p53 resulting in cell death.

This is a problematic study with serious flaws, conceptually and experimentally. Conceptually, nutlin-3 is an extremely potent p53 activator by blocking MDM2-p53 interaction resulting in either cell cycle arrest or apoptosis in p53+ cancer cells whereas the primary function of p19ARF is to inhibit MDM2 ubiquitin E3 ligase activity and stabilizes p53. It is difficult to understand why this group could not show nutlin-3 activating p53, as many labs in the filed are able to show.

We too were surprised to find that nutlin-3 did not activate p53 in B16 cells. However, we are not alone. Several groups, as cited in the text of the manuscript, have shown that nutlin-3 may fail to activate p53 if the cells overexpress mdmx (mdm4). I do not believe that we have a problem since nutlin-3 treatment was quite successful in the C6 cell line included for comparison.

With regard to p19ARF, it is possible that the levels of p19ARF overexpression are inadequate. If these are cell type-specific, controls cancer cells should be used to convincingly show that same treatment (p19ARF overexpression or nutlin-3) activates p53.

Control cancer cells (C6, rat glioma, p53wt, p19Arf-deficient) were used in our study. In this case, individual p19Arf and nutlin-3 treatments were sufficient to activate p53, reduce proliferation, reduce viability and alter the cell cycle. These cells were included to show exactly this point and we believe that our vector, the level of expression and the drug treatments were successful, at least in C6 cells.

Besides, it makes no sense that nutlin-3, a potent p53 stabilizer needs help from p19ARF, which also functions to stabilize p53.

We agree that this is surprising. However, our principle aim was to see if p19Arf, when delivered by our novel vector, would be able to arrest tumor cells. Though this clearly worked in the case of C6, we wondered what may have been the problem in B16 cells. Since the delivery of p19Arf for the activation of p53 was insufficient to alter B16 cell proliferation, we proposed that the problem may still be a lack of p53 activity. For this reason, we ventured to treat with the combination of p19Arf and nutlin-3.

We also agree that both agents should stabilize p53. However, the mechanism for each agent is unique. Whereas nutlin-3 is quite specific for the interaction of mdm2 with p53, delivery of p19Arf may have an impact on several pathways that intersect with mdm2. For example, Hif1α, PML, HDAC and many more factors are known to interact with mdm2. The possible impact of p19Arf on...
these interactions could explain why the combination of treatments was effective, though we are not able to show a specific mechanism at this time.

Overall, the data as presented were in low quality and the controls were missing or inadequate. This study is clearly premature and the authors need to use proper controls, some of which are listed below. It is also evident that the deficiency of scientific writing in English made the reading difficult.

Below we respond to each of the comments.

The manuscript has been revised and improved with regard to the writing.

Specific comments:
1. In the abstract. What is meant by: “The p53-responsive pCLPG retroviral vector was used to transfer p189Arf, potentiating interplay of the vector, transgene and endogenous p53 in the B16 cell line”. It is confusing. Does it mean: to study the effects of retroviral-mediated expression of p19Arf on p53 in B16 cells?

We agree that the original version of the abstract was confusing. For this reason it has been rewritten to better explain the features of our novel vector, pCLPG.

2. Fig. 1. It should be described in the Materials and Methods, not as a figure. We feel that Fig 1 is essential to the reader’s understanding of our work. For this reason the figure remains and its legend has been expanded. Comments 1 and 2 make it clear that we did not adequately address the important role that this vector played in this study. The text, especially the abstract, has been modified to better describe this p53-responsive vector. The central focus of our work is the use of viral vectors that employ a p53-responsive promoter (that is, a promoter that is directly bound and activated by p53) in order to drive transgene expression. In this way, endogenous p53 may drive viral expression of p19Arf which can complement p53 activity, resulting in increased vector expression and tumor suppression.

3. Fig. 2. The data showing that expression of p19Arf failed to activate p53 in B16 cells. There are numbers of reasons. Were expression levels of p19Arf too low? Is p53 truly wild type in the cells used?

We cannot rule out the possibility that p19Arf expression levels were insufficient to activate p53 in B16 cells. However, both the immunofluorescence assay and the western blot show readily detectable levels of p19Arf in B16, levels that appear to be equivalent to those seen in the C6 cells. In the case of C6, the p19Arf expression levels were sufficient to confer the activation of p53.

We are quite sure that p53 is truly wild type in the B16 cells used. As shown in the manuscript, p53 can be activated when proper stimulus was provided resulting in the increase in p53-responsive reporter activity. In addition, we have
treated B16 cells with pifithrin-α, a specific inhibitor of transactivation by p53 and observed that this can reduce the activity provided by our p53-responsive reporter construct, as shown in the figure below. This figure also confirms the activation of p53 upon treatment with the DNA-damaging drug doxorubicin. Sequencing of the p53 transcript has not yet been completed.

![Graph showing relative eGFP activity](image)

**Test of p53 phenotype**: B16 cells were transduced with the indicated vector and then selected for G418 resistance. These cells were then treated with pifithrin-α (30 μM) for 6h and then the medium was changed for fresh medium or medium containing 100 ng/ml doxorubicin. The cells were then incubated for an additional 16 hours before harvesting and analysis by FACS. The intensity of eGFP activity was determined by the FACS software. These values were then normalized considering pCLeGFP with no drug treatment as 1. Shown in the graph is the mean and standard deviation of the normalized values obtained from three independent experiments.

4. **Fig. 4.** It is essential to show that doxorubicin and nutlin-3 activate p53 in B16 cells. Why the levels of p21 protein were extremely low upon drug treatment? There seemed something fundamentally wrong for the drug treatment and/or western blotting for p21.

   We agree that the p21 levels seem to be quite low. We have altered the text to reflect that p21 expression may be induced by p53-independent mechanisms.

5. **Fig. 5.** If p19Arf failed to activate p53 (see #3 above), it is no surprising that it did not inhibit cell growth. Again, were expression levels of p19Arf too low in B16 cells?
As mentioned above, p19Arf levels seem to be quite consistent between B16 and C6 cells, so we do not have a basis on which to say that the expression level was too low. Even so, we cannot rule out this possibility. And it is clear that growth B16 was not inhibited by p19Arf alone. However, we feel that an especially interesting point in this work is the fact that the combination of p19Arf plus nutlin-3 was sufficient to reduce viability as measured in the MTT assay.

6. Fig. 6. It is difficult to understand why nutlin-3 failed to activate p53 in B16 and C6 cells. Did the same batch of nutlin-3 activate p53 in published cancer cells such as U2-OS, SJSA-1 cells?

Figure 6 clearly shows that nutlin-3 treatment of C6 resulted in a more than 4-fold increase in reporter activity. Note that treatment of C6 cells with doxorubicin also resulted in a 4-fold induction of reporter activity, implying that nutlin-3 was just as effective as doxorubicin in stimulating p53 function. All experiments were performed with the same lot of nutlin-3 and doxorubicin. In addition, both cell lines were studied simultaneously, thus minimizing differences in handling. Since we have no reason to believe that the batch of nutlin-3 is defective, we did not test additional cell lines.