Author's response to reviews

Title: Par-3 partitioning defective 3 homolog (C. elegans) and androgen-induced prostate proliferative shutoff associated protein genes are mutationally inactivated in prostate cancer cells.

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Author's response to reviews: see over
Dear Editor:

Please consider our revised manuscript entitled “Par-3 partitioning defective 3 homolog (C. elegans) and androgen-induced prostate proliferative shutoff associated protein genes are mutationally inactivated in prostate cancer cells.” by Kunnev, et al for publication in BMC Cancer. We would like to thank the reviewers for critical reviewing the manuscript which, we hope, allowed us to improve its quality. In the revised manuscript we made following changes to address the questions raised by the reviewers.

Reviewer 1

1) We should see data that the mRNA and/or protein (qPCR or western blotting) is reduced for these genes

On page 12 line 19 of the manuscript we addressed this question by describing the results of RT-PCR analysis for the PARD3 and AS3 genes in the LNCaP and 22Rv1 cells, respectively. We modified the Figure 1 by showing the image of RT-PCR analysis (Figure 1A). We believe that RT-PCR instead of qPCR is sufficient since the exact values of mRNA level fold changes produced by mutations that trigger NMD is not that important. It is the identified mutations that matters. Microarray analysis of mRNA level alterations produced by NMD inhibition is used only for selecting candidates for sequencing analysis. If sequencing does not confirm the presence of nonsense or frameshift mutations the exact mRNA levels become irrelevant. We also modified Figure 2 by adding the image of western blot analysis with anti-PAR3 antibodies in addition to the western blotting with anti-flag antibodies of LNCaP cells transfected or not transfected with PARD3-expression construct. We could not demonstrate the lower level of Par3 protein in LNCaP cells possibly due to the fact that frameshift and nonsense mutations produce not only lower mRNA levels but they also produce truncated proteins which also can be degraded at the protein levels due to abnormal folding. We also tried to use commercial anti-Par3 antibodies to analyze the protein arrays of prostate tumors and normal tissues. Possibly due to cross-reactivity of the available anti-Par3 antibodies with the PARD3B protein product, the homolog of Par3 protein, the results of our analysis were inconclusive and were not included in the manuscript.

2) Does expression of Par3 alter the cellular junctions (tight or adherens) in the cancer cells? Without Par3 the junctions could be defective.

On page 15 of the revised manuscript we describe the effect of Par3 expression in the LNCaP cells on the intracellular distribution of vinculin, E-cadherin and zona occludens 1 (zo-1), markers for focal adhesion, adherens junction and tight junction protein complexes, respectively. On Figure 3 we present images of LNCaP cells staining with corresponding antibodies. It can be concluded from these images that tight junction but not the adherens junction or the focal adhesion is affected by introduction of functional copy of the PARD3 gene.

3) Are these genes mutated in any other prostate cancer cell lines or tumors?
Prostate cancer cell lines DU145 and PC-3 available from ATCC do not have mutations in these genes. We analyzed genomic DNA from 11 prostate tumor samples trying to identify mutations in PARD3 gene. We did not find any mutations. However, since prostate tumors usually contain large amounts of stromal tissue we could miss the mutations due to possible presence of large amounts of normal DNA in our samples.

Reviewer 2

Additional data not shown indicated PARD3 did not enhance growth in soft agar or nude mice, although details not given. Since the results of figure 3 were unexpected, it would be helpful if authors would include enough information regarding the methods to assess the significance of results not shown (DISCRETIONARY).

To address this question we re-evaluated our results for the effect of PARD3 expression on the growing of LNCaP cells in soft agar taking into consideration the size of the growing colonies. We found that number of colonies with cut-off diameter for counting > 0.2 mm did not differ significantly between LNCaP cells expressing and not expressing Par3 protein. However, the number of larger colonies with cut-off diameter for counting > 0.5 mm was significantly lower in the cells expressing PARD3 gene. New Figure 5 shows the images of colonies and the bar graphs representing the number of colonies of different size in the LNCaP cells expressing or not expressing Par3 protein.

Given the unexpected proliferative effect of PARD3, the authors also asked whether it would enhance growth of LNCaP in matrigel suspension cultures. Surprisingly, the authors concluded that PARD3 impaired growth of LNCaP in matrigel suspension cultures. However, the results in figure 4B need quantification (number of colonies, of what sizes, how many times reproduced, etc) and clarification of the methods (the cut-off size for counting colonies not given). It is also not clear how these results support the authors conclusion that there was a higher rate of cell death in 3D cultures. Data demonstrating apoptosis was not obtained and reduced colony size or number could be due to reduced proliferation rates, not just apoptosis. The methods, results and discussion supporting Figure 4B should be clarified (DISCRETIONARY).

Since we did not demonstrate the increased cell death in 3D cultures we eliminated these words about cell deaths or apoptosis. On page 15 line 24 we wrote instead: “Also we found that expression of PARD3 affected the growth of LNCaP cells in 3D culture in Matrigel. Figure 5C shows that colonies formed by LNCaP cells not expressing Par3 protein are larger and look more condensed, while the colonies formed by Par3 expressing cells look dispersed at the periphery.” The Figure 5C thus is for illustrating the differences in the appearance of colonies in 3D culture.

Since PARD3 plays a role in cell-cell adherence, the authors assessed the effect of PARD3 expression on attachment of LNCaP cells to collagen and fibronectin coated wells. Quantitative results given in Figure 4A show that PARD3 enhanced attachment at 4 hours after seeding, but the methodology for counting unstained cells needs clarification (were 900 cells counted per field?). If available, inclusion of an image would be convincing (DISCRETIONARY).
We included the images of the cells attached to the collagen and to the fibronectin for the LNCaP cells expressing or not expressing Par3 protein into the new Figure 4.

The Affymetrix gene expression data were submitted to Gene Expression Omnibus (GEO) database under accession number GSE16856. Thank you for considering the revised manuscript for publication in BMC Cancer.

Sincerely,

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