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14. ABSTRACT Mutations in mitochondrial DNA (mtDNA) are frequent in prostate cancer and they seem to occur early during prostate malignant transformation. Depletion of mtDNA in prostate cancer cells has been linked to acquisition of androgen-independence, progression to an invasive phenotype that is resistant to conventional chemotherapies, as well as induction of epithelial-mesenchymal transition leading to cancer metastasis. Using long-range genomic polymerase chain reaction, large deletion of mtDNA can be detected in prostate cancer tissues but not benign or normal prostate tissues. Noticeably, our study excludes the germ-line origin of the mutant mtDNA pattern in prostate cancer patient through analysis of the blood of the corresponding patient. Our data conclude that mtDNA deletion is due to carcinogenesis process in somatic prostate cells. In addition, our data have unveiled the molecular alteration in prostate cancer cells resulted from mtDNA deletion. For example, Skp2 protein elevation is often associated in prostate cells with loss of mtDNA. Also, the presence of Skp2 expression can decrease the expression of BRCA2 protein as an early biomarker of prostate neoplastic transformation, which is due to BRCA2 proteolysis.					
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INTRODUCTION

Mitochondrial DNA (mtDNA) depletion has been shown to promote malignant progression of prostate cancer cells. However, the molecular mechanisms underlying the association between mutant mtDNA and prostate cancer progression remain obscure. Mutant mtDNA has been associated with increased genomic DNA double-strand breaks. The resulting genomic instability could account for the multiple phenotypic effects observed in prostate cells harboring mutations/depletion of mtDNA, i.e. increased migration, acquisition of androgen-independence and progression to an invasive phenotype that is resistant to conventional chemotherapies. *BRCA2* is among the few genes known to be involved in repair of DNA double-strand breaks, and its loss confers a significantly elevated risk to develop aggressive, rapidly progressing, high-grade prostate carcinoma. During the first two years we have identified the presence of large mtDNA deletions in prostate cancer specimens but not in age-matched benign prostate hyperplasia, and we have correlated their presence with loss of BRCA2 protein in cancer. We have also provided evidence for an inverse correlation between BRCA2 protein levels, the number of mtDNA deletions and Gleason grade in prostate carcinoma, suggesting that accumulation of mtDNA deletions may occur with progression of prostate cancer and further decrease BRCA2 levels in PCa. Furthermore, we have identified a translational and post-translational mechanism of down-regulation of BRCA2 expression by mutant mtDNA, which implicates up-regulation of both Skp2 and miR-1245.

BODY

During the third year we have accomplished most of the planned experimental tasks.

Aim 2. Identify the molecular mechanisms of down-regulation of BRCA2 expression by mutant mtDNA (Months 10-24)

Task 2. Identify proteins differentially regulated by mutant mtDNA that may regulate loss of BRCA2 expression (Months 19-24)

2.a Identify proteins differentially regulated by mutant mtDNA using Clontech antibody microarrays and confirm results by immunoblotting (*Months 19-21*)

2.b Determine the role of differentially expressed molecules in regulating BRCA2 loss using gain/loss of-function genetic approaches and/or pharmacological inhibition (*Months 21-24*)

We have found that mtDNA depletion promotes an increase in cytoplasmic calcium concentration and that pharmacological inhibition of calcium elevation is able to recover the expression levels of Skp2 and miR-1245, two negative regulators of BRCA2 expression in mtDNA-depleted cells, thereby restoring BRCA2 protein levels (Figure 1).

Aim 3. Investigate the role of BRCA2 in preventing/hindering mtDNA-related prostate cancer progression (Months 19-36)

Task 1. Generate clones of C4-2, LNCaP Rho-, PNT1A/C4-2 cybrids and PNT1A Rho0 cells stably overexpressing BRCA2 (Months 19-21)

1.a Stably transfect mtDNA mutant cells with BRCA2 cDNA or empty vector. Eventually, first subclone BRCA2 cDNA in an inducible system of expression (*Months 19-21*)

We were able to generate PNT1A Rho(0) clones overexpressing BRCA2. These cells have been used to understand the role of BRCA2 loss in the migratory and apoptotic-resistant properties of mtDNA-depleted cells by performing *in vitro* experiments. However, PNT1A are not able to form tumors in nude mice as they are normal immortalized cells, thus they can be used for *in vitro* but not *in vivo* experiments. We were also able to generate C4-2 cells overexpressing BRCA2, but they show a mitotic arrest and it is impossible to perform experiments. Thus we have subcloned BRCA2 cDNA in a doxycycline-regulated system (pTet-OFF from Clontech) and generated C4-2 and LNCaP Rho- stable clones overexpressing BRCA2 upon removal of doxycycline. Unfortunately, BRCA2 expression in C4-2 cells caused mitotic arrest after a few days in culture also when using a doxycycline-inducible system of expression.

Task 2. Assess the role of BRCA2 in preventing tumor growth and metastasis in pre-clinical animal models (Months 21-32)

2.a To assess the role of BRCA2 in preventing tumor growth, inject athymic nude mice (Balb/c) subcutaneously with mock- and BRCA2-expressing mtDNA-mutant cells [C4-2 and LNCaP Rho-, 2 injecting sites *per* mouse, 10 animals/cell type; mock LNCaP and mock-“mtDNA reverted” LNCaP used as control, 2 injecting sites *per* mouse, 10 animals/cell type], measure tumor growth, harvest tumors for histological examination and immunohistochemical analysis of BRCA2 expression. Total: 60 mice requested. (*Months 21-30*)

Unfortunately, BRCA2 caused mitotic arrest after a few days in culture also when using a doxycyclin-inducible system of expression (task 1.a). Therefore, we were not able to perform experiments of tumor growth in animals.

2.b Generate stable clones expressing luciferase (*Months 22-23*)

We tried to generate stable mtDNA-mutated clones overexpressing luciferase without success. We now think that it would be unlikely the generation of these clones because luciferase uses ATP for its activity, thus it depletes mtDNA-mutated cells of the energy required for anabolic processes and survival. Of note, mtDNA-mutated cells have reduced ATP levels due to dysfunctional oxidative phosphorylation.

2.c Assess the role of BRCA2 in preventing tumor metastasis in pre-clinical animal models. Inject luciferase-expressing human prostate cells into the right lobe of nude mice's prostate (orthotopic model), and measure occurrence of micro- and macro-metastasis by imaging (detection of cellular bioluminescence after injecting luciferin). Harvest affected tissues/organs for histological examination and immunohistochemical analysis of BRCA2 levels. Cells: mock and BRCA2-expressing C4-2, LNCaP Rho-. Controls: mock LNCaP, mock “mtDNA-reverted” LNCaP (10 animals/cell type). Total: 60 animals requested (*Months 24-32*)

We were not able to perform these experiments because mtDNA-depleted cells overexpressing BRCA2 exhibit a marked inhibition in cell growth. In addition, luciferase-transfected mtDNA-depleted cells did not survive (see tasks 2.a and 2.b).

Task 3. Investigate the ability of BRCA2 to suppress mtDNA-related neoplastic and metastatic phenotype in vitro (Months 31-36)

3.a Assess the ability of wild-type and BRCA2-expressing mtDNA-mutated prostate cells to resist to anoikis induced by loss of anchorage in poly-HEMA coated-plates, resist to apoptosis induced by chemotherapeutic drugs, migrate along extracellular matrix proteins and through reconstituted basement membrane matrix, and respond to androgen stimulation (Months 31-36)

We have demonstrated that reconstitution of BRCA2 expression in mtDNA-depleted PNT1A cells is able to suppress anoikis resistance and migration (Figure 2). In addition, we found that mtDNA-depleted cells become more sensitive than wild-type cells to apoptosis induced by PARP inhibitors and that recovery of BRCA2 protein restores sensitivity to PARP inhibitors to control levels (Figure 3).

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of a BRCA2-dependent increase in migration, anoikis resistance and sensitivity to PARP inhibitors in mtDNA-depleted cells
- Demonstration of a calcium-dependent mechanism of suppression of BRCA2 expression in mtDNA-depleted cells

REPORTABLE OUTCOMES

Arbini, A.A., Greco, M., Yao, J.L., Bourne, P., Marra, E., Hsieh, J.T., di Sant'Agnese, P.A., Moro, L. (2011) Loss of BRCA2 protein expression is associated with elevated Skp2 levels in human prostate cancer. *Am. J. Pathol.*, 178:2367-2376.

Ro, S., Ma, H-Y., Park, C., Ortogero, N., Song, R., Hennig, G.W., Zheng, H., Lin, Y-M., Moro, L., Hsieh, J.T., Yan W (2013) The mitochondrial genome encodes abundant small noncoding RNAs. *Cell Res.*, 1-16.

Arbini, A., Guerra F., Greco, M., Marra, E., Gandee, L., Xiao, G., Lotan, Y., Gasparre, G., Hsieh, J.T., Moro, L. (2013) Mitochondrial DNA mutations sensitize cancer cells to PARP inhibitors. *Cancer Res.* (submitted).

CONCLUSION

Our results provide evidence for a calcium-dependent mechanism of regulation of BRCA2 protein loss following mtDNA mutations, which implicates both Skp2 and miR-1245 upregulation upon a mtDNA-dependent increase in cytosolic calcium levels. In addition, we provide evidence that BRCA2 reduction in mtDNA-depleted cells is

involved in increased migration and anoikis resistance as well as in increased sensitivity to PARP inhibitors of mtDNA-depleted cells.

APPENDICES

Arbini, A.A., Greco, M., Yao, J.L., Bourne, P., Marra, E., Hsieh, J.T., di Sant'Agnese, P.A., Moro, L. (2011) Loss of BRCA2 protein expression is associated with elevated Skp2 levels in human prostate cancer. *Am. J. Pathol.*, 178:2367-2376. (<http://www.ncbi.nlm.nih.gov/pubmed/21514447>)

Ro, S., Ma, H-Y., Park, C., Ortogero, N., Song, R., Hennig, G.W., Zheng, H., Lin, Y-M., Moro, L., Hsieh, J.T., Yan W (2013) The mitochondrial genome encodes abundant small noncoding RNAs. *Cell Res.*, 1-16. (<http://www.ncbi.nlm.nih.gov/pubmed/23478297>)

SUPPORTING DATA

Figures 1-3

Figure legends

Fig. 1 BRCA2 protein is reduced in mtDNA-depleted cells through a calcium-dependent mechanism. **A**, Cytosolic calcium concentration was quantified in PNT1A wild-type, Rho(0) and cybrids cells using the fluorescent indicator Fura-2AM. **B**, Cells were treated with the calcium chelator BAPTA-AM for 3 h, then total proteins and miRNAs extracted and analyzed for Skp2 and BRCA2 protein, and for miR-1245 levels, respectively.

Fig. 2 Increased migration and anoikis resistance in mtDNA-depleted cells is reduced after reconstitution of BRCA2 protein levels. **A**, Migration was assessed by plating 100,000 cells onto Transwells coated with laminin-1, a basement membrane protein. After 4 h, migrated cells were fixed, stained with crystal violet and counted. **B**, Anoikis resistance was assessed by plating the cells on poly-HEMA-coated plates. After 48 h, cells were collected and analyzed for caspase 3/7 activity using a fluorometric-based system.

Fig. 3. MtDNA-depletion sensitizes cells to PARP inhibitors through loss of BRCA2 protein. **A**, Cells were incubated with 10 μ M rucaparib, a PARP inhibitor, for 24 h, then assessed for caspase-3 activity (apoptotic marker). Apoptosis is reported as percentage of untreated controls. **B**, Wild-type and mtDNA-mutant cells treated with 10 μ M AG014699 for 24 h were cultured in drug-free medium for 21 days, fixed, and counted. Cell survival was calculated as percentage of untreated controls. Where indicated, Rho(0) cells had been transiently transfected with BRCA2 cDNA before rucaparib treatment (+BRCA2).

Figure 1

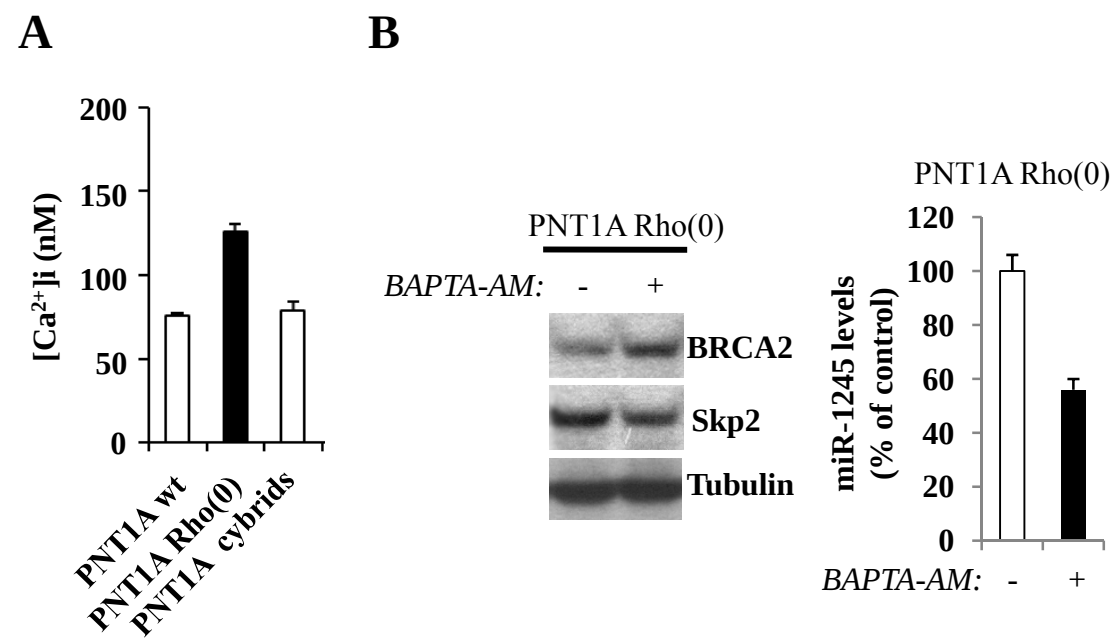
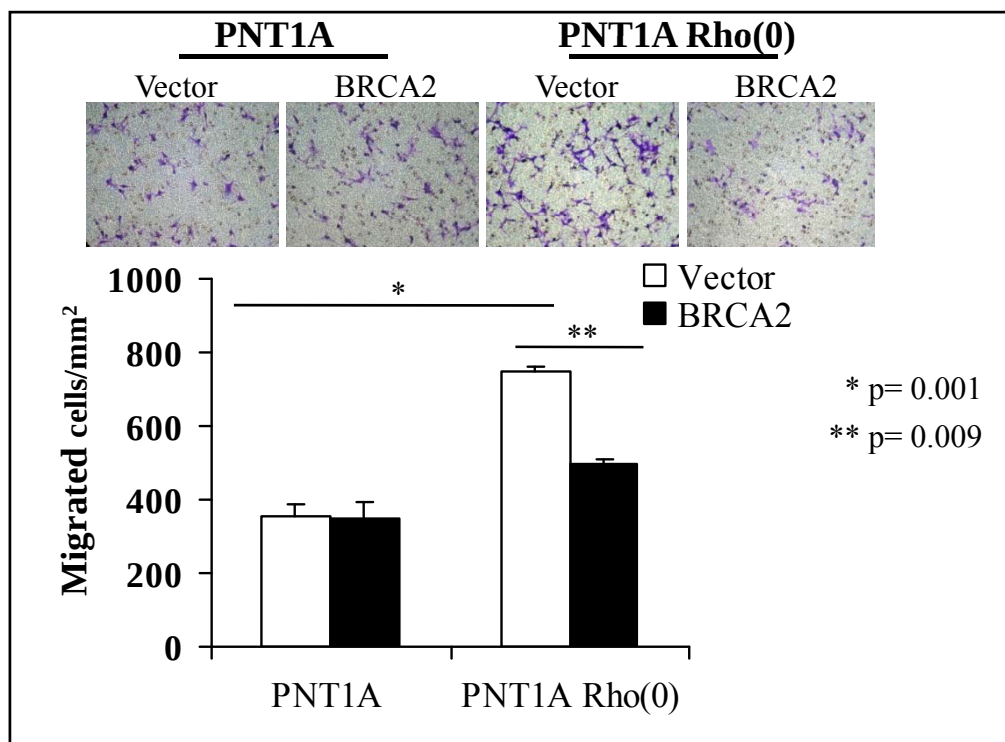


Figure 2

A



B

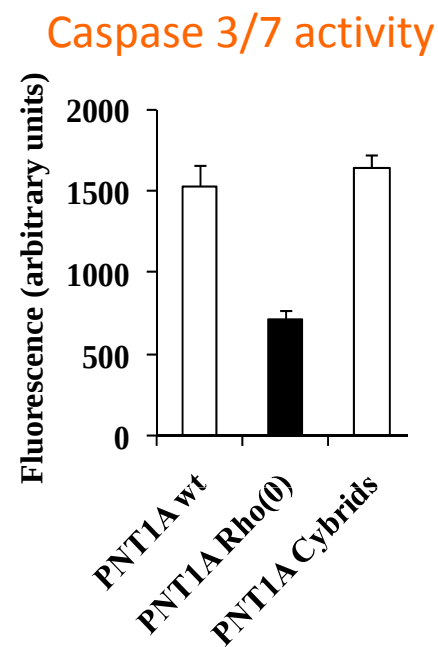
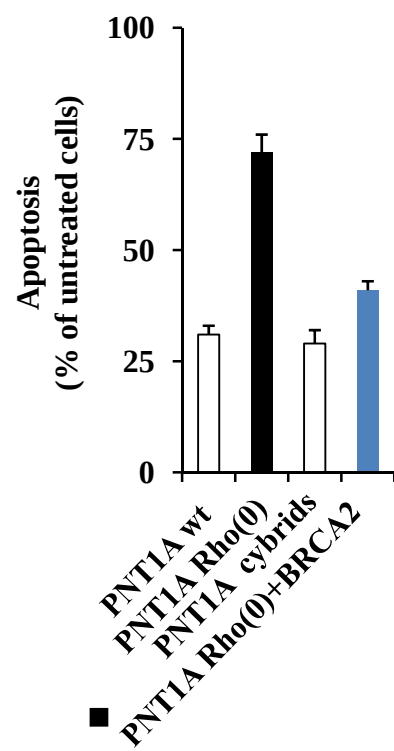


Figure 3

A



B

